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# A 90-Kilobase Conjugative Chromosomal Element Coding for Biphenyl and Salicylate Catabolism in *Pseudomonas putida* KF715

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**The biphenyl and salicylate metabolic pathways in *Pseudomonas putida* KF715 are chromosomally encoded. The *bph* gene cluster coding for the conversion of biphenyl to benzoic acid and the *sal* gene cluster coding for the salicylate *meta*-pathway were obtained from the KF715 genomic cosmid libraries. These two gene clusters were separated by 10-kb DNA and were highly prone to deletion when KF715 was grown in nutrient medium. Two types of deletions took place at the region including only the *bph* genes (ca. 40 kb) or at the region including both the *bph* and *sal* genes (ca. 70 kb). A 90-kb DNA region, including both the *bph* and *sal* genes (termed the *bph-sal* element), was transferred by conjugation from KF715 to *P. putida* AC30. Such transconjugants gained the ability to grow on biphenyl and salicylate as the sole sources of carbon. The *bph* and *sal* element was located on the chromosome of the recipient. The *bph-sal* element in strain AC30 was also highly prone to deletion; however, it could be mobilized to the chromosome of *P. putida* KT2440 and the two deletion mutants of KF715.**

A number of biphenyl-utilizing bacteria have been isolated to date. They include gram-negative species of *Pseudomonas*, *Achromobacter*, *Sphingomonas*, *Comamonas*, *Burkholderia*, *Ralstonia*, and *Alcaligenes* and gram-positive *Rhodococcus* spp. (1, 3, 11, 37). Because these biphenyl-utilizing strains cometabolize polychlorinated biphenyls (PCB), the biochemistry of PCB degradation has been extensively studied (12). A gene cluster coding for biphenyl-PCB degradation (termed *bph*) was first cloned from *Pseudomonas pseudoalcaligenes* KF707 (14). Since then, a number of *bph* genes have been cloned and sequenced. Southern and sequence analyses of the *bph* genes revealed that some biphenyl-utilizing strains possess *bph* genes that are very similar, if not identical, to one another, but some share various degrees of homology (13).

The *bph* genes are present on bacterial chromosomes (8, 14, 16, 35), plasmids (18, 39), and transposons (22, 28, 34). The presence of similar genes in different strains implies that even chromosomal *bph* genes have or used to have a mechanism for mobilization to other strains. The *bph* genes of *Pseudomonas* sp. strain CB406 were mobilized following the construction in vivo of a cointegrate plasmid inserted into the broad-host-range plasmid RP4 (22). Springael and coworkers (23, 34) identified a transposon, Tn*4371*, carrying the *bph* genes encoding conversion of biphenyl to benzoic acid from *Ralstonia eutrophus* A5 (formerly *Alcaligenes eutrophus* A5) in which Tn*4371* first transposed from the chromosome to indigenous IncP1 plasmid, and the plasmid carrying Tn*4371* can be transferred to other strains by conjugation. A recent study shows that Tn*4371* is a kind of conjugative transposon of 55 kb (24, 29). Interestingly, another smaller conjugative transposon Tn-*bph* coding for biphenyl catabolism resides within Tn*4371*. This can be transferred to the recipient strain by conjugation independently. Divergence of the *bph* genes among various biphenyl-utilizing strains indicates that the *bph* genes might be very ancient and thus have accumulated many mutations over a long historical period. In fact, some *bph* operons are highly rearranged and shuffled in soil bacteria (12, 26).

*P. putida* KF715 *bphABCD* genes specify conversion of biphenyl to benzoic acid and 2-hydroxypenta-2,4-dienoic acid, where *bphA* is composed of four subunit genes (*bphA1*, *bphA2*, *bphA3*, and *bphA4*) encoding multicomponent biphenyl dioxygenase, *bphB* encoding dihydrodiol dehydrogenase, *bphC* encoding 2,3-dihydroxybiphenyl dioxygenase (DHB dioxygenase), and *bphD* encoding 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (HOPDA hydrolase) (16, 35). The KF715 *bph* gene cluster is very similar to the well-characterized *bphABCXD* operon of *P. pseudoalcaligenes* KF707 in terms of gene organization as well as the restriction enzyme profiles. However, the *bphX* region (3.5 kb), which exists between *bphC* and *bphD* and which is involved in the conversion of 2-hydroxypenta-2,4-dienoic acid to acetyl-coenzyme A (16, 17), is missing in the KF715 *bph* gene cluster (Fig. 1). In the course of further study of the KF715 catabolic genes, we found that KF715 easily loses the biphenyl-utilizing ability when the cells are grown in nutrient medium. Lee et al. have cloned and sequenced the three *sal* genes coding for the conversion of salicylate to 2-hydroxymuconic semialdehyde in KF715 (20, 21). Furthermore, we found that the catabolic capabilities of biphenyl and salicylate are transferred from KF715 to other *P. putida* strains.

Here we report on the deletion and mobilization of the large chromosomal *bph-sal* element in *P. putida* KF715.

## MATERIALS AND METHODS

**Bacterial strains and cultivation.** *P. putida* KF715, used throughout this study, was originally isolated from soil in Kitakyushu, Japan, together with the well-characterized *P. pseudoalcaligenes* KF707 (14, 35). Some characteristics of KF715 were previously described (13, 16). *P. putida* KF715M1 is the KF715 mutant strain in which both the *bphABCD* and *sal* genes are deleted. *P. putida* KF715M2 is another KF715 derivative in which only the *bph* genes are deleted, but the *sal* genes are retained. *Pseudomonas graminis* KF701 (formerly *Achromobacter xy-*

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*P. pseudoalcaligenes* KF707

deletion

*P. putida* KF715

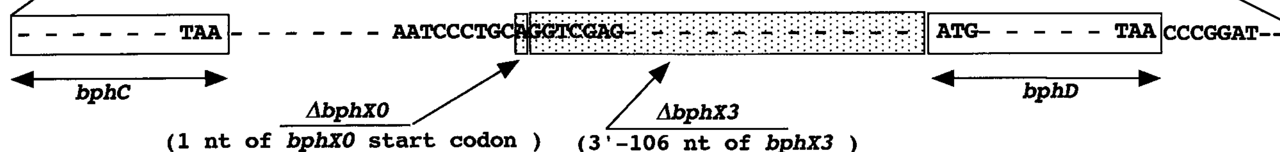


FIG. 1. Analysis of the deletion of *bphX* region within the *P. putida* KF715 *bph* operon in comparison with the corresponding region of *P. pseudoalcaligenes* KF707. The intervening sequence between *bphC* and *bphD* is schematically depicted. The deletion point is shown by a vertical arrow, indicating that only one nucleotide A of the start codon of *bphX0* and the 3′ region of 106 nucleotides of *bphX3* remain. The nucleotide sequences right after the stop codon of *bphD* from KF707 and KF715 are presented in boxes. nt, nucleotide(s).

