**Versatile Transcription of Biphenyl Catabolic bph Operon in Pseudomonas pseudoalcaligenes KF707**

Received for publication, April 6, 2000, and in revised form, July 6, 2000  
Published, JBC Papers in Press, July 18, 2000, DOI 10.1074/jbc.M003023200

**Takahito Watanabe, Ryuichi Inoue, Nobutada Kimura, and Kensuke Furukawa‡**  
From the Laboratory of Applied Microbiology, Graduate School of Bioresource and Environmental Sciences, Kyushu University, Hakozaki 6-10-1 Fukuoka 812-8581, Japan

*Pseudomonas pseudoalcaligenes* KF707 possesses a chromosomally encoded *bph* gene cluster responsible for the catabolism of biphenyl/polychlorinated biphenyls. The gene cluster consists of *(orf0) bphA1A2(orf3) bphA3A4BCX0X1X2X3D*. We studied the role of *orf0* and transcription in the *KF707* *bph* operon. Primer extension analysis revealed that at least as many as six transcriptional initiation sites exist upstream of *orf0*, *bphA1*, *bphX0*, *bphX1*, and *bphD*, including two upstream of *bphD*. The *orf0*-disruptant failed to grow on biphenyl but accumulated large amounts of the biphenyl ring *meta*-cleavage yellow compound (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate). Western blot analysis revealed that ORF0 protein is inducibly expressed in *KF707* in the presence of biphenyl. Gel shift assay revealed that ORF0 directly binds to the *orf0* promoter region. This binding was greatly enhanced by addition of the biphenyl ring *meta*-cleavage yellow compound. These results indicate that *orf0*, *bphA1A2(orf3) bphA3A4BC* and *bphX0X1X2X3D* are independently transcribed, and that ORF0 protein belonging to the GntR family is involved in the regulation of the *bph* operon in *KF707* and is absolutely required for the expression of *orf0* and *bphX0X1X2X3D*.

The biphenyl-utilizing bacteria have been widely isolated from various environmental samples, which include both Gram-negative and Gram-positive bacteria. Because these organisms are able to degrade polychlorinated biphenyls, xenobiotic compounds known as one of the most serious environmental pollutants, biochemical and genetic bases of polychlorinated biphenyl degradation have been extensively studied by many workers. The biphenyl/polychlorinated biphenyl degradative genes, termed *bph*, were first cloned from *Pseudomonas pseudoalcaligenes* *KF707* (1–3) and then from many Gram-negative and Gram-positive strains (4–17).

The *bph* gene cluster of *KF707* is organized as *(orf0)-bphA1A2(orf3) bphA3A4BCX0X1X2X3D* (Fig. 1). The *bphA1* and *bphA2* genes encode a large subunit and a small subunit of the terminal dioxygenase, respectively, *bphA3* encodes a ferredoxin reductase, and *bphA4* encodes a ferredoxin reductase. These four gene products associate to form a multicomponent biphenyl dioxygenase that is involved in the initial oxygenation of the biphenyl ring. The *bphB* gene encodes a biphenyl dihydrodiol dehydrogenase. The *bphC* gene encodes a 2,3-dihydroxybiphenyl (23DHBP) 1 dioxygenase that is involved in the ring *meta*-cleavage. The *bphD* gene encodes a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPD) hydrolase to obtain benzoate and 2-hydroxypenta-2,4-dienoate. The *bphX0* gene encodes a putative glutathione S-transferase, but the role of this enzyme is yet unknown in the catabolism of biphenyl. The *bphX1*, *bphX2*, and *bphX3* genes, encoding 2-hydroxypenta-2,4-dienoate hydratase, acetaldehyde dehydrogenase, and 4-hydroxy-2-oxovalerate aldolase, respectively, are involved in the catabolism of 2-hydroxy-penta-2,4-dienoate to acetyl coenzyme A (Fig. 1). The roles of *orf0* and *orf3* in this operon remain to be elucidated.

Despite the detailed biochemical and genetic analyses of *bph* genes of various soil bacteria, the knowledge concerning regulation have remained unclear. Recently, it was suggested that the *bph* gene cluster in *Tn4371* found from *Ralstonia eutropha* A5, *bphEFGA1A2A3BCD*, forms an operon transcribed from a *σ*70 promoter and that the *bphS* gene product negatively regulates the transcription of the *bph* gene cluster as a repressor (18). On the other hand, the *bph* operon in Gram-positive *Rhodococcus sp.* M5, *bpdC1C2BADEF*, is regulated by the two-component signal transduction system of *bpdS* and *bpdT*. These *bpd* genes are inducibly transcribed by biphenyl. In this system, *BpdS* and *BpdT* function as a sensor histidine kinase and a response regulator, respectively (19). Here we report the versatile transcription of the *KF707* *bph* gene cluster, indicating that the ORF0 protein is an activator and is absolutely involved in the expressions of its own *orf0* and *bphX0X1X2X3D* and that at least six transcriptional initiation sites are present in the *KF707* *bph* gene cluster.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The strains and plasmids used in this study are listed in Table I. The biphenyl-utilizing strain *P. pseudoalcaligenes* KF707 was grown in basal salt medium (BSM) supplemented with 0.2% (w/v) biphenyl as a sole source of carbon as described previously (1). Biphenyl-utilizing defective derivatives of KF707 such as KF730 (*bphA1*: *Tn5*-B21), KF748 (*bphB*: *Tn5*-B21), and KF744 (*bphC*: *Tn5*-B21) were previously constructed (20), and KF7095 (*orf0*: *Tc5*) was constructed in this study. They were grown in BSM supplemented with 0.1% (w/v) sodium succinate and tetracycline (*Tc*)

