Analysis of transcription of the *bph* locus of *Burkholderia* sp. strain LB400 and evidence that the ORF0 gene product acts as a regulator of the *bphA1* promoter

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Although gene clusters for the degradation of biphenyls and polychlorobiphenyls have been extensively characterized, comparatively little is known about the regulation of their expression. In the present work, different aspects of transcription of the *bph* locus of the potent polychlorobiphenyl degrader *Burkholderia* sp. strain LB400 were investigated. An RNA blot analysis of the entire gene cluster revealed that the transcription of all genes encoding biphenyl catabolic enzymes responded similarly to the presence of biphenyl, succinate or a mixture of the two. One region of the locus, encompassing ORF0, was separately transcribed and differently regulated. A single start position was mapped for this monocistronic transcript. Synthesis of the adjacent RNA, encoding subunits of biphenyl dioxygenase, was strongly biphenyl-inducible. In this case, four major 5'-ends were mapped between 25 and 70 bp upstream of the start codon of gene *bphA1*. Sequence elements between approximately positions 710 and 1080 upstream were required *in cis* for full functioning of the respective promoter(s) (P_{bphA1}). ORF0⁻ mutants of strain LB400 retained the ability to grow on biphenyl, but showed decreased concentrations of *bphA1A2* RNA and decreased *lacZ* expression in strains harbouring a reporter system with a *bphA1-lacZ* transcriptional fusion. This effect was compensated by the introduction of an intact ORF0 *in trans*, indicating that the ORF0 gene product mediates activation of P_{bphA1} .

Keywords: aerobic bacteria, biphenyl catabolism, *bph* genes, transcriptional regulation

INTRODUCTION

The *bph* locus of *Burkholderia* sp. strain LB400 encodes a pathway with the potential to break down a wide range of polychlorobiphenyls. The biphenyl dioxygenase of this catabolic route is able to attack a relatively broad spectrum of congeners including hexachlorobiphenyls (Bopp, 1986; Nadim *et al.*, 1987; Haddock *et al.*, 1995; Seeger *et al.*, 1995, 1999), and many of the dioxygenation products are further catabolized (Bedard & Haberl, 1990; Seeger *et al.*, 1995, 1999).

The *bph* gene cluster has been extensively characterized

(Erickson & Mondello, 1992; Hofer *et al.*, 1993, 1994). The locus contains ten cistrons encoding enzymes for the degradation of biphenyls to benzoates, pyruvates and acetyl-CoA. It further harbours a glutathione transferase gene (*bphK*) and two ORFs, ORF0 and ORF1, of unknown function. This and similar gene clusters are frequently termed operons, but in most cases information on their transcription is scarce, and the formation of a single polycistronic mRNA is merely an assumption. One transcriptional study of the *bph* locus of strain LB400 identified three RNA 5'-ends in the 5'-terminal region of the gene cluster (Erickson & Mondello, 1992). On the basis of these data, the existence of three promoters (p1, p2 and p3) was postulated. p1 and p2, located directly upstream of the gene encoding the large subunit of biphenyl dioxygenase (*bphA1*), were reported to direct constitutive transcrip-

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Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Reference or source
<i>E. coli</i>		
INVαF'	F' <i>endA1 recA1 hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 thi-1 gyrA96 relA1 φ80 lacZΔM15 Δ(lacZYA-argF)U169</i>	Invitrogen
CC118(<i>λpir</i>)	Δ(<i>ara-leu</i>) <i>araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE</i> (Am) <i>recA1 Tn7 λpir</i>	Herrero <i>et al.</i> (1990)
HB101	Sm ^r ; <i>recA thi pro leu hsdR</i> ⁻ M ⁺	Boyer & Roulland-Dussoix (1969)
BL21(DE3)[pLysS]	Cm ^r ; BL21 derivative modified for the transcription of heterologous genes by the phage T7 RNA polymerase	Studier (1991)
<i>Burkholderia</i> sp.		
LB400	Prototrophic, biphenyl degrader	Bopp (1986)
LB400ORF0FS	LB400 derivative with a 4 bp insertion in ORF0 of its <i>bph</i> locus	This study
LB400[Tn <i>lacZ</i>]	Km ^r ; LB400 derivative carrying a reporter transposon derived by insertion of <i>trp'</i> - <i>lacZ</i> of pUJ8 into pUTminiTn5 <i>Km</i>	This study
LB400[Tn120]	Km ^r ; LB400 derivative carrying a <i>lacZ</i> reporter transposon containing bp 364–1605 of the LB400 <i>bph</i> locus	This study
LB400[Tn <i>EE28</i>]	Km ^r ; LB400 derivative carrying a <i>lacZ</i> reporter transposon containing bp 1–2855 of the LB400 <i>bph</i> locus	This study
LB400[Tn <i>Bg2000</i>]	Km ^r ; LB400 derivative carrying a <i>lacZ</i> reporter transposon containing bp 785–2839 of the LB400 <i>bph</i> locus	This study
LB400[Tn <i>FS24</i>]	Km ^r ; LB400 derivative carrying a <i>lacZ</i> reporter transposon containing bp 90–2437 of the LB400ORF0FS <i>bph</i> locus	This study
LB400[Tn <i>PC55</i>]	Km ^r ; LB400 derivative carrying a <i>lacZ</i> reporter transposon containing bp 90–664 of the LB400 <i>bph</i> locus	This study
LB400[Tn <i>PI29</i>]	Km ^r ; LB400 derivative carrying a <i>lacZ</i> reporter transposon containing bp 1139–1431 of the LB400 <i>bph</i> locus	This study
LB400[Tn <i>PI45</i>]	Km ^r ; LB400 derivative carrying a <i>lacZ</i> reporter transposon containing bp 1139–1605 of the LB400 <i>bph</i> locus	This study
LB400[Tn <i>PI75</i>]	Km ^r ; LB400 derivative carrying a <i>lacZ</i> reporter transposon containing bp 724–1431 of the LB400 <i>bph</i> locus	This study
LB400[Tn <i>PI87</i>]	Km ^r ; LB400 derivative carrying a <i>lacZ</i> reporter transposon containing bp 724–1605 of the LB400 <i>bph</i> locus	This study
LB400ORF0FS [Tn <i>EE28</i>]	Km ^r ; LB400ORF0FS derivative carrying a <i>lacZ</i> reporter transposon containing bp 1–2855 of the LB400 <i>bph</i> locus	This study
LB400ORF0FS [Tn <i>FS24</i>]	Km ^r ; LB400ORF0FS derivative carrying a <i>lacZ</i> reporter transposon containing bp 90–2437 of the LB400ORF0FS <i>bph</i> locus	This study
Plasmids		
pCR2.1	Ap ^r Km ^r ; high-copy-number vector for overhang cloning and expression of PCR products	Invitrogen
pUC19	Ap ^r ; high-copy-number cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pUJ8	Ap ^r ; multicopy promoter probe vector to generate fusions with a promoterless <i>lacZ</i> gene	de Lorenzo <i>et al.</i> (1990)
pUTminiTn5 <i>Km</i>	Ap ^r Km ^r ; <i>ori</i> of R6K; mobilizable suicide plasmid carrying a Tn5 derivative conferring Km ^r	de Lorenzo <i>et al.</i> (1990)
pK18 <i>mobsacB</i>	Km ^r ; <i>ori</i> of R6K, <i>oriT</i> of RP4, mobilizable suicide plasmid carrying the <i>sacB</i> gene of <i>Bacillus subtilis</i>	Schäfer <i>et al.</i> (1994)
pRK600	Cm ^r ; <i>ori</i> of ColE1; RK2-Mob ⁺ ; RK2-Tra ⁺ ; conjugational helper plasmid	Ditta <i>et al.</i> (1980)
pDD5301	pUC18 derivative containing the <i>bph</i> locus of strain LB400	Dowling <i>et al.</i> (1993)
pUTTn <i>lacZ</i>	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing the <i>trp'</i> - <i>lacZ</i> fusion of pUJ8 inserted in the <i>NotI</i> restriction site of plasmid pUTminiTn5 <i>Km</i>	This study
pCR120	Ap ^r Km ^r ; pCR2.1 derivative containing bp 364–1605 of the LB400 <i>bph</i> locus	This study
pUJ120	Ap ^r ; pUJ8 derivative containing a transcriptional fusion between bp 364–1605 of the LB400 <i>bph</i> locus and <i>trp'</i> - <i>lacZ</i>	This study
pUTTn120	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing a transcriptional fusion between bp 364–1605 of the LB400 <i>bph</i> locus and <i>trp'</i> - <i>lacZ</i>	This study