*losoxidans* KF701), which possesses *bph* genes very similar to those of KF715, was described previously (13). *P. putida* AC30 was obtained from A. M. Chakrabarty (University of Illinois at Chicago), and a rifamcin-resistant and tryptophan-requiring mutant (AC30Rif$^r$Trp) was obtained in this study. *P. putida* KT2440 was donated by K. N. Timmis (GBF-National Research Center for Biotechnology, Braunschweig, Germany), and a streptomycin-resistant (Sm$^r$) and methionine-requiring mutant (KT2440Sm$^r$Met) was obtained in this study. A plasmid pCNU516 containing the *salA* gene was donated from Y. Kim (Chungbuk National University, Cheongju, Korea). Other strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) broth (40) was used as a nutrient medium. Basal salt medium (BSM) (14) was also used. Rifampin or streptomycin was used at 300 or 500 μg/ml, respectively, when needed.

**Conjugation.** Transfer of the KF715 Bph$^+$ phenotype by conjugation into the recipient strain *P. putida* AC30Rif$^r$Trp was carried out by filter mating. Donor and recipient cells were grown overnight in LB agar medium, and both cultures ($2 \times 10^9$) were suspended in 1 ml of LB broth. The cell suspensions (0.5 ml each) were mixed and put on a nitrocellulose filter and incubated on an LB agar plate for 6 h. The cells on the filter were suspended in sterilized distilled water and plated onto BSM plates containing 300 μg of rifampin and 20 μg tryptophan per ml, providing solid biphenyl in the inverted lid to select transconjugants.

**Detection of loss of Bph$^+$ and Sal$^+$ phenotypes.** After sequential batch growth on LB medium, biphenyl-, salicylate-, and benzoate-utilizing abilities were scored for every 100 colonies.

**Southern blot analysis.** Digoxigenin-11-dUTP was employed to label DNA by using a digoxigenin DNA labeling and detection kit according to the instructions of the manufacturer (Boehringer Mannheim, Mannheim, Germany). The genomic DNA was digested with appropriate restriction enzymes and subjected to electrophoresis through 0.7% agarose gels. The digested DNA fragments were transferred onto nylon membranes (Biodyne B; PALL, Port Washington, N.Y.). Hybridization was performed with the digoxigenin-labeled DNA probes.

**PCR.** PCR was performed with a total volume of 50 μl which contained PCR buffer (Takara Shuzo Co., Ltd., Kyoto, Japan), template DNA (0.5 μg), 100 mM (each) deoxyribonucleotide triphosphate, 1 mM (each) oligoprimer, and 0.5 U of *Taq* DNA polymerase. Amplification of DNA was carried out as described elsewhere (19). The oligoprimers used for the amplification of the *bphC*, *bphD*, and *salA* DNA were as follows: *bphC*, 5′-ATGTGCATTAAAAGTTTG-3′ for the upstream sequence and 3′-AACGCCTTGTTTCGTATT-5′ for the downstream sequence; *bphD*, 5′-ATGACAGCGCTCACTGAA-3′ and 3′-AAAAAT GCCGTCCGGATT-5′; and *salA*, 5′-ATGAACGCTAAGAAACCA-3′ and 3′-CGCGACGCAGTTCCCATT-5′.

**Construction of cosmid libraries.** Genomic DNA of *P. putida* strains KF715 and AC30Bph$^+$ were partially digested by *Sau*3AI. The DNA was subjected to 20% sucrose density gradient centrifugation at 85,000 × *g* for 16 h. After fractionation, the DNA sizes were examined by 0.3% agarose gel electrophoresis, and the 35- to 45-kb DNA was collected. The purified DNA was ligated at the *Bam*HI site of a cosmid vector Super Cos1 and packaged in vitro into lambda phage particles by using a GIGAPACK III Gold kit (Stratagene, La Jolla, Calif.), which were infected into *Escherichia coli* DH5α (38). The genomic libraries were amplified by growing the cells in LB broth supplemented with ampicillin. Amplified genomic libraries were preserved in 20% glycerol at −80°C until use.

**PFGE.** Agarose-embedded DNA suitable for separation by pulsed-field gel electrophoresis (PFGE) was prepared in accordance with instructions provided by the manufacturer (Bio-Rad Laboratories AG, Glattbrugg, Switzerland) with some modifications. PFGE was performed by clamped homogeneous electric-field (CHEF) electrophoresis for 24 h by the CHEF-DRII system (Bio-Rad). The gel (1%) was subjected to electrophoresis in 45 mM Tris-borate and 1 mM EDTA (pH 8.3) at 6 V/cm and 14°C.