---

* This work was supported in part by CREST (Core Research for Evolutionary Science and Technology) of the Japan Science and Technology Corporation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

* The abbreviations used are: 23DHBP, 2,3-dihydroxybiphenylyl; *Bph* 1−, phenotype able/unable to grow on biphenyl as a sole carbon source; BSM, basal salt medium; DIG, digoxigenin; HOPD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (biphenyl ring *meta*-cleavage yellow compound); MOPS, 3-((N-morpholino)propanesulfonic acid; RT, reverse transcriptase; PCR, polymerase chain reaction; *Tc*, tetracycline; bp, base pair(s); kb, kilobase(s).
### Pseudomonas pseudoalcaligenes KF707 bph gene cluster

| orf0 | A1 | A2 | orf3 | A3 | A4 | B | C | X0 | X1 | X2 | X3 | D |
|------|----|----|------|----|----|---|---|----|----|----|----|---|

**FIG. 1.** Catabolic pathway for degradation of biphenyl and organization of the bph gene cluster in *P. pseudoalcaligenes KF707.* Compound I, biphenyl; compound II, 2,3-dihydroxy-4-phenylhexa-4,6-diene (dihydriodiol compound); compound III, 2,3-dihydroxybiphenyl; compound IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (biphenyl meta-cleavage compound); compound V, benzoic acid; compound VI, 2-hydroxypenta-2,4-dienoic acid.

**RT-PCR**—RT-PCR was performed using the RT-PCR kit according to the manufacturer's instruction (TOYOBO, Osaka). For the detection of the orfo-mRNA, the following primers were used for 8 μg of total RNA: 5'-ATGAATACGAGAACGCTTGCAAGGCGACG-3' and 5'-CCGGTCTGGCCTGCAAG-3' within orfo coding sequence. To examine whether orfo and *bphA1* could be co-transcribed, the following primers encompassing the both genes were used: 5'-CGGATGCGCGCCGCTGTCG-3' within orfo coding sequence and 5'-CTCTTCTTGGTATGACCTCATG-3' within bphA1 coding sequence. The PCR products were subjected to electrophoresis through 2.0% agarose gel.

**Construction of orfo Expression Plasmid**—The orfo of KF707 was amplified from chromosomal DNA using the following primers: 5’-ACCTTACATGGGAGATACGCTGATACGAGAC-3’ and 5’-CTCTTCTTGGTATGACCTCATG-3’ for the forward sequence, where the Ncol site is underlined and the start codon ATG is in bold type. For the reverse primer sequence, 5’-AAGGCTGTCCGACCTTCGACAAAGCCGATCCAG-3’ was used, where the SalI site is underlined. Amplification of orfo was carried out for 25 cycles under the following conditions: denaturation, 94 °C for 1 min; primer annealing, 52 °C for 1.5 min; and primer extension, 72 °C for 1.5 min. The PCR products were digested by Ncol and SalI and inserted at the Ncol site of the plasmid, which contains an isopropyl-β-D-thiogalactopyranoside-inducible T7 promoter. The orfo was designed to be fused to Trx (thioredoxin protein) (Novagen), which contains an isopropyl-β-D-thiogalactopyranoside-inducible T7 promoter. The orfo was designed to be fused to Trx (thioredoxin protein) (Novagen), which contains an isopropyl-β-D-thiogalactopyranoside-inducible T7 promoter. The orfo was designed to be fused to Trx (thioredoxin protein) (Novagen), which contains an isopropyl-β-D-thiogalactopyranoside-inducible T7 promoter. The orfo was designed to be fused to Trx (thioredoxin protein) (Novagen), which contains an isopropyl-β-D-thiogalactopyranoside-inducible T7 promoter. The orfo was designed to be fused to Trx (thioredoxin protein) (Novagen), which contains an isopropyl-β-D-thiogalactopyranoside-inducible T7 promoter.
subjected to SDS-polyacrylamide gel electrophoresis and transferred to Trans-Blot 
Transfer Medium (Bio-Rad). Anti-ORF0 antibody was prepared by the method described by Imajoh-Ohmi et al. (25). Western blot analysis using anti-ORF0 antibody was carried out to detect the expression of ORF0 protein in pSUP707 by the method described by Imajoh-Ohmi et al. (25). Signal intensities were quantified with a densitometer.