Table 1 (cont.)

Strain or plasmid	Relevant genotype or characteristics	Reference or source
pUC120	Ap ^r ; pUC19 derivative carrying bp 364–1605 of the LB400 <i>bph</i> locus	This study
pUC120FS	Ap ^r ; pUC120 derivative with a 4 bp insertion in ORF0 of the LB400 <i>bph</i> locus	This study
pK18120FS	Km ^r ; pK18 <i>mobsacB</i> derivative carrying bp 364–1605 of the LB400 <i>bph</i> locus with a 4 bp insertion in ORF0 (positions 785a–d)	This study
pUJEE28	Ap ^r ; pUJ8 derivative containing a transcriptional fusion between bp 1–2855 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pUTTnEE28	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing a transcriptional fusion between bp 1–2855 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pUJBg2000	Ap ^r ; pUJ8 derivative containing a transcriptional fusion between bp 785–2839 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pUTTnBg2000	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing a transcriptional fusion between bp 785–2839 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pCRFS24	Ap ^r Km ^r ; pCR2.1 derivative containing bp 90–2437 of the LB400ORF0FS <i>bph</i> locus	This study
pUJFS24	Ap ^r ; pUJ8 derivative containing a transcriptional fusion between bp 90–2437 of the LB400ORF0FS <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pUTTnFS24	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing a transcriptional fusion between bp 90–2437 of the LB400ORF0FS <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pCRPC55	Ap ^r Km ^r ; pCR2.1 derivative containing bp 90–664 of the LB400 <i>bph</i> locus	This study
pUJPC55	Ap ^r ; pUJ8 derivative containing a transcriptional fusion between bp 90–664 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pUTTnPC55	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing a transcriptional fusion between bp 90–664 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pCRPI29	Ap ^r Km ^r ; pCR2.1 derivative containing bp 1139–1431 of the LB400 <i>bph</i> locus	This study
pUJPI29	Ap ^r ; pUJ8 derivative containing a transcriptional fusion between bp 1139–1431 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pUTTnPI29	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing a transcriptional fusion between bp 1139–1431 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pCRPI45	Ap ^r Km ^r ; pCR2.1 derivative containing bp 1139–1605 of the LB400 <i>bph</i> locus	This study
pUJPI45	Ap ^r ; pUJ8 derivative containing a transcriptional fusion between bp 1139–1605 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pUTTnPI45	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing a transcriptional fusion between bp 1139–1605 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pCRPI75	Ap ^r Km ^r ; pCR2.1 derivative containing bp 724–1431 of the LB400 <i>bph</i> locus	This study
pUJPI75	Ap ^r ; pUJ8 derivative containing a transcriptional fusion between bp 724–1431 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pUTTnPI75	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing a transcriptional fusion between bp 724–1431 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pCRPI87	Ap ^r Km ^r ; pCR2.1 derivative containing bp 724–1605 of the LB400 <i>bph</i> locus	This study
pUJPI87	Ap ^r ; pUJ8 derivative containing a transcriptional fusion between bp 724–1605 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study
pUTTnPI87	Ap ^r Km ^r ; pUTminiTn5 <i>Km</i> derivative containing a transcriptional fusion between bp 724–1605 of the LB400 <i>bph</i> locus and <i>trp'–lacZ</i>	This study

tion, whereas p3, located upstream of ORF0, was described to be activated in the presence of biphenyl. In contrast to these findings, Furukawa and coworkers (Taira *et al.*, 1992) reported the mapping of a single RNA start site within the 3'-terminal region of ORF0 of the highly similar *bph* locus of *Pseudomonas pseudoalcaligenes* KF707. However, no positive proof for functioning of any of the deduced promoters has been given. A two-component system regulating biphenyl catabolism in the Gram-positive strain *Rhodococcus* sp. M5 and a GntR-like transcriptional repressor of the *bph* gene cluster located on transposon Tn4371 have recently been identified (Labbé *et al.*, 1997; Mouz *et al.*, 1999), but data on the transcription of the *bph* gene cluster of strain LB400 remain scarce.