## RESULTS

**Organization of the *bph* and *sal* genes in *P. putida* KF715.** We previously reported that a region named *bphX* (3.5 kb) between *bphC* and *bphD* is missing in KF715 (16), where four genes are present in *P. pseudoalcaligenes* KF707 (GenBank accession no. D85853) and *Burkholderia cepacia* LB400 (17). In KF707 they are *bphX0* (encoding glutathione *S*-transferase; *bphK* in LB400), *bphX1* (encoding 2-hydroxypenta-2,4-dienoate hydratase; *bphH* in LB400), *bphX2* (acetaldehyde dehydrogenase [acylating]; *bphJ* in LB400), and *bphX3* (encoding 4-hydroxy-2-oxovalerate aldolase; *bphI* in LB400). Sequence analyses of the intervening region of *bphC* and *bphD* in KF715 revealed that only one nucleotide (nucleotide A) of the start codon of *bphX0* and that the 3′-terminal 106 nucleotides of *bphX3* remain (Fig. 1). Thus, most of the 3.5-kb *bphX* region is deleted in the KF715 *bph* genes. The KF715-*bphD* and KF707-

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant description[a] | Source or reference |
|---|---|---|
| Strains | | |
| *P. putida* KF715 | Wild type, Bph$^+$ Sal$^+$ Ben$^+$ | 16 |
| *P. putida* KF715M1 | Bph$^-$ Sal$^-$ Ben$^+$ (Δ*bph*/Δ*sal*) | This study |
| *P. putida* KF715M2 | Bph$^-$ Sal$^+$ Ben$^+$ (Δ*bph*) | This study |
| *P. putida* KF715M1Bph$^+$ | Bph$^+$ Sal$^+$ Ben$^+$ | This study |
| *P. putida* KF715M2Bph$^+$ | Bph$^+$ Sal$^+$ Ben$^+$ | This study |
| *P. putida* AC30 | Ben$^+$ | A. M. Chakrabarty |
| *P. putida* AC30Rif$^r$Trp | Ben$^+$ Rif$^r$ *trp* | This study |
| *P. putida* AC30Bph$^+$ | Bph$^+$ Sal$^+$ Ben$^+$ Rif$^r$ *trp* (transconjugant acquired the *bph-sal* element) | This study |
| *P. putida* KT2440 | Ben$^+$ | K. N. Timmis |
| *P. putida* KT2440Sm$^r$Met | Ben$^+$ Sm$^r$ *met* | This study |
| *P. putida* KT2440Bph$^+$ | Bph$^+$ Sal$^+$ Ben$^+$ Sm$^r$ *met* (transconjugant acquired the *bph-sal* element) | This study |
| *P. pseudoalcaligenes* KF707 | Wild type, Bph$^+$ Ben$^+$ | 14 |
| *P. graminis* KF701 | Wild type, Bph$^+$ Ben$^+$ | 13 |
| *E. coli* S17-1 | *pro thi recA hsdR*; chromosomally integrated RP4-2-Tc::Mu-Km::Tn*7* | 33 |
| *E. coli* DH5α | *supE44* Δ*lacU169*(ϕ80 *lacZ* ΔM15) *hsdR recA1 endA1 gyrA96 thi relA1* | 40 |
| Plasmids | | |
| pMFB2 | pKT230-*bphABC*(KF707); Sm$^r$; 19.7 kb | 14 |
| pNHF715 | pKT230-*bphABCD*(KF715); Sm$^r$; 22.3 kb | 16 |
| pCNU516 | pUC18-*salA*(KF715); Ap$^r$; 4.6 kb | 20, 21 |
| Super Cos1 | Ap$^r$ Nm$^r$; cosmid vector; 7.6 kb | Stratagene |
| pKTF6246 | Cosmid containing the *sal* genes | This study |
| pKF6337 | Cosmid containing upstream of the *bph* genes | This study |
| pKF6465 | Cosmid containing downstream of the *bph-sal* element from AC30Bph$^+$ | This study |
| pKF6500 | Cosmid containing upstream of the *bph* genes | This study |

[a] Abbreviations: Bph, biphenyl; Sal, salicylate; Ben, benzoate; Rif, rifampin; Tc, tetracycline; Km, kanamycin; Sm, streptomycin; Ap, ampicillin; Δ, deletion; $^r$, resistance.

*bphD* genes share an identity as high as 96.0%, and both are ended with TAA in common, but their nucleotide sequence right after the stop codon is entirely different (Fig. 1).

Another catabolic gene cluster coding for the conversion of salicylate to 2-hydroxymuconic semialdehyde was cloned (termed *salA*-encoding salicylate hydroxylase, *salB*-encoding ferredoxin, and *salC*-encoding catechol 2,3-dioxygenase [C23O]) (20, 21). We determined the location of the *sal* genes. For this purpose we constructed a genomic cosmid library for KF715. First, we screened a cosmid clone expressing both DHB dioxygenase (encoded by *bphC*) and C23O (encoded by *salC*) by spraying with 2,3-dihydroxybiphenyl and catechol, respectively. From the restriction maps of the cosmids and the following Southern blot analyses with *bph* and *sal* DNA probes, it was revealed that the *sal* gene cluster lies 10 kb downstream of the *bphABCD* gene cluster.

**Appearance of the Bph$^-$ and Sal$^-$ phenotypes and their characteristics.** When *P. putida* KF715 was sequentially subcultured in LB broth, it was found that colonies unable to grow on biphenyl (Bph$^-$) appeared at high frequency. After 80 generations, 91% of the cells tested had lost the Bph-utilizing ability. About half of such Bph$^-$ mutants derived from KF715 also had lost their ability to grow on salicylate. These results indicated that two types of deletion occurred within the KF715 genome around the *bph-sal* gene clusters. The genomic Southern analyses with the *bphA1* DNA (see TNF-IV in Fig. 3) and *salA* DNA (TNF-VI), together with other parts of DNA (TNF-II and TNFVII) since the probes revealed that strain KF715M1 had lost both the *bph* and *sal* genes, including the intervening region (ca. 10 kb), and that KF715M2 had lost only the *bph* genes (Table 2 and Fig. 3). Shown in Fig. 2 are the PFGE profiles of *Spe*I digests and the following Southern blot analysis of the genomic DNA from KF715, KF715M1, and KF715M2. No hybridization was observed for the genomic DNA of both KF715M1 and KF715M2 when *bphA1* was used as the probe, while KF715M2 DNA hybridized with the *salA* probe only. PCR experiments revealed that the *bphC*, *bphD*, and *salA* genes were amplified from the genomic DNA of the original KF715 and that only the *salA* gene was amplified from KF715M2 (data not shown). Neither *bphC* and *bphD* nor *salA* were amplified from KF715M1. These results are in agreement with those from the Southern blot analyses.