Construction of pSUPB101—The TeC gene (4.3 kb) was removed from pSUP102::Tn5-B30 by XhoI digestion. A 2.5-kb SacI fragment containing the orf0 gene was inserted into the XhoI site of pSUP102::Tn5-B30. The resultant plasmid has the unique XhoI site in the middle of the inserted fragment, a purified TeC gene from pSUP102::Tn5-B30 was then inserted into the XhoI site within orf0 to generate pSUPB101 (Table I). pSUPB101 containing orf0 disrupted by the TeC gene was introduced into E. coli S17–1 (27) by transformation.

Southern Blot Analysis—Southern blot analysis was performed using the DIG DNA Labeling and Detection Kit according to the manufacturer’s instructions (Roche Diagnostics). The chromosomal DNA of the Pseudomonas strains listed in Table I were digested with XhoI and subjected to electrophoresis through 1.0% agarose gels. The digested DNA fragments were transferred onto Biodyne B (PALL). Hybridization was performed with the DIG-11-dUTP-labeled BamHI-EcoRI fragment (3.9 kb) from pSUP102::Tn5-B30 vector as a probe.

RNAse Protection Assay—RNAse protection assay was performed using the RNase protection kit according to the manufacturer’s instructions (Roche Diagnostics). The RNA probe used in this assay was labeled with DIG-11-UTP by the methods of Roche Diagnostics. The RNA probe used in this assay was prepared by the method described by Imajoh-Ohmi et al. (25). Signal intensities were quantified with a densitometer.

TABLE I

Bacterial strains and plasmids used in this study

| Strain/plasmid | Genotype/description | Source/reference |
|----------------|----------------------|-----------------|
| P. pseudoalcaligenes |                       |                 |
| KF707 | Bph’, wild type | Ref. 1 |
| KF730 | Bph’, bphA1; Tn5-B21; TeC | Ref. 20 |
| KF748 | Bph’, bphB; Tn5-B21; TeC | Ref. 20 |
| KF744 | Bph’, bphC; Tn5-B21; TeC | Ref. 20 |
| KF7095 | Bph’, orf0; TeC, TeR | This study |
| E. coli |                       |                 |
| JM109 | Host strain for DNA manipulation | Takara Shuzo |
| BL21(DE3) | Host strain for expression | Novagen |
| S17–1 | pro, thi, recA, hsdR, chromosomally integrated RP4-2- | Ref. 27 |
| pSUP102::Tn5-B30 | TeC | Ref. 27 |
| pSUP102::Tn5-B30ΔTeC | (4.3-kb XhoI fragment); orf0bphA1 | This study |

Plasmids

| Name | Description | Source |
|------|-------------|--------|
| pBluescriptII® KS(+) | Cloning vector; AmpR | Stratagene |
| pET32b(+) | Expression vector; AmpR | Novagen |
| pUC118 | Cloning vector; AmpR | Takara Shuzo |
| pTV118N | Cloning vector; AmpR | Takara Shuzo |
| pUC19 | Cloning vector; AmpR | Takara Shuzo |
| pTWF1 | 1.7-kb Psfl fragment from KF707 in pUC19; orf0bphA1 | This study |
| pTWF2 | 5.2-kb Safl fragment from KF707 in pUC118; bphBCX0X1X2X3D | This study |
| pTWF3 | 0.75-kb Ncol-SalI PCR fragment in pET32b(+) | This study |
| pTWF6 | 2.1-kb Ncol-PstI fragment from pTWF3 in pTV118N; orf0 | This study |
| pTWF7 | 0.98-kb ClaI-PstI fragment in pBluescriptII® KS(+) | This study |
| pSUP102::Tn5-B30 | pACYC184 derivative with RP4-specific Mob site and transposon Tn5-B30; CmR, GmR, TeR | Ref. 26 |
| pSUP102::Tn5-B30ΔTeC | 2.5-kb Safl fragment in pSUP102::Tn5-B30 without TeC | This study |
| pSUP101B | TeC is inserted in XhoI site of pSUP102::Tn5-B30ΔTeC, orf0, TcR; CmR, GmR, TcR | This study |

RESULTS

Disruption of orf0—It was previously shown that orf0 is not directly responsible for the enzymatic activity of biphenyl di-oxygenase (encoded by bphA1A2A3A4) (28). The orf0 encoded a putative protein of 245 amino acids, and the data base search revealed that the ORF0 protein shares 28% identity to GntR, a regulatory protein known as the transcriptional repressor of the gluconate operon in Bacillus subtilis (29). These two proteins exhibited a high similarity of amino acid sequence in the N-terminal region (Fig. 2A). Furthermore, a helix-turn-helix motif involved in DNA-binding was predicted in the N-terminal
region of ORF0 (Fig. 2B). Therefore, ORF0 was considered to be a possible regulatory protein belonging to the GntR family (30).