In this report we describe a Northern blot analysis of transcription of the entire *bph* locus, an exact mapping of RNA 5'-ends in the 5'-terminal region by primer extension analysis, and the demonstration of promoter function using a mono-copy promoter-probe system in strain LB400. Furthermore, we investigated the expression of ORF0 and its involvement in the regulation of transcription from the promoter upstream of *bphA1*, the first gene of the locus encoding a polypeptide of a biphenyl catabolic enzyme.

METHODS

Bacterial strains, plasmids, media and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *Burkholderia* strains were grown at a temperature of 30 °C in L medium (Bopp *et al.*, 1983) or M9 minimal medium (Sambrook *et al.*, 1989) supplemented with trace elements (Bauchop & Elsdén, 1969) and either succinate (20 mM) or biphenyl (5 mM nominal concentration), or both, as carbon sources. *Escherichia coli* strains were grown at 37 °C on LB (Sambrook *et al.*, 1989). Media were supplemented with ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹) or chloramphenicol (10 µg ml⁻¹) when required.

Constructions of plasmids and strains. Recombinant DNA techniques were performed according to standard methods (Sambrook *et al.*, 1989). Plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen) and sequenced using a *Taq* Dyedexy terminator cycle sequencing kit and an automatic DNA sequencer model 373A (Applied Biosystems), according to the manufacturer's instructions. Oligonucleotides (Table 2) were synthesized by GIBCO. For cloning, *E. coli* strain INVαF' was used, unless otherwise indicated. For conjugal transfers *E. coli* CC118[λpir] was used as donor and *E. coli* HB101(pRK600) as helper strain (Young & Poulis, 1978). In the newly constructed plasmids, the orientation of the cloned genes was the same as the orientation of the *lacZ* gene (for pCR2.1, pUC19, pK18*mobsacB* and pUJ8 derivatives) or of the Km^r gene (for pUTminiTn5*Km* derivatives). In detail, plasmids and strains were constructed as follows.

LB400ORF0FS. The 1.2 kb *EcoRI* fragment of pCR120 (construction described below) containing ORF0 was cloned in *EcoRI*-digested pUC19, yielding pUC120. Linearization of pUC120 with *Bgl*II, filling-in of the ends, and religation generated pUC120FS. The *EcoRI* fragment of pUC120FS was then cloned into the *EcoRI* site of pK18*mobsacB* (Schäfer *et al.*, 1994), yielding plasmid pK18120FS. The wild-type copy of

ORF0 in strain LB400 was replaced by homologous recombination after transfer of this suicide plasmid by a triparental mating (Young & Poulis, 1978). Km^r colonies able to utilize glutamate were selected. Km^s double-crossover recombinants were selected after addition of 10% sucrose to agar plates as previously described (Schäfer *et al.*, 1994). Successful exchange of ORF0 was verified by PCR amplification with primers CIOP1R and CIOP4RBIS (which anneal to regions external to the cloned fragment) followed by incubation with *Bgl*II. For further analysis, the PCR fragment of a *Bgl*II⁻ clone was sequenced with primers CIOP1R and CIOP3. Additionally, a 2.35 kb PCR fragment encoding ORF0 and part of *bphA1* was amplified from LB400ORF0FS genomic DNA by use of primers CIOP0 and CIOP5 and cloned in pCR2.1, generating pCRFS24. The integrity of the insert was verified by sequencing. The lack of a full-length ORF0 protein was verified by SDS-PAGE analysis of ³⁵S-labelled proteins of *E. coli* BL21[DE3](pLysS, pCRFS24) (data not shown).

LB400[TnEE28] and LB400ORF0FS[TnEE28]. (See also Fig. 1 and Table 4.) The 2.8 kb *EcoRI* fragment containing the ORF0 and *bphA1* genes of strain LB400 was excised from pAIA1 (B. Hofer & S. Backhaus, unpublished) and inserted into the *EcoRI* site upstream of the promoterless *trp-lacZ* fusion in plasmid pUJ8, yielding pUJEE28. This transcriptional fusion was excised as a 6.9 kb *NotI* fragment and cloned downstream of a transcriptional terminator into the respective site of pUTminiTn5*Km*, generating pUTTnEE28. This suicide plasmid was used for monocopy insertion of the reporter gene fusion into the genomes of strains LB400 and LB400ORF0FS as described by de Lorenzo *et al.* (1990). Km^r colonies able to utilize glutamate were selected.

LB400[TnBg2000]. (See also Fig. 1 and Table 4.) A 2.05 kb *bph* fragment was excised from pUJEE28 with *Bgl*II, filled in and inserted into the *SmaI* site of pUJ8, generating pUJBg2000. This transcriptional fusion was then subcloned, yielding pUTTnBg2000, and finally integrated into strain LB400, as described above.