TABLE 2. Growth characteristics and genomic Southern analyses of *P. putida* KF715 and *P. putida* AC30 and their derivatives[a]

| Strain | Growth on: | | | Hybridization with: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bph | Sal | Ben | TNF-I | TNF-II | TNF-III | TNF-IV | TNF-V | TNF-VI | TNF-VII | TNF-VIII | TNF-IX |
| KF715 | + | + | + | + | + | + | + | + | + | + | + | (+) |
| KF715M1 | − | − | + | + | + | − | − | (+) | − | + | + | (+) |
| KF715M2 | − | + | + | + | + | − | − | + | + | + | + | (+) |
| AC30Rif$^r$Trp | − | − | + | (+) | − | − | − | − | − | − | − | + |
| AC30Bph$^+$ | + | + | + | + | + | + | + | + | + | + | + | + |

[a] Abbreviations are as defined in Table 1. The DNA probes used in this experiment are shown in Fig. 3. (+), positive bands due to the unknown DNA homologous to the respective probe used.

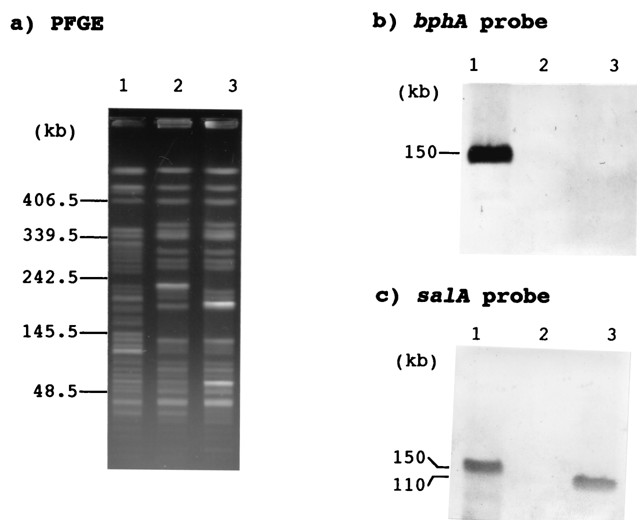


FIG. 2. PFGE of the genomic DNA of *P. putida* KF715, KF715M1, and KF715M2 and the subsequent Southern blot analysis of *bph* and *sal* genes. The *bphA1* and *salA* DNAs were used as the probe. The genomic DNAs were digested by *Spe*I. Lanes: 1, KF715; 2, KF715M1; 3, KF715M2. Molecular size markers are shown on the left.

**Recovery of the Bph$^+$ phenotype by introducing the *bph* genes.** The two Bph$^-$ mutant strains KF715M1 and KF715M2 were mated with *E. coli* S17-1 carrying pMFB2, containing the *bphABC* genes of *P. pseudoalcaligenes* KF707 (14), or pNHF715 containing the *P. putida* KF715 *bphABCD* genes (16), respectively. Both KF715M1 and KF715M2 carrying pNHF715 restored the ability to grow on biphenyl; however, the same mutant strains carrying pMFB2 failed to grow on biphenyl but produced a large amount of yellow *meta*-cleavage compound (HOPDA) from biphenyl (Table 2).

**Deletion regions in KF715M1 and KF715M2.** From the analyses of genomic DNAs by Southern blot and PCR, it was revealed that KF715M1 had lost *bph* and *sal* genes, including the intervening region (10 kb), and that KF715M2 had lost the *bph* genes but retained the *sal* genes. Based on these findings, we analyzed KF715 cosmid DNAs carrying both the *bphABCD* and *sal* genes in comparison with the corresponding regions from KF715M1 and KF715M2. By using various DNA probes that include the upstream of *bphA*, the downstream of *bphD*, and the downstream of *sal*, DNA fragments which hybridized with these probes, but with different sizes, were screened (Fig. 3). In order to determine the upstream of deletion sites in KF715M1 and KF715M2, the *Eco*RI 13-kb DNA (TNF-II in Fig. 3) was prepared from cosmid pKF6337, which was derived from the KF715 chromosome. The TNF-II DNA probe hybrid-