To investigate the function, we disrupted orf0 in strain KF707. For this purpose, the plasmid pSUPB101 in which the TcR gene was inserted in orf0 was constructed as described under “Experimental Procedures.” E. coli S17–1 cells carrying pSUPB101 were filter-mated with KF707 overnight. pSUPB101 cannot replicate in Pseudomonas strains; therefore, single cross-over recombinants were first screened on BSM agar plates supplemented with 0.1% (w/v) succinate, Tc (30 μg/ml), and gentamicin (20 μg/ml). Such single cross-over recombinants were repeatedly subcultured in LB broth to obtain double cross-over recombinants. These were checked for growth on BSM plates supplemented with succinate, Tc, and gentamicin. The loss of the vector-borne gentamicin resistance gene was confirmed by Southern blot analysis (data not shown).

Strain KF7095 (orf0-disruptant) failed to grow on biphenyl and accumulated large amounts of the biphenyl ring meta-cleavage yellow compound (HOPD) (Fig. 3). In the case of strain KF707 (wild type), HOPD accumulated temporarily, but reduced gradually because of the conversion to benzoic acid by the BphD enzyme (HOPD hydrolase). These results strongly suggested that the bphA1A2A3A4BC genes are expressed in KF7095, but the bphD gene is not.

Enzyme Activities of BphC, BphX0, BphX2, and BphD in KF707 Derivatives—We previously obtained the following KF707 transposon mutant strains; KF730 in which Tn5-B21 is inserted in bphA1 (bphA1::Tn5-B21), KF748 in which Tn5-B21 is inserted in bphB (bphB::Tn5-B21), and KF744 in which Tn5-B21 is inserted in bphC (bphC::Tn5-B21) (20). Using these transposon mutants and the orf0-disruptant (KF7095), we measured the enzyme activities of BphC, BphX0, BphX2, and BphD (Fig. 4). No activity of BphC (3DHBP dioxygenase) was detected in KF730 because the bphB and bphC genes were not expressed by a polar effect of Tn5-B21 inserted in the bphA1 gene. Likewise, no activity of BphC in KF748 and KF744 was detected. These mutants failed to grow on biphenyl. However, these three strains exhibited the enzyme activities of BphX0

---

Fig. 2. Analysis of the amino acid sequence of ORF0. A, multiple alignment between ORF0 (KF707; DDBJ/EMBL/GenBank accession number D85852), ORF0 (Pseudomonas sp. LB400; Swiss-Prot number P37335), BphS (Tn4371 originally isolated from R. eutropha A5; DDBJ/EMBL/GenBank accession number AJ012075), AphS (silencer of the aph genes in C. testosteroni TA441 involved in utilization of phenol; DDBJ/EMBL/GenBank accession number AB008787), and GntR (repressor of the gluconate operon in B. subtilis; Swiss-Prot number P10585). The multiple alignment was done using the GENETYX-MAC Ver.10.1 software (Software Development, Tokyo). B, multiple sequence alignment for helix-turn-helix (HTH) motif of ORF0 (KF707), ORF0 (LB400), BphS (Tn4371), AphS (TA441), and GntR (B. subtilis) (30, 36). Fully conserved residues are in bold type. The number of amino acid residues in each protein is indicated in parentheses.
To examine how the orf0 is transcribed, we performed primer extension analysis. One transcriptional initiation site was found at 106 nucleotides upstream of the start codon of orf0 (Fig. 5A). The possible promoter sequence was assigned to TTGACA (−35 region) and TTAGTT (−10 region) (Fig. 5B). This promoter sequence is similar to the consensus sequence of the E. coli σ70-dependent promoter. The extension signal from the biphenyl-grown cells (Fig. 5A, lane B) was much (2.5 times) stronger than that of the succinate-grown cells (Fig. 5A, lane S), indicating that the transcription of orf0 is induced by biphenyl. We also performed Northern blot analysis using antisense orf0 RNA probe but failed to obtain a clear signal corresponding to the orf0 (data not shown).

In addition, we performed RT-PCR against the total RNAs of KF707 and KF7095 using the primers designed to anneal the orf0-coding region from the start codon to the upstream of the unique XhoI site in which the TrR gene is inserted in KF7095. The results clearly show that 200-bp RT-PCR products were detected when KF707 was grown on either biphenyl or succinate (Fig. 5C, lanes 1 and 2). The amount of RT-PCR product was much higher in the biphenyl-grown cells than that of the succinate-grown cells. However, no amplified DNA was detected in KF7095 (Fig. 5C, lane 3). The result clearly indicated that orf0 is not transcribed in KF7095. We also performed RT-PCR to examine whether orf0 and bphA1 are co-transcribed using the primers encompassing the both genes, but no amplified DNA was detected (Fig. 5C, lane 4). This result indicated that orf0 and bphA1 are independently transcribed.