LB400[Tn120], -[TnPC55], -[TnPI87], -[TnPI45], -[TnPI75] and -[TnPI29]. (See also Fig. 1 and Table 4.) The promoter fragments 120, PC55, PI87, PI45, PI75 and PI29 were generated by PCR using as template pDD5301 and as primer pairs CIOP1R and CIOP4R, CIOP0 and CIOPPC, CIOP2 and CIOP4R, CIOP3R and CIOP4R, CIOP2 and CIOP10, or CIOP3R and CIOP10, respectively. The amplification products were cloned in pCR2.1 using the TA cloning Kit (Invitrogen) according to the manufacturer's instructions. The resulting plasmids were pCR120, pCRPC55, pCRPI87, pCRPI45, pCRPI75 and pCRPI29. The integrity of the inserts was verified by sequencing. The amplification products were excised from the respective pCR plasmids as *EcoRI* fragments, subcloned and integrated into strain LB400 as described above. The resulting subclones were pUJ120, pUJPC55, pUJPI87, pUJPI45, pUJPI75 and pUJPI29 in the pUJ series and pUTTn120, pUTTnPC55, pUTTnPI87, pUTTnPI45, pUTTnPI75 and pUTTnPI29 in the pUTTn series.

LB400[TnFS24] and LB400ORF0FS[TnFS24]. (See also Fig. 1 and Table 5.) The 2.4 kb *bph* segment of pCRFS24 (described above) was excised with *EcoRI*, subcloned as above, yielding pUJFS24 and pUTTnFS24, and finally integrated into the genomes of the two recipients.

LB400[TnlacZ]. The 4.3 kb *NotI* fragment of pUJ8 was cloned into *NotI*-digested pUTminiTn5*Km*, yielding pUTTn*lacZ*. The mini-transposon was then integrated into the genome of LB400 as described above.

Table 2. Oligonucleotides used in this study

Name	Sequence and position in the <i>bph</i> locus*
CIOP0	90 5'-CGATCTGCGGCTGGACGGGCTCAT-3' 113
CIOP1	388 5'-AGATTCGTCGCTGTCGTGCACCTC-3' 365
CIOP1R	365 5'-GAGGTGCACGACAGCGACGAATCT-3' 388
CIOP2	724 5'-GTTGCGGGCGGACATCGTGGAGGG-3' 747
CIOP3	1163 5'-AATACAGGATAGACAGGAACCGCT-3' 1140
CIOP3R	1140 5'-AGCGGTTCCCTGTCTATCCTGTATT-3' 1163
CIOP4	1583 5'-GAGCTTGAGCGGGTTTTTGGTC-3' 1604
CIOP4R	1604 5'-GACCAAAAACCCGCTCAAGCTCCA-3' 1581
CIOP4RBIS	1631 5'-TCTCGTGCCCAAGTAACAGCCAAG-3' 1608
CIOP5	2437 5'-CAGGAATGAACAGGTCGGGAAG-3' 2416
CIOP6	3592 5'-ACCTCATTGGCACGCTTCCCTTCA-3' 3615
CIOP7	3809 5'-CACTTCCGTTGCGATGACCACTGG-3' 3786
CIOP8	4246 5'-CGTTCGCACGGGCAAGGTCAAATC-3' 4269
CIOP9	5063 5'-ACCAAGACCACATCGGCGGGCACC-3' 5040
CIOP10	1431 5'-CGTCTCCGTTGAAATGCCCTTGGC-3' 1408
CIOP11	6049 5'-CACGATCTCCAATGCGGGCTTCTA-3' 6072
CIOP12	6417 5'-GAGAAGAATCCACGAACGCCCAAG-3' 6394
CIOP13	11039 5'-CAACGAGGCGGGTAACGGCGAAAC-3' 11062
CIOP14	11568 5'-AGTTCTTCAGGTGTTCCGGTTGGC-3' 11545
CIOP15	2702 5'-CACCTGATTTTCCCTGGCAACG-3' 2723
CIOP16	3251 5'-GGTCTCCGCCACGCCACGTCCG-3' 3228
CIOP17	6534 5'-TAGCTGCTTGGCGTTTCGTTTCTGA-3' 6557
CIOP18	7074 5'-GTGTGGTGGCGTTTCGTTGCAGTGC-3' 7051
CIOP19	7885 5'-ATCTGTTTCGTCGTGCTCGGTTGGA-3' 7908
CIOP20	8102 5'-CTCATTCAGGTCGGGGCGTTCAT-3' 8079
CIOP21	8215 5'-GAGGACGCCTACCACATTCAGCAA-3' 8238
CIOP22	8560 5'-TCTTGATTTTCCAGTCGCCGATGC-3' 8537
CIOP23	9190 5'-CTCGGCGCCCTGATGATAGACCTG-3' 9213
CIOP24	9786 5'-TTCCTCGGCAAACATCTCGGCGGT-3' 9763
CIOP25	10207 5'-GTCGGAGCAGCACATCACCCAATC-3' 10230
CIOP26	10707 5'-GCATAGCCCAGCGTGAGCGAGTCG-3' 10684
CIOPPC	664 5'-CGCAAATGACTTGTCGCCTCCCAT-3' 641

* Numbers indicate the position of the terminal nucleotides in the *bph* locus of strain LB400 (Erickson & Mondello, 1992; Hofer *et al.*, 1993, 1994). Numbering of the gene cluster starts at the first base pair of the 5'-terminal *Eco*RI site.

Northern blot analysis. Strain LB400 and its derivatives were grown on biphenyl or succinate as described above. At OD₆₀₀ 0.5, total RNA was extracted with the RNeasy Midi Kit (Qiagen) according to the supplier's instructions. Aliquots of 30 µg of RNA (determined photometrically) were denatured at 100 °C in the presence of formaldehyde (2 M) and 50% formamide, separated on 1% agarose/10% formaldehyde gels (Sambrook *et al.*, 1989), and blotted onto a Bydine B Transfer Membrane 0.45 µm (PALL) (Sambrook *et al.*, 1989). Probes were generated by PCR using pDD5301 as template and the primer pairs given in Table 3. Digoxigenin-labelled nucleotides were incorporated into gel-purified probes using the Random Primed DNA Labelling Kit Dig (Boehringer Mannheim) according to the manufacturer's instructions. Membranes were hybridized at 50 °C and signals were detected with anti-digoxigenin antibodies conjugated with alkaline phosphatase (Boehringer Mannheim) according to the supplier's instructions. RNA lengths were estimated using as reference the Dig-labelled RNA molecular weight marker I (Boehringer Mannheim).