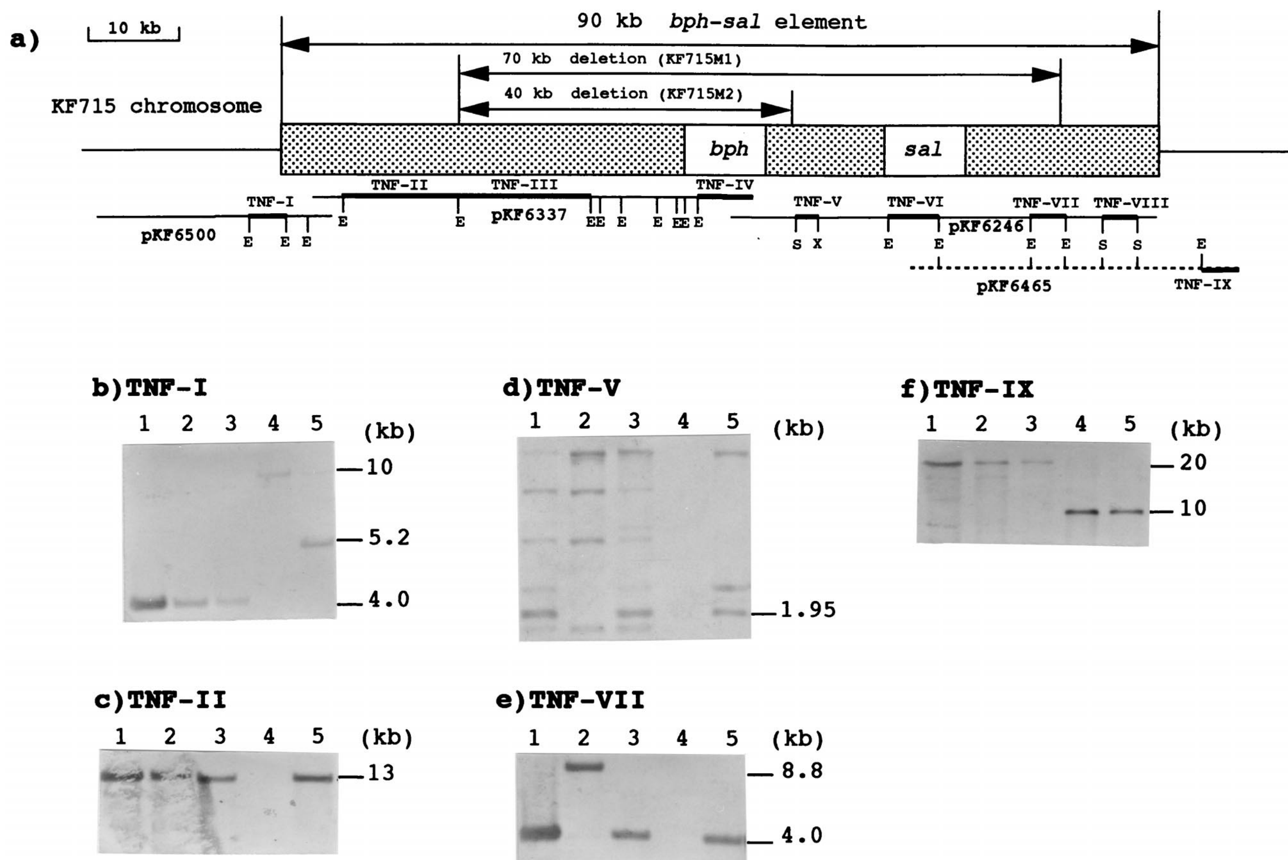


FIG. 3. Analyses of terminal ends of the *bph-sal* element in *P. putida* KF715 and the deletion regions within the same element in KF715M1 and KF715M2. The following four cosmids were analyzed: pKT6500 containing the upstream of the *bph-sal* element, pKF6337 containing the *bph* gene cluster and its upstream region, pKF6246 containing the *sal* genes, and pKF6465 containing the *salC* gene and the downstream of the *bph-sal* element. The former three cosmids (pKF6500, pKF6337, and pKF6246) are derived from *P. putida* KF715, and pKF6465 is derived from *P. putida* AC30Bph$^+$. These cosmids were digested by *Eco*RI, and some fragments were further digested by *Sal*I and *Xho*I. The restriction sites are shown, where E, S, and X indicate *Eco*RI, *Sal*I, and *Xho*I, respectively. The DNA fragments used as the probes are indicated by heavy bars (TNF-I to IX). The estimated sizes and the positions of the *bph-sal* element and the deletion regions of KF715M1 and KF715M2 are indicated by arrows. Lanes: 1, KF715; 2, KF715M1; 3, KF715M2; 4, AC30Rif$^r$Trp; 5, AC30Bph$^+$.

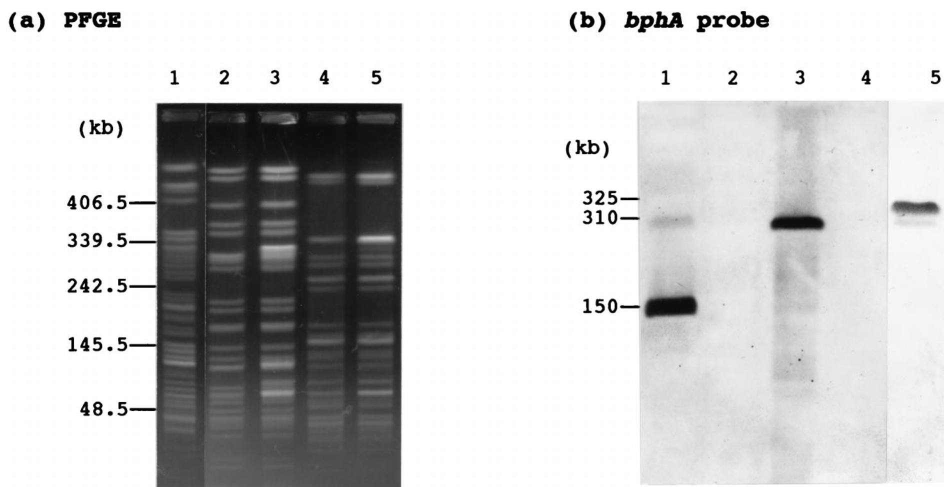


FIG. 4. PFGE of the genomic DNA of *P. putida* KF715, *P. putida* AC30Bph$^+$, and *P. putida* KT2440Bph$^+$ (a) and the subsequent Southern blot analysis of the *bph-sal* element with the *bphA1* probe (b). The genomic DNA was digested by *Spe*I. Lanes: 1, KF715; 2, AC30Rif$^r$Trp; 3, AC30Bph$^+$; 4, KT2440Sm$^r$Met; 5, KT2440Bph$^+$. Molecular size markers are shown on the left.

ized with the same size of DNA from both KF715M1 and KF715M2 (Fig. 3c). The downstream 15-kb *Eco*RI DNA (TNF-III) fragment adjacent to TNF-II failed to hybridize with both KF715M1 and KF715M2 DNA (data not shown). These results indicate that the upstream deletion end of these two mutants lies just inside or just downstream of the 3′ end of the TNF-II DNA (Fig. 3). The downstream deletion site of KT715M1 was found within the 4.0-kb *Eco*RI fragment (TNF-VII) because this fragment was hybridized with the DNA fragment of a different size (8.8 kb) in KF715M1, while this probe hybridized with the same 4.0-kb DNA in KF715 and KF715M2 (Fig. 3e). The downstream deletion site of KF715M2 was estimated to lie just upstream of the 1.95-kb *Sal*I-*Xho*I fragment (TNF-V). The TNF-V probe hybridized with DNA fragments of the same size in KF715 and KF715M2 but hybridized differently in KF715M1 (Fig. 3d). The same probe hybridized with several other DNA fragments (Fig. 3d). Preliminary sequence data showed that the 1.95-kb TNF-V DNA contains an IS-like sequence similar to IS*5* (EMBL-DDBJ-GenBank database under accession no. AJ249209). We found that several copies of this IS-like sequence are present in KF715 and its derivatives (data not shown). This may be the reason why several bands appeared with the TNF-V probe. The upstream 4.6-kb DNA fragment (TNF-IV) adjacent to TNF-V DNA did not hybridize with the KF715M2 DNA (Table 2), indicating that the downstream deletion site lies just upstream of the 1.95-kb DNA. Based on these results, we estimate that ~70-kb DNA is deleted in KF715M1, which includes the *bphA* upstream region (30 kb), the entire *bphABCD* region (10 kb), the intervening region between the *bph* and *sal* genes (10 kb), and the 20-kb region including the *sal* genes (Fig. 3). On the other hand, an ~40-kb DNA region is deleted in KF715M2, which includes the *bphA* upstream region (30 kb) and the entire *bphABCD* region (10 kb). Furthermore, PFGE revealed that the *Spe*I digest profiles of the genomic DNA of KF715, KF715M1, and KF715M2 are different (Fig. 2a). This indicates that at least part of the genome of KF715 is highly prone to rearrangement, which may include deletion and recombination.