Western Analysis of ORF0 Protein—We were successful in expressing the ORF0 protein as His-tagged form in E. coli. After purification, we prepared antibody against the ORF0 protein. Using the anti-ORF0 antibody, we detected the ORF0 protein of 27.7 kDa in size from KF707 cell extracts by Western blot analysis (Fig. 6). These data allowed us to conclude that orf0 is absolutely expressed in KF707. The signal from the biphenyl-grown cells (Fig. 6, lane 1) was much stronger (3.1 times) than that of the succinate-grown cells, indicating that the expression of ORF0 is induced in the presence of biphenyl. This is consistent with the result obtained in the primer extension analysis and the RT-PCR analysis (Fig. 5, A and C).

Binding of ORF0 Protein to the Operator Region—The results obtained in primer extension analysis and Western analysis strongly indicated that orf0 is inducibly expressed in the presence of biphenyl (Figs. 5A and 6). In addition, the ORF0 protein could be considered to be a regulatory protein in the KF707 bph operon because ORF0 exhibits a helix-turn-helix DNA binding motif in the N-terminal half (Fig. 2B). Furthermore, the sequence boxed in Fig. 5B could be a possible ORF0-binding region (termed an operator region). This sequence is located between the transcriptional initiation site and the start codon of orf0 and is similar to the binding sites of some GntR-type regulators such as AphS in the aph operon of Comamonas testosteroni TA441 (Fig. 7 and Ref. 31).

To confirm whether ORF0 directly binds to the operator region, we performed gel shift assay. As described under “Experimental Procedures,” a 38-bp fragment located between the transcriptional initiation site and start codon of orf0 was labeled with DIG-11-ddUTP. The ORF0 protein fused to HisTag® at the C terminus was expressed in E. coli carrying pTV118N-orf0 (pTWF6). The purified ORF0 protein (28 kDa) was used in gel shift assay. When the ORF0 protein was mixed with the labeled DNA fragment, its mobility was retarded (Fig. 8, shifted band I in lane 2). The labeled DNA fragment was not shifted when an excess amount of cold target DNA was added (Fig. 8, lane 3). The addition of an unrelated DNA did not affect the binding of ORF0 to the target DNA (Fig. 8, lane 6). The shifted band was not detected when the cell extract of E. coli carrying pTV118N was used as a control (Fig. 8, lane 7). These results strongly indicated that the ORF0 protein specifically

**FIG. 3.** Accumulation of HOPD (yellow compound) in KF7095 (orf0-disruptant). These strains were grown in BSM supplemented with biphenyl and succinate, and their cell extracts were used as the crude enzyme in this assay. Although KF707 accumulated HOPD temporarily, HOPD was simultaneously hydrolyzed by the BphD enzyme activity. The crude enzyme in this assay. Although KF707 accumulated HOPD temporarily, HOPD was simultaneously hydrolyzed by the BphD enzyme activity. The crude enzyme in this assay. Although KF707 accumulated HOPD temporarily, HOPD was simultaneously hydrolyzed by the BphD enzyme.

**FIG. 4.** Enzyme activities of BphC, BphX0, BphX2, and BphD in KF707 and KF707 mutants. Transposon Tn5-B21 shown as a stem-loop structure and the tetracycline-resistant (TcR) gene shown as a filled box are schematically drawn. The shaded boxes show the inactive genes. +, enzyme activity identical to that of wild type KF707; −, no enzyme activity. BphC activity of KF707 could not be measured quantitatively as described in Fig. 3 legend. In addition, we failed to perform quantitative assay of the BphX0 and BphX2 activities because of the instability of their substrates (1-chloro-2,4-dinitrochlorobenzene and glutathione for BphX0; NAD+ and acetaldehyde for BphX2). The data of the BphD enzyme activity represent the means of triplicate assays.

(glutathione S-transferase), BphX2 (acetaldehyde dehydrogenase), and BphD (HOPD hydrolase). More interestingly, the KF7095 (orf0-disruptant) exhibited the BphC activity but no BphX0, BphX2, nor BphD activity. The same strain accumulated large amounts of the biphenyl ring meta-cleavage yellow compound (HOPD) (Fig. 3). These results suggest that the bphaIA2(orf3)/bphaA4ABC and the bphaX0X1X2X3D are independently transcribed.

**FIG. 2.** DNA binding motif in the N-terminal half (Fig. 2B). Furthermore, the sequence boxed in Fig. 5B could be a possible ORF0-binding region (termed an operator region). This sequence is located between the transcriptional initiation site and the start codon of orf0 and is similar to the binding sites of some GntR-type regulators such as AphS in the aph operon of Comamonas testosteroni TA441 (Fig. 7 and Ref. 31).