Primer extension analysis. Strain LB400 was grown and total RNA was extracted as described under Northern blot analysis. Primers CIOPPC and CIOP4R (Table 2) (each 20 µg) were end-labelled by incubation with [γ -³²P]rATP and 1 U T4 polynucleotide kinase (Promega) at 37 °C for 40 min (Sambrook *et al.*, 1989). Then 10–20 µg of the labelled primers were hybridized with 25 µg RNA at 50 °C for 20 min and extended with 1 U AMV reverse transcriptase (Promega) at 42 °C for 40 min (Sambrook *et al.*, 1989). Reference sequence ladders were generated using the same primers with the Deaza G/A ³²Sequencing Mixes Kit (Pharmacia Biotech) in accordance with the supplier's instructions. Primer extension products and sequence ladders were run on sequencing gels (Sambrook *et al.*, 1989).

β-Galactosidase assays. Bacterial cultures were grown on succinate or biphenyl to an OD₆₀₀ of 0.5. Aliquots (500 µl) were permeabilized with chloroform and SDS and processed as described by Miller (1972).

Detection of proteins. ³⁵S-labelling of plasmid-encoded gene

Table 3. Probes used for detection of potential *bph* transcripts

Name	Primers used for synthesis	Positions of termini*	Length (bp)	Target gene
S1	CIOP0/CIOP1	90–388	299	none
S2	CIOP2/CIOP3	724–1163	440	ORF0
S3	CIOP4/CIOP5	1583–2437	855	<i>bphA1</i>
SA2	CIOP15/CIOP16	2702–3251	550	<i>bphA2</i>
S4	CIOP6/CIOP7	3592–3809	218	ORF1
S5	CIOP8/CIOP9	4246–5063	818	<i>bphA3/bphA4</i>
S6	CIOP11/CIOP12	6049–6417	369	<i>bphB</i>
S7	CIOP17/CIOP18	6534–7074	541	<i>bphC</i>
S8	CIOP19/CIOP20	7885–8102	218	<i>bphK</i>
S9	CIOP21/CIOP22	8215–8560	346	<i>bphH</i>
S10	CIOP23/CIOP24	9190–9786	597	<i>bphJ</i>
S11	CIOP25/CIOP26	10207–10707	501	<i>bphI</i>
S12	CIOP13/CIOP14	11039–11568	530	<i>bphD</i>

*Numbers indicate the position of the terminal nucleotides in the *bph* locus (Erickson & Mondello, 1992; Hofer *et al.*, 1993, 1994). Numbering of the gene cluster starts at the first base pair of the 5'-terminal *EcoRI* site.

products was carried out in *E. coli* BL21[DE3](pLysS) as previously described (Hofer *et al.*, 1993). Plasmids used were pCR120, pCRPI29 and pCRFS24. SDS-PAGE analysis has also been described (Hofer *et al.*, 1993).

Computational methods. Database searches and sequence alignments were performed with the BLASTN, BLASTP (Altschul *et al.*, 1990) and BESTFIT (Devereux *et al.*, 1984) programs.

RESULTS

Analysis of transcription of the *bph* locus by probe hybridization

Transcription of the entire *bph* locus was investigated by Northern analysis using RNA extracted from strain LB400 after growth in minimal medium containing succinate, biphenyl or a mixture of the two as sole carbon source(s). Thirteen double-stranded probes complementary to all genes or ORFs of the *bph* locus (Table 3) were generated by PCR. Only probe S1, which is complementary to the 5'-end of the locus upstream of ORF0 (Fig. 1), gave no signal, indicating that this DNA region is not expressed. The ORF0 probe S2 (Fig. 1) detected a transcript of about 1.35 kb (Fig. 2a) which was designated ORF0 RNA. Probes S3, SA2 and S4, containing parts of *bphA1*, *bphA2* and ORF1, respectively (Fig. 1), all detected the same RNA with a length of about 2.4 kb (Fig. 2b). This transcript is subsequently designated *bphA1A2* RNA. The other probes visualized a variety of relatively short RNAs. The majority of these transcripts were even shorter than the corresponding genes. This finding strongly suggests a degradation of larger transcripts. The concentrations of all *bph* transcripts, with the single exception of ORF0 RNA, responded similarly to growth of strain LB400 on the different carbon sources. Large differences in amounts were observed in the order biphenyl-grown > biphenyl/succinate-grown > succinate-grown (Fig. 2b).

Fig. 1. Overview of the transcriptional analysis of the 5'-terminal region of the *bph* locus. A, genes and ORFs. B, location of probes used. C, location of elongation primers used. D, location of mapped transcripts: a, ORF0 RNA; b, *bphA1A2* RNA. E, transposon constructs employed to identify *bph* segments important for promoter function. GATC indicates a 4 bp insertion in ORF0. The numbers refer to positions in the *bph* locus (Erickson & Mondello, 1992). Numbering of the gene cluster starts at the first base-pair of the 5'-terminal *EcoRI* site. Abbreviations: E, *EcoRI*; Bg, *Bgl*III; *lacZ*, β -galactosidase-encoding gene.

Fig. 2. Northern blot analysis of *Burkholderia* sp. strain LB400 transcripts. RNA samples were extracted from cells grown on succinate (S), biphenyl (B) or a mixture of the two (S+B). Aliquots of 30 µg were separated on 10 % formaldehyde/1 % agarose gels and visualized by hybridization with different probes. The sizes of the main transcripts are indicated. RLM: RNA length marker. (a) Probe S2 (ORF0); (b) probe S3 (*bphA1*).

These results suggest that transcription of the entire *bph* locus downstream of ORF0 is similarly regulated and that at least two mechanisms operate, up-regulation by biphenyl (and possibly other carbon sources) and down-regulation by succinate (and probably other carbon sources). ORF0 RNA was found in approximately equal concentrations in succinate- and biphenyl/succinate-grown cells and in moderately enhanced concentration in biphenyl-grown cells. The different biphenyl responses of the ORF0 and *bphA1A2* RNAs indicate that ORF0 is transcribed independently from the other *bph* genes and that the two RNAs do not represent processing products of the same precursor.