**Self-mobilization of the *bph* and *sal* genes.** From the evidence that *P. graminis* KF701 possesses a *bphABCD* gene cluster nearly identical to that of KF715 (14), a question arises regarding whether the *bph* gene cluster of KF715 can be transferred to other strains. *P. putida* KF715 cells were filter mated with *P. putida* AC30Rif$^r$Trp, and Bph$^+$ colonies were screened on a BSM agar plate containing rifampin and tryptophan in the presence of biphenyl as a sole source of carbon. As a result, Bph$^+$ AC30 transconjugants were obtained at a frequency of as high as $10^{-6}$ per recipient cells. No colony was obtained when only donor or recipient cells were spread on the same plates as the control. All Bph$^+$ Rif$^r$ colonies thus obtained grew on salicylate as well and were confirmed for the requirement of tryptophan. Shown in Fig. 4 are PFGE profiles of DNA fragments of one such Bph$^+$Rif$^r$Trp strain (AC30Bph$^+$). The *Spe*I digestion profile of the AC30Bph$^+$ genome was essentially the same as that of the AC30Rif$^r$Trp strain but was different from that of KF715. Genomic Southern analyses confirmed that AC30Bph$^+$ gained both the *bph* and *sal* genes. The two gene clusters were located on the same *Spe*I fragment since identical hybridization profiles were obtained when the *bphA* probe (Fig. 4b) and the *salA* probe (data not shown) were used, respectively. The *Spe*I DNA fragment which hybridized to the *bph-sal* DNAs was ca. 150 kb for KF715 and ca. 310 kb for AC30Bph$^+$ (Fig. 4b). These results indicate that both the *bph* and *sal* gene clusters were transferred into AC30Rif$^r$Trp. We named this conjugative chromosomal DNA segment the *bph-sal* element.

**Mobilization of the *bph-sal* element from AC30Bph$^+$.** The *bph-sal* genes of AC30Bph$^+$ were also highly prone to deletion. The deletion frequency of the *bph-sal* genes was comparable to that of KF715, but the deletion always took place in the region including both the *bph* and *sal* genes. This result is in contrast to the original KF715, in which two types of deletions, as seen in KF715M1 and KF715M2, occurred at almost the same frequency. The conjugative transfer of the Bph$^+$ and Sal$^+$ phenotypes from AC30Bph$^+$ was also observed with *P. putida* KT2440Sm$^r$Met as the recipient, at a frequency of as high as $2 \times 10^{-6}$ per recipient cell. Genomic Southern analyses of KT2440Bph$^+$ confirmed the presence of both the *bph* and *sal* genes at the *Spe*I 325-kb DNA (Fig. 4b). AC30Bph$^+$ was also mated with KF715M1 and KF715M2, respectively, resulting in the appearance of KF715M1Bph$^+$ and KF715M2Bph$^+$ at a frequency of $10^{-6}$ per recipients. The *Spe*I digests of genomic DNA from KF715M1Bph$^+$ and KF715M2Bph$^+$ exhibited

bands hybridizing at 250- and 320-kb DNA, respectively, when the *bph* probe was used (data not shown).

**Size of the *bph-sal* element.** We attempted to determine the size of the *bph-sal* element by using the cosmids obtained from KF715 and AC30Bph$^+$. We constructed the physical map of this element with the restriction enzyme *Eco*RI (Fig. 3). The 4.0-kb *Eco*RI fragment (TNF-I) in a cosmid pKF6500 which is located at the 5′ region far upstream from the *bph* genes hybridized, with the same size, with the KF715 chromosome and with those of the two deletion mutants KF715M1 and KF715M2 as well (Fig. 3b). However, the same probe hybridized with the 5.2-kb DNA of AC30Bph$^+$. These results indicate that the 4.0-kb DNA (TNF-I DNA) contains the upstream end of the *bph-sal* element and that the two deletion mutants retain the upstream end of the *bph-sal* element. On the other hand, cosmid pKF6465 which is from AC30Bph$^+$ and contains the *salC* gene was used to determine the downstream end of the *bph-sal* element (Fig. 3f). The 3.7-kb *Eco*RI fragment (TNF-IX) which is located far downstream of the *sal* genes and includes the vector-borne *Eco*RI site was strongly hybridized with the chromosomes of AC30Rif$^r$Trp and AC30Bph$^+$ at the same 10.0-kb DNA and hybridized with the ~20-kb DNA of the KF715 chromosome to a lesser extent. The reason why the TNF-IV hybridized with the KF715 and the derivatives has yet to be elucidated, but the *bph-sal* element may be inserted within the region conserved between KF715 and AC30, such as tRNA structural genes as seen in *clc* element (28). The *Sal*I 3.0-kb DNA fragment (TNF-VIII) upstream from TNF-IX DNA hybridized with both KF715 and AC30Bph$^+$ DNA of the same size (Table 2). These results indicate that the 3′ end of the *bph-sal* element is located just upstream of the TNF-IX DNA fragment in pKF6465. Thus, the hybridization results allow us to assume that the size of the *bph-sal* element would be ca. 90 kb, which includes 40 kb upstream from the *bph* genes, a 10-kb *bph* gene cluster, a 10-kb intervening region between *bph* and *sal*, and the 30-kb region including the *sal* genes (Fig. 3a).