To confirm whether ORF0 directly binds to the operator region, we performed gel shift assay. As described under “Experimental Procedures,” a 38-bp fragment located between the transcriptional initiation site and start codon of orf0 was labeled with DIG-11-ddUTP. The ORF0 protein fused to HisTag® at the C terminus was expressed in E. coli carrying pTV118N-orf0 (pTWF6). The purified ORF0 protein (28 kDa) was used in gel shift assay. When the ORF0 protein was mixed with the labeled DNA fragment, its mobility was retarded (Fig. 8, shifted band I in lane 2). The labeled DNA fragment was not shifted when an excess amount of cold target DNA was added (Fig. 8, lane 3). The addition of an unrelated DNA did not affect the binding of ORF0 to the target DNA (Fig. 8, lane 6). The shifted band was not detected when the cell extract of E. coli carrying pTV118N was used as a control (Fig. 8, lane 7). These results strongly indicated that the ORF0 protein specifically
binds to the operator region that lies between its transcrip-
tional initiation site and the start codon. More interestingly, in the presence of biphenyl-metabolites such as 23DHBP and HOPD, the retardation of shifted bands were much more notable, and the signal intensities were stron-
ger, particularly in the presence of HOPD (Fig. 8, shifted band II in lanes 4 and 5). These observations indicated that the ORF0 protein strongly binds to the operator region in the presence of biphenyl-metabolites.

Determination of Transcriptional Initiation Sites of bphX0, bphX1, and bphD—Because KF7095 did not have any enzyme activities of BphX0, BphX2, and BphD, it is conceivable that there are at least two independent transcripts of bphX0X1X2X3D. The transcriptional initiation site was found far upstream of 452 nucleotides from the start codon of bphX0 (Fig. 9A, lane B). This site lies within the center of the coding sequence of bphC. No extension signal was detected when the cells were grown in the absence of the orf0 mRNA by RT-PCR using the total RNAs from biphenyl-grown KF707 cells (lane 1), succinate-grown KF707 cells (lane 2), and KF7095 cells (lane 3). Lane 4 indicates the result of RT-PCR against the total RNA of biphenyl-grown KF707 cells using the primers encompassing orf0 and bphA1 coding sequences.

![B](https://example.com/b.png)

**B**

CAGCCACCACCATGAGAGGCTCTGGCGTTCCGCTGAG
TGATTGCAGCAGCGCTGACGGCTCAGCGCTCAGCTCAGCCGAG

**RBS**

CCAGCCGCCTTGGTGCATGCTGACCGCCGAGAAGTCATATTCCG

**PSGVMAGEKFS**

Fig. 5. Primer extension analysis of orf0 and RT-PCR analysis of RNA transcripts. A, the end-labeled primer was complementary to a 24-bp sequence located downstream of the orf0 start codon. Lanes A, G, C, and T correspond to the dideoxy sequencing reaction carried out with the same primer. Lane B represents the primer extension reactions with RNA prepared from KF707 biphenyl-grown cells. Lane S represents succinate-grown cells. The arrow indicates the transcriptional initiation site, and the number represents the upstream nucleotide position from the start codon of orf0. B, the putative promoter sequence of orf0 is shown in bold type. The putative ribosome-binding site (RBS) is underlined. One transcriptional initiation site had been previously determined at 104 bp upstream of bphA1 (3). C, detection of

![C](https://example.com/c.png)

![A](https://example.com/a.png)
respectively.

C
in vitro
of arrow
determination of transcriptional initiation site of bphX0 by primer extension. Notation is the same as in the legend for Fig. 5A except that lane M represents the primer extension reaction with RNA prepared from KF7095 (orf0-disruptant) that was grown with biphenyl and succinate. B, RNase protection assays for the detection of the bphX0-mRNA against the total RNA of KF707 and KF7095, respectively. C, physical map of the bphC and bphX0 region. The +1 indicates the transcriptional initiation site of bphX0. The direction of transcription is indicated by an arrow. The antisense RNA (the corresponding region indicated in a dotted arrow) was synthesized by the method of in vitro transcription and used as a probe for RNase protection assay.

of biphenyl (Fig. 9A, lane S), indicating that the expression of bphX0 is induced by biphenyl or its metabolites. In the case of KF7095, a faint band was detected (Fig. 9A, lane M), but this was concluded to be an artificial band from the following experiments. Using the antisense RNA probe of bphX0 (Fig. 9C), we performed RNase protection assay to detect the mRNA of bphX0. As shown in Fig. 9B, the mRNA of bphX0 was detected in KF707 but not in KF7095. Furthermore, we did primer extension analysis using the primer complementary to the sequence located 24 nucleotides downstream of the bphX0 start codon. No extension signal was detected in KF7095 (data not shown). These results were consistent with the fact that no BphX0 enzyme activity is detected and allowed us to conclude that bphX0 is not expressed in KF7095 and that orf0 is involved in the transcription of bphX0.

Further primer extension analyses revealed one transcriptional initiation site at 48 nucleotides upstream of the start codon of bphX1 (Fig. 10A, lane B). However, no signal was detected from succinate-grown cells (Fig. 10A, lane S) nor from KF7095 (Fig. 10A, lane M). These results suggest that BphX1 is induced by biphenyl or its metabolites and that the orf0 product is absolutely required in the expression.