Mapping of RNA 5'-ends

RNA 5'-ends were mapped in the 5'-terminal region of the *bph* locus by primer extension on RNA extracted from cells grown in minimal medium with the addition of either biphenyl or succinate. Since ORF0 RNA was not detected with probe S1, its 5'-end should be located within a few hundred base-pairs upstream of ORF0. As shown in Fig. 3(a), primer CIOPPC detected a single 5'-terminus located 89 bp upstream of the start codon of ORF0, which corresponds to C₅₂₂ of the published sequence (Erickson & Mondello, 1992). The signal was moderately enhanced by growth on biphenyl. Promoter activity of a fragment encompassing this region (see Table 4, below) and the absence of transcripts hybridizing with the probe S1 (Table 3) are consistent with the interpretation that the mapped 5'-end repre-

sents a genuine transcriptional start site rather than an RNA terminus formed by processing.

The different biphenyl responses of the *bphA1A2* and ORF0 RNAs and the annealing of *bphA1A2* RNA with probes S3, S2A and S4, but not with probe S2 (Fig. 1), indicate that the promoter for this transcript is located within a region of 200–300 bp upstream of the *bphA1* gene. As shown in Fig. 3(b), primer extension experiments performed with oligonucleotide CIOP4R detected four major 5'-ends located 25, 41, 47 and 70 bp upstream of the start codon of *bphA1* [corresponding to G₁₄₁₄, T₁₃₉₈, G₁₃₉₂ and T₁₃₆₈ in the published sequence (Erickson & Mondello, 1992)]. All four signals were strongly enhanced by growth on biphenyl. An overview of the location of the ORF0 and *bphA1A2* RNAs is given in Fig. 1(D).

Demonstration of promoter function and characterization of required DNA lengths

In order to verify and quantitate the activity of the promoters suggested by primer elongation and to identify the lengths of the DNA regions required for full promoter function, we fused different fragments (shown in Fig. 1) containing the probable start sites of ORF0 and *bphA1A2* RNA to a *lacZ* reporter gene located on an artificial transposon. The resulting transposons were then inserted into the LB400 genome. The use of transposons ensures that the reporter system closely mimics the natural situation by increasing the promoter

Fig. 3. Mapping of RNA 5'-ends upstream of ORF0 and *bphA1*: autoradiograms of extensions of primers CIOPPC (a) and CIO4R (b). RNA was extracted from strain LB400 growing in minimal medium supplemented with either succinate (lane S) or biphenyl (lane B). Lanes T through A contain the respective sequencing reactions. The position of the first base of each major mRNA species relative to the first base-pair of the respective cistron is indicated at the left margin.

Table 4. Promoter activity of transcriptional *bph*/reporter gene fusions in strain LB400 background

Strain	<i>bph</i> segment upstream of reporter gene*	β -Galactosidase activity (Miller units)† after growth on:	
		Biphenyl	Succinate
LB400[Tn <i>lacZ</i>]	none	150	550
LB400[TnPC55]	90–664	1500	1000
LB400[Tn120]	364–1605	6600	1300
LB400[TnPI75]	724–1431	150	140
LB400[TnPI87]	724–1604	260	360
LB400[TnBg2000]	785–2839	570	430
LB400[TnPI29]	1139–1431	200	320
LB400[TnPI45]	1139–1604	220	260
LB400[TnEE28]	1–2855	4200	560

* Numbers indicate the position of the terminal nucleotides in the *bph* locus (Erickson & Mondello, 1992; Hofer *et al.*, 1993, 1994). Numbering of the gene cluster starts at the first base-pair of the 5'-terminal *EcoRI* site.

† Each value is the mean of measurements of at least three isolates of each mutant strain. The SD was less than 20% of the mean.

copy number only from one to two or leaving it unchanged, if mutants are used in which the wild-type promoter is inactivated. At least three individual clones resulting from the insertion of each reporter transposon were used for the measurement of LacZ activity, which was determined after growth of the strains on either biphenyl or succinate (Table 4). Only gross values are listed because a true background subtraction is not possible. The fusion of *bph* bp 90–664, harbouring the mapped P_{ORF0} (LB400[TnPC55]) showed a low level of

increase in promoter activity after growth on biphenyl. This result is in accordance with the data obtained in the Northern blot and primer extension analyses. In contrast, LB400[TnEE28], a fusion with *bph* bp 1–2855, harbouring the mapped P_{ORF0} and P_{bphA1} (which may consist of several overlapping promoters), showed a significantly higher biphenyl-responsiveness of transcription. Transcription from P_{ORF0} is probably not measured with this construct, as our results suggest its termination within the cloned fragment (cf. above).

Fig. 4. SDS-PAGE analysis of expression of ORF0. The products of genes downstream of a phage T7 late promoter of the vector were visualized by labelling with ^{35}S -labelled amino acids and autoradiography of the gel. Lanes 1 and 4, size markers (molecular masses indicated); lane 2, plasmid pCRPI29; lane 3, plasmid pCR120 harbouring ORF0.

Somewhat higher LacZ activities were measured for biphenyl and succinate when the 5'- and 3'-ends of the cloned fragment were shifted to positions 364 and 1605, respectively (LB400[Tn120]). This may be due to deletion of the terminator for ORF0 RNA or a consequence of the different fusions between *bph* DNA and reporter gene (see following section). In any case, all of the other subfragments fused to the reporter system showed no significant promoter activity. Surprisingly, this includes the large segments encompassing bp 724–1605 and 785–2839. The similar behaviour of shorter segments renders it improbable that silencer-like sequences are responsible for the inactivity of long segments. This suggests that the presence *in cis* of sequences between positions 364 and 724 is important for proper functioning of P_{bphA1} .