## DISCUSSION

In previous studies (16, 35), we reported that the *bphX* region (ca. 3.5 kb conferring the conversion of 2-hydroxypenta-2,4-dienoic acid to acetyl-coenzyme A) is lacking in the KF715 *bph* gene cluster. In a sequence comparison with the well-characterized *bph* genes of *P. pseudoalcaligenes* KF707 (35) and *B. cepacia* LB400 (17), only one nucleotide (nucleotide A) of the start codon of *bphX0* and the 3′-terminal 106 nucleotides of *bphX3* remained, suggesting that KF715 used to have the entire *bphABCXD* genes as in the case of KF707 and LB400. In the present study, we have also demonstrated that two chromosomal regions, including the *bph* and/or *sal* gene clusters, are highly prone to deletion. The KF715 *bph* and *sal* gene clusters are separated by 10-kb DNA. Deletion occurs at the region that includes both the *bph* and *sal* genes (ca. 70 kb) or at the region that includes only the *bph* genes (ca. 40 kb). The upper deletion sites seem to be identical. No deletion was observed at the lower region containing only the *sal* region, and strain KF715M2, which retained only the *sal* genes, was stable. In contrast to KF715, other biphenyl-utilizing strains, such as *P. pseudoalcaligenes* KF707 and *P. graminis* KF701, maintain the *bph* genes stably. The genome of *P. putida* KF715 seems to be highly rearranged, not only at the *bph-sal* regions but at the entire genome, because the restriction profiles by *Spe*I digestion and following PFGE were significantly different between KF715, KF715M1, and KF715M2 (Fig. 2a). Instability of the *bph-sal* element may be one of such rearrangement events in strain KF715, where insertional elements could be highly involved, as shown in *Yersinia pestis*. A 102-kb unstable region of *Y. pestis* comprising a so-called high-pathogenicity island undergoes internal rearrangement, which is a consequence of the presence of numerous insertional elements in the chromosome (5).

The *bph-sal* element behaves like a conjugative transposon. Conjugative drug-resistant transposons were first discovered from gram-positive *Enterococcus faecalis* (9, 15) and *Streptococcus pneumoniae* (2, 32) and, later, from gram-negative *Bacteroides* spp., whose conjugative transposons range in size from 65 to 150 kb (4, 25). Most of them carry tetracycline resistance genes. These conjugative transposons can excise themselves from the genome in which they are integrated, transfer themselves by conjugation into a recipient cell, and integrate into the recipient's genome (4, 6, 30, 31). Recently, two conjugative catabolic transposons were also reported (24, 27). The *clc* gene cluster of *Pseudomonas* sp. strain B13 conferring 3-chlorobenzoate degradation (7, 10) is present on the B13 chromosome as a 105-kb mobile genetic element (*clc* element) (27, 28). This element is self-transmissible and integrates into the chromosomes of various bacterial recipients, with glycine tRNA structural gene (*glyV*) as the integration site (28). It has not been clear whether the insertion site of the *bph-sal* element is specific as seen in the *clc* element. However, the fact that the 4.0-kb TNF-I covering the upstream end of the *bph-sal* element (Fig. 3b) hybridized with ca. The 10-kb fragment of AC30 DNA (Fig. 3b) and that the 10-kb TNF-IX fragment of AC30 downstream of the *bph-sal* element (Fig. 3f) hybridized with ca. 20-kb fragment of KF715 DNA suggest that there may exist a hot spot of insertion, such as the conserved tRNA structural genes. The *bph-sal* element can be transferred from the AC30Bph$^+$ transconjugant to another *P. putida* KT2440. This implies that all factors necessary for mobilization are located on the 90-kb *bph-sal* element. The DNA sequencing of this element is currently under way to find the genes involved in the transfer and thereby to reveal the mechanism of conjugal transfer. Occurrence of the mobile *bph* elements may explain why biphenyl-utilizing bacteria are widely distributed and possess *bph* genes that are very similar, if not identical, to one another.

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## REFERENCES

1. **Abramowicz, D. A.** 1990. Aerobic and anaerobic biodegradation of PCBs: a review. Crit. Rev. Biotechnol. **10:**241–251.
2. **Ayoubi, P., A. Kilic, and M. Vijayakumar.** 1991. Tn*5253*, the pneumococcal omega (*cat tet*) BM6001 element, is a composite structure of two conjugative transposons, Tn*5251* and Tn*5252*. J. Bacteriol. **173:**1617–1622.
3. **Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51:**761–768.
4. **Bedzyk, L. A., N. B. Shoemaker, K. E. Young, and A. A. Salyers.** 1992. Insertion and excision of *Bacteroides* conjugative chromosomal elements. J. Bacteriol. **174:**166–172.
5. **Buchrieser, C., M. Prentice, and E. Carniel.** 1998. The 102-kilobase unstable region of *Yersinia pestis* comprises a high-pathogenicity island linked to a pigmentation segment which undergoes internal rearrangement. J. Bacteriol. **180:**2321–2329.
6. **Clewell, D. B., and S. E. Flannagan.** 1993. The conjugative transposons of gram-positive bacteria, p. 369–393. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.
7. **Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss.** 1974. Isolation and