In addition, two transcriptional initiation sites were found at 30 and 29 nucleotides upstream of the start codon of bphD (Fig. 10B, lane B). The other faint bands detected in lane B were ignored because they were not detected in several primer extension analyses. These two extension signals were detected, but weakly, even in the absence of biphenyl (Fig. 10B, lane S). The intensities of the extension signals detected in the presence of biphenyl (Fig. 10B, lane B) were much stronger (2.7 times) than those in the absence of biphenyl (Fig. 10B, lane S), indicating that bphD is inducibly transcribed in the presence of biphenyl and its metabolites. On the other hand, KF7095 exhibited no extension signal of the bphD gene (Fig. 10B, lane M), indicating that orf0 is absolutely required for the expression of bphD as in the case of bphX0 and bphX1.

**DISCUSSION**

In this study, it was found that the ORF0 protein belonging to the GntR family is involved in the regulation of the bph operon in *P. pseudoalcaligenes* KF707. The orf0 was inducibly expressed in the presence of biphenyl (Figs. 5A and 6). It was further demonstrated that ORF0 itself directly binds to the operator region (Figs. 5B, 7, and 8) by gel shift assays. The retardation was greatly enhanced in the presence of biphenyl ring meta-cleavage compound (HOPD) (Fig. 8). These results indicate that the ORF0 protein may change the conformation in the presence of HOPD and strongly binds to the operator region and promotes the transcription of its own gene.

It was first reported that the GntR protein negatively regulates the gnt operon in *B. subtilis* (29). The GntR-type regulatory proteins involved in the degradation of aromatic compounds are also reported to be repressors (18, 31). However, ORF0 in KF707 is not likely to be a repressor (silencer) but more likely to be an activator being required for the expression of the lower pathway genes of bphX0X1X2X3D (Figs. 4, 9B and 10A and B).

We have preliminary data demonstrating that ORF0 protein also binds to the upstream region (about 200 bp) of bphX0 and bphX1, respectively (data not shown). Thus, it seems that ORF0 controls its own expression and acts as an activator for the expression of bphX0X1X2X3D. However, the same protein is not involved in the expression of the bphAIA2(orf3)bphA3A4ABC genes, because...
the orf0-disruptant (KF7095) converts biphenyl to the HOPD (Fig. 3), indicating that these genes are expressed without the ORF0 protein. Along with this finding, we previously reported that one transcriptional initiation site is located 104 nucleotides upstream of the start codon of bphA1 (3). Furthermore, the result obtained in RT-PCR (Fig. 5, lane 4) revealed that no mRNA encompassing both orf0 and bphA1 is present. These results allowed us to conclude that the orf0 is transcribed alone but not co-transcribed with other degradative genes. The bphS and aphp regulatory genes are independently transcribed with respect to their degradative genes (18, 31). On the other hand, the gntR gene in the gnt operon of B. subtilis is inducibly co-transcribed with the other genes. In this case, the expression of the GntR repressor is posttranscriptionally regulated (32).

In this and the previous studies (3), primer extension analyses revealed that transcriptional initiation sites exist upstream of orf0, bphA1, bphX0, bphX1 and bphD, respectively. These results revealed that the bph gene cluster has at least six transcriptional initiation sites, including two for the bphD gene. It was reported previously that three transcriptional initiation sites are found upstream of bpha in Pseudomonas sp. strain LB400 whose bph gene cluster is nearly identical to that of KF707 (6). However, the transcription and regulation of LB400 bph genes have not yet been studied in detail since then.

As shown in Figs. 3 and 4, KF7095 expressed BphC but not BphX0, BphX2, and BphD. Primer extension analysis identified the transcriptional initiation site upstream of bphX0 in wild type KF707 (Fig. 9A). These results strongly indicate that the bphaA1B2orf3A3ABC and the bphaX0X1X2X3D are independently transcribed. However, no typical transcriptional termination signal was found downstream of bphC. At the present, we could determine six transcriptional initiation sites in the KF707 bph operon by primer extension analyses but failed to obtain information about the promoters except for orf0 and the transcriptional terminator(s) in this study. No typical terminator sequences were found in the operon. To investigate the sizes of mRNA transcripts, we performed Northern blot analyses using the antisense probes of bph genes. However, we failed to detect the clear transcript signals because they were smeared (data not shown).

A number of studies in the past revealed that many of the gene clusters involved in the degradations of aromatic compounds including biphenyl are present on movable elements such as plasmids and transposons. Such elements permit microorganisms to degrade aromatic compounds by horizontal gene transfer. It was reported that the bph gene cluster involved in the metabolism of biphenyl/4-chlorobiphenyl is located in the 55-kb transposon Tn4371 (17, 18, 33, 34). Our recent study also revealed that the 90-kb bph-sal element coding for biphenyl and salicylate metabolism behaves like a conjugative transposon in Pseudomonas putida KF715 whose bph gene cluster is nearly identical to that of KF707 except that the bphaX0X1X2X3 genes were deleted (35). If this is the case in KF707, the bph genes could be foreign genes derived from other strains. Such genes might be regulated and expressed in different fashions because the sigma factor(s) in KF707 may recognize certain regions as promoters. This could be the reason why so many transcriptional initiation sites were detected in the KF707 bph gene clusters.