Analysis of ORF0 translation and mutational analysis of the role of the ORF0 gene product for transcription from the *bphA1* promoter

ORF0 encodes a hypothetical protein of 245 amino acids and 27.8 kDa. Having demonstrated the synthesis of a specific ORF0 RNA in strain LB400, we investigated the translation of ORF0. A PCR-generated fragment containing ORF0 was cloned in *E. coli* in a phage T7 RNA polymerase-dependent expression system (plasmid pCR120). This allows the specific visualization of insert-encoded proteins, as their synthesis proceeds under conditions that suppress host transcription. Using ^{35}S -

labelling, we were able to visualize after SDS-PAGE a protein with a mobility corresponding to 27.5 kDa whose synthesis was dependent on the cloned fragment (Fig. 4).

The results presented above suggest that parts of the ORF0 region may fulfil an important function for P_{bphA1} *in cis*. Therefore, a potential role of the ORF0 protein for this promoter was not investigated by deletion analysis, but by introduction of a frameshift mutation (insertion of 4 bp) at codon 59. This should lead to premature termination of the mutant ORF0 after 63 codons, i.e. within the segment encoding its potential helix–turn–helix motif. The wild-type ORF0 was exchanged in strain LB400 for the mutant analogue, yielding strain LB400ORF0FS. The replacement was verified by PCR amplification followed by restriction and DNA sequencing (for details see Methods). This mutant retained the ability to grow on biphenyl. For a quantitative analysis of *bph* expression, the mutant ORF0/ P_{bphA1} region was fused to the *lacZ* reporter gene (yielding TnFS24, Fig. 1) and introduced into the genomes of strains LB400 and LB400ORF0FS. For comparison, *lacZ* expression from the reporter transposon carrying a wild-type ORF0/ P_{bphA1} region (TnEE28) was measured in the same two backgrounds. Three individual transposon mutants of each strain were examined. The mean values of LacZ activity are given in Table 5. The levels of *lacZ* expression from TnEE28 were not significantly different in the two recipients. In both types of mutants, growth on biphenyl relative to succinate led to a significant increase in LacZ activity. This was also observed for mutants LB400[TnFS24] which possess an intact ORF0 allele in the chromosome. This demonstrates that the frameshift does not significantly interfere with the functioning of P_{bphA1} . Similar to LB400[Tn120] (Table 4), LB400[TnFS24] showed higher LacZ activity than LB400[TnEE28] after growth on both carbon sources, indicating that the additional *bph* DNA in TnEE28 influences the level of *lacZ* expression. TnEE28 contains a complete *bphA1* gene in front of *lacZ* while TnFS24 contains a truncated *bphA1* gene. This or other features of the additional DNA segment, e.g. leading to formation of different RNA secondary and tertiary structures, may very well influence such phenomena as mRNA processing or ribosome binding which ultimately would affect the efficiency of translation. Relative to the LB400 background, the LB400ORF0FS background reduced *lacZ* expression from TnFS24 approximately twofold after growth on succinate, and five- to sixfold after growth on biphenyl. This indicates a positive influence of the ORF0 protein on P_{bphA1} particularly during biphenyl catabolism. These results were confirmed by analysis of the concentration of *bphA1A2* RNA in strain LB400 and in mutants LB400ORF0FS and LB400ORF0FS[TnEE28] after growth on biphenyl (data not shown). The concentration was strongly reduced in strain LB400ORF0FS, but was similar to wild-type levels in strain LB400ORF0FS[TnEE28]. The results of both experimental approaches show that the ORF0 gene

Table 5. Promoter activity of transcriptional *bph*/reporter gene fusions in ORF0 diploid LB400 mutants with different ORF0 genotypes

Strain	ORF0 genotype of <i>bph</i> locus/miniTn5	<i>bph</i> segment upstream of reporter gene*	β -Galactosidase activity (Miller units)† after growth on:	
			Biphenyl	Succinate
LB400[Tn <i>lacZ</i>]	+ / –	none	150	550
LB400[Tn <i>EE28</i>]	+ / +	1–2855	4200	770
LB400ORF0FS[Tn <i>EE28</i>]	– / +	1–2855	4000	1200
LB400[Tn <i>FS24</i>]	+ / –	90–2437‡	7300	2500
LB400ORF0FS[Tn <i>FS24</i>]	– / –	90–2437‡	1300	1200

* Numbers indicate the position of the terminal nucleotides in the *bph* locus (Erickson & Mondello, 1992; Hofer *et al.*, 1993, 1994). Numbering of the gene cluster starts at the first base-pair of the 5'-terminal *EcoRI* site.

† Each value is the mean of measurements of at least three isolates of each mutant strain. The SD was less than 20% of the mean.

‡ Contains four additional base-pairs (positions 785a–d).

product mediates, either directly or indirectly, up-regulation of transcription from P_{bphA1} .

DISCUSSION

Northern analysis of transcription of the *bph* locus of *Burkholderia* sp. strain LB400 revealed that the transcription of all genes encoding biphenyl catabolic enzymes responded similarly to the presence of biphenyl, succinate or a mixture of the two. It also showed that transcription of these genes was subject to at least two types of regulatory mechanisms, up-regulation by growth on biphenyl and down-regulation by growth on succinate (and probably other carbon sources). This was indicated by the intermediate concentrations of *bph* RNAs in the presence of both carbon sources. The analysis furthermore demonstrated that one region of the locus, encompassing ORF0, was separately transcribed and differently regulated. The RNA blotting analysis detected a variety of relatively small transcripts. With the exception of ORF0, *bphA1*, *bphA2*, ORF1, *bphK* and *bphD* the major transcripts appeared to have sizes even smaller than the genes from which the respective probes were derived. Moreover, sometimes a given probe detected relatively high amounts of additional shorter RNAs. These observations suggest a processing of longer transcripts and incomplete inhibition of RNase activity during work-up. The formation of numerous *bph* transcripts may in part also be due to the existence of several promoters and terminators. We note that evidence has recently been presented for the existence of at least five promoters in the closely related *bph* locus of *P. pseudoalcaligenes* KF707 (Watanabe *et al.*, 2000).