characterization of a 3-chlorobenzoate degrading pseudomonad. Arch. Microbiol. **99:**61–70.
8. **Erickson, B. D., and F. J. Mondello.** 1992. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated biphenyl-degrading enzyme in *Pseudomonas* strain LB400. J. Bacteriol. **174:**2903–2912.
9. **Franke, A. E., and D. B. Clewell.** 1981. Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145:**494–502.
10. **Frantz, B., and A. M. Chakrabarty.** 1987. Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation. Proc. Natl. Acad. Sci. USA **84:**4460–4464.
11. **Furukawa, K.** 1982. Microbial degradation of polychlorinated biphenyls, p. 33–57. *In* A. M. Chakrabarty (ed.), Biodegradation and detoxification of environmental pollutants. CRC Press, Inc., Boca Raton, Fla.
12. **Furukawa, K.** 1994. Molecular genetics and evolutionary relationship of PCB-degrading bacteria. Biodegradation **5:**289–300.
13. **Furukawa, K., N. Hayase, K. Taira, and N. Tomizuka.** 1989. Molecular relationship of chromosomal genes encoding biphenyl/polychlorinated biphenyl catabolism: some soil bacteria possess a highly conserved *bph* operon. J. Bacteriol. **171:**5467–5472.
14. **Furukawa, K., and T. Miyazaki.** 1986. Cloning of gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **166:**392–398.
15. **Gawron-Burke, C., and D. B. Clewell.** 1982. A transposon in *Streptococcus faecalis* with fertility properties. Nature **300:**281–284.
16. **Hayase, N., K. Taira, and K. Furukawa.** 1990. *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. J. Bacteriol. **172:**1160–1164.
17. **Hofer, B., S. Backhaus, and K. N. Timmis.** 1994. The biphenyl/polychlorinated biphenyl-degradation locus (*bph*) of *Pseudomonas* sp. LB400 encodes four additional metabolic enzymes. Gene **144:**9–16.
18. **Hooper, S. W., T. C. Dockendorff, and G. S. Sayler.** 1989. Characteristics and restriction analysis of the 4-chlorinated biphenyl/polychlorinated biphenyl catabolic plasmid pSS50. Appl. Environ. Microbiol. **55:**1286–1288.
19. **Kimura, N., A. Nishi, A. Goto, and K. Furukawa.** 1997. Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. J. Bacteriol. **179:**3936–3943.
20. **Lee, J., K. R. Min, Y.-C. Kim, C.-K. Kim, J.-Y. Lim, H. Hoon, K.-H. Min, K.-S. Lee, and Y. Kim.** 1995. Cloning of salicylate hydroxylase gene and catechol 2,3-dioxygenase gene and sequencing of an intergenic sequence between the two genes of *Pseudomonas putida* KF715. Biochem. Biophys. Res. Commun. **211:**382–388.
21. **Lee, J., J. Oh, K. R. Min, and Y. Kim.** 1996. Nucleotide sequence of salicylate hydroxylase genes and its 5′-flanking region of *Pseudomonas putida* KF715. Biochem. Biophys. Res. Commun. **218:**544–548.
22. **Lloyd-Jones, G., C. De Jong, R. C. Ogden, W. A. Duetz, and P. A. Williams.** 1994. Recombination of the *bph* (biphenyl) genes from pWW100 and their deletion during growth on benzoate. Appl. Environ. Microbiol. **60:**691–696.
23. **Merlin, C., D. Springael, M. Mergeay, and A. Toussaint.** 1997. Organization of the *bph* gene cluster of transposon Tn*4371*, encoding enzymes for the degradation of biphenyl and 4-chlorobiphenyl compounds. Mol. Gen. Genet. **253:**499–506.
24. **Merlin, C., D. Springael, and A. Toussaint.** 1999. Tn*4371*: a modular structure encoding a phage-like integrase, a *Pseudomonas*-like catabolic pathway, and RP4-like transfer function. Plasmid **41:**40–54.
25. **Nikolich, M. P., N. B. Shoemaker, and A. A. Salyers.** 1994. Characterization of a new type of *Bacteroides* conjugative transposon, Tc$^r$Em$^r$ 7853. J. Bacteriol. **176:**6606–6612.
26. **Pflugmacher, U., B. Averhoff, and G. Gottschalk.** 1996. Cloning, sequencing, and expression of isopropylbenzene degradation genes from *Pseudomonas* sp. strain JR1: identification of isopropylbenzene dioxygenase that mediates trichloroethene oxidation. Appl. Environ. Microbiol. **62:**3967–3977.
27. **Ravatn, R., S. Studer, D. Springael, A. J. B. Zehnder, and J. R. van der Meer.** 1998. Chromosomal integration, tandem amplification, and deamplification in *Pseudomonas putida* F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from *Pseudomonas* sp. strain B13. J. Bacteriol. **180:**4360–4369.
28. **Ravatn, R., S. Studer, A. J. B. Zehnder, and J. R. van der Meer.** 1998. Int-B13, an unusual site-specific recombinase of the bacteriophage P4 integrase family, is responsible for chromosomal insertion of the 105-kilobase *clc* element of *Pseudomonas* sp. strain B13. J. Bacteriol. **180:**5505–5514.
29. **Salyers, A. A., N. B. Shoemaker, A. M. Stevens, and L.-Y. Li.** 1995. Conjugative transposons: an unusual and divers set of integrated gene transfer elements. Microbiol. Rev. **59:**579–590.
30. **Scott, J. R.** 1992. Sex and the single circle: conjugative transposon. J. Bacteriol. **174:**6005–6010.
31. **Shoemaker, N. B., and A. A. Salyer.** 1990. A cryptic 65-kilobase-pair transposonlike element isolated from *Bacteroides unifornis* has homology with *Bacteroides* conjugal tetracycline resistance elements. J. Bacteriol. **172:**1694–1702.
32. **Shoemaker, N. B., M. D. Smith, and W. R. Guild.** 1980. DNase resistant transfer of chromosomal *cat* and *tet* insertions by filter mating in pneumococcus. Plasmid **3:**80–87.
33. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
34. **Springael, D., S. Kreps, and M. Mergeay.** 1993. Identification of a catabolic transposon, Tn*4371*, carrying biphenyl and 4-chlorobiphenyl degradation genes in *Alcaligenes eutrophus* A5. J. Bacteriol. **175:**1674–1681.
35. **Taira, K., J. Hirose, S. Hayashida, and K. Furukawa.** 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. **267:**4844–4853.
36. **Tsuda, M., H.-M. Tan, A. Nishi, and K. Furukawa.** 1999. Mobile catabolic genes in bacteria. J. Biosci. Bioeng. **87:**401–410.
37. **Unterman, R.** 1996. A history of PCB biodegradation, p. 209–253. *In* R. L. Crawford and D. L. Crawford (ed.), Bioremediation: principles and applications. Cambridge University Press, New York, N.Y.
38. **Wahl, G. M., K. A. Lewis, J. C. Ruiz, B. Rotherberg, J. Zhao, and G. A. Evans.** 1987. Cosmid vectors for rapid genomic walking, restriction mapping, and gene transfer. Proc. Natl. Acad. Sci. USA **84:**2160–2164.
39. **Yamada, A., H. Kishi, K. Sugiyama, T. Hatta, K. Nakamura, E. Masai, and M. Fukuda.** 1998. Two nearly identical aromatic compound hydrolase genes in a strong polychlorinated biphenyl degrader, *Rhodococces* sp. strain RHA1. Appl. Environ. Microbiol. **64:**2006–2012.
40. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.