REFERENCES
1. Furukawa, K., and Miyazaki, T. (1986) J. Bacteriol. 166, 392–398
2. Furukawa, K., Arimura, N., and Miyazaki, T. (1987) J. Bacteriol. 169, 427–429
3. Taira, K., Hirose, J., Hayashida, S., and Furukawa, K. (1992) J. Biol. Chem. 267, 1844–1853
4. Kimbara, K., Hashimoto, T., Fukuda, M., Koana, T., Takagi, M., Oishi, M., and Yano, K. (1989) J. Bacteriol. 171, 2740–2747
5. Hayase, N., Taira, K., and Furukawa, K. (1990) J. Bacteriol. 172, 1160–1164
6. Erickson, B. D., and Mondello, F. J. (1992) J. Bacteriol. 174, 2903–2912
7. Hofer, B., Hett, L. D., Dohling, D. N., and Timmis, K. N. (1993) Gene (Amst.) 130, 47–55
8. Hofer, B., Buckhause, S., and Timmis, K. N. (1994) Gene (Amst.) 144, 9–16
9. Kikuchi, Y., Nagata, Y., Hinata, M., Kimbara, K., Fukuda, M., Yano, K., and Takagi, M. (1994) J. Bacteriol. 176, 1689–1694
10. Kikuchi, Y., Yasukochi, Y., Nagata, Y., Fukuda, M., and Takagi, M. (1994) J. Bacteriol. 176, 4269–4276
11. Masai, E., Yamada, A., Healy, J. M., Hatta, T., Kimbara, K., Fukuda, M., and Yano, K. (1995) Appl. Environ. Microbiol. 61, 2079–2085
12. Masai, E., Sugiyama, I., Iwashita, N., Shimizu, S., Hanischl, J. E., Hatta, T., Kimbara, K., Yano, K., and Fukuda, M. (1997) Gene (Amst.) 187, 141–149
13. Wang, Y., Gannon, J., Labbe, D., Bergeron, H., and Loo, P. C. (1995) Gene (Amst.) 164, 117–122
14. Kim, E., and Zelista, G. J. (1995) J. Bacteriol. 177, 3095–3103
15. Kim, E., Kim, Y., and Kim, C. K. (1996) Appl. Environ. Microbiol. 62, 262–265
16. Loo, P. C., Gannon, J., Labbe, D., and Wang, Y. (1996) Gene (Amst.) 171, 53–57
17. Merlin, C., Springael, D., Mercey, M., and Toussaint, A. (1997) Mol. Gen. Genet. 253, 499–506
18. Mouz, S., Merlin, C., Springael, D., and Toussaint, A. (1999) Mol. Gen. Genet. 262, 790–799
19. Labbe, D., Gannon, J., and Loo, P. C. (1997) J. Bacteriol. 179, 2722–2726
20. Furukawa, K., Hayashida, S., and Taira, K. (1991) Gene (Amst.) 98, 21–28
21. Habig, W. H., and Jakoby, W. B. (1981) Methods Enzymol. 77, 395–405
22. Shingler, V., Powlowski, J., and Marklund, U. (1992) J. Bacteriol. 174, 711–724
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. D., Smith, J. A., and Struhl, K. (1994) Current Protocols in Molecular Biology, Unit 4.4, John Wiley & Sons, Inc., New York
25. Imajoh-Ohmi, S., Tsujimura, K., and Inagaki, M. (1994) Kousuputidoakouta- jiken (in Japanese) pp. 47–118, Shujunsha, Tokyo
26. Simon, R., Quandt, J., and Klipp, W. (1989) Gene (Amst.) 80, 161–169
27. Simon, R., Priester, U., and Puhler, A. (1983) Bio/Technology 1, 784–795
28. Kimura, N., Nishi, A., Goto, M., and Furukawa, K. (1997) J. Bacteriol. 179, 3936–3943
29. Fujita, Y., and Fujita, T. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 4524–4528
30. Haydon, D. J., and Guest, J. R. (1993) Bios Microbiol. Lett. 63, 291–295
31. Arai, H., Akahira, S., Ohishi, T., and Kudo, T. (1999) Mol. Microbiol. 33, 1132–1140
32. Fujita, Y., Fujita, T., and Miwa, Y. (1999) FERS Lett. 267, 71–74
33. Merlin, C., Springael, D., and Toussaint, A. (1999) Pflaum 41, 40–54
34. Springael, D., Kreps, S., and Mercey, M. (1993) J. Bacteriol. 175, 1674–1681
35. Nishi, A., Tominaga, K., and Furukawa, K. (2000) J. Bacteriol. 182, 1949–1955
36. Yoshida, K., Miwa, Y., Ohmori, H., and Fujita, Y. (1995) Mol. Gen. Genet. 248, 583–591
37. Allison, S. L., and Phillips, A. T. (1990) J. Bacteriol. 172, 5470–5476