Primer extension analysis mapped the 5'-end of the monocistronic ORF0 RNA to C₅₂₂, 89 bp upstream of the start codon of ORF0. This deviates by only 6 bp from the approximate position determined by Erickson

& Mondello (1992) using S1 nuclease mapping and makes it unlikely that the detected RNA terminus is an artefact of either mapping technique. Moreover, the lack of transcription further upstream of ORF0 is consistent with the location of a promoter in this region. Thus we conclude that ORF0 RNA is initiated at C₅₂₂. Inspection of the sequence around this site shows that the region from position –13 to position +8 has a very high AT content of 86 mol%. Watanabe *et al.* (2000) found that also the ORF0 of the closely related *bph* locus of *P. pseudoalcaligenes* KF707 is transcribed into a monocistronic RNA and mapped its 5'-end to position 106 upstream of the ORF0 start codon. A comparison of the two promoter regions (Fig. 5a) shows an overall sequence identity of about 30%. In both cases, at appropriate spacings upstream of the mapped RNA start positions, limited similarities, typical for positively regulated promoters, are found to the –10 and –35 consensus motifs of σ^{70} promoters. Whilst KF707 shows a perfect match only with the –35 consensus sequence, LB400 shows an almost perfect match only with the –10 consensus motif. An unusually high local sequence identity of 60% exists in the region between the mapped RNA start sites. Within this region, the binding motif for GntR-type transcriptional regulators as defined by Watanabe *et al.* (2000) is perfectly conserved. Indeed, these authors demonstrated *in vitro* binding of the ORF0 protein of strain KF707 to a 38 bp DNA fragment encompassing this region. This suggests that ORF0 of strain LB400 could be subject to the same type of (auto-) regulation. In agreement with this, a similar two- to threefold enhancement of ORF0 transcription by growth on biphenyl relative to succinate was observed with both organisms. We note that this contrasts with previous data for LB400, which indicated a much larger difference in favour of biphenyl-grown cells (Erickson & Mondello, 1992).

Using a transcriptional fusion with *lacZ*, we demon-

Fig. 5. Sequence alignments of promoter regions of the *bph* loci of *Burkholderia* sp. strain LB400 and *P. pseudoalcaligenes* KF707. Asterisks between sequences indicate nucleotide identity. (a) Promoter regions upstream of ORF0. The ORF0 start codons are boxed. Mapped start nucleotides are marked by arrows. Potential -10 and -35 recognition motifs are over- and underlined, respectively; matches with their consensus sequences are highlighted by dots. Highly conserved nucleotides of GntR-type regulator recognition sites are marked by three connected boxes. (b) Promoter regions upstream of *bphA1*. The ORF0 stop codons are boxed. Mapped start nucleotides are marked by arrows; the numbers indicate their distances from the *bphA1* start codons. The RpoN (σ^{54}) recognition motif is marked by two connected boxes; note that the correct spacing of 10 nucleotides is only found in the LB400 sequence.

strated in strain LB400 promoter activity of the region upstream of *bphA1*. This is the first gene of the locus that encodes a catabolic enzyme. The respective primer extension experiment yielded four major signals corresponding to positions -25 , -41 , -47 and -70 upstream of the *bphA1* start codon (Fig. 5b). Erickson & Mondello (1992) determined the approximate positions of RNA 5'-ends of this region by S1 nuclease mapping. They did not observe the terminus at position -25 , but otherwise their results agree well with ours. Thus at least three of the termini are unlikely to be artefacts of the specific mapping technique used. Only a single RNA 5'-end was found in the corresponding *bph* region of strain KF707, which contains about 85% of identical residues. It was mapped at position -99 relative to the start of *bphA1* (Fig. 5b). Unfortunately, we were unable to narrow down the promoter region much by deletion analysis. Thus, it cannot be said with certainty whether the mapped RNA 5'-ends originate from processing or transcription or both. We note, however, that the length and localization of *bphA1A2* RNA and the absence of RNA of the same biphenyl responsiveness annealing to probe S2 are consistent with the view that the mapped ends represent transcriptional start sites. Overlapping

promoters, depending on different regulatory factors, have previously been described and appear to be useful in maintaining appropriate levels of mRNA under various physiological conditions (Nørregaard-Madsen *et al.*, 1994; Kallipolitis & Valentin-Hansen, 1998). Inspection of the regions upstream of the mapped start sites revealed no high degree of similarity with known prokaryotic promoter consensus sequences. The RpoN (σ^{54}) recognition motif (GG-N₁₀-GC) (Ishimoto & Lory, 1989; Totten *et al.*, 1990), positioned 1 bp closer to the putative start site than normally observed, is found only in strain LB400 (Fig. 5b). However, a detailed comparison with the well-characterized RpoN-dependent *mop* (Schirmer *et al.*, 1997), *xyl* (Abril *et al.*, 1991; Perez-Martin & de Lorenzo, 1996) and *dmp* (Shingler *et al.*, 1993; Fernandez *et al.*, 1994) promoters showed no further similarities. When strain LB400 was grown on succinate as sole carbon source, the same 5'-ends were detected for *bphA1A2* RNA. This indicates that the change in carbon sources does not lead to a switch in promoter(s) used and is in agreement with the results of Erickson & Mondello (1992). However, a major difference from this previous work is once more the biphenyl/succinate-responsiveness of transcription. We

HTH consensus	LP E L FGVSR TVREAL L	
Orf0-KF707	TFQRLRADIVEGRLAAGSF IGVELLKSRYFVSGGTMREALSI VAENLVQTQAQRGFHVT	94
Orf0-B4	TFQRLRADIVEGRLAAGSF IGVELLKSRYFVSGGTMREALSI VAENLVQTQAQRGFHVT	94
Orf0-LB400	TFQRLRADIVEGRLAAGSF IGVELLKSRYFVSGGTMREALSI VAENLVQTQAQRGFHVT	94
BphS-Tn4371	PYATLRDCILRGSHPGQGS FIELLKSSLGVSGGTMREALSI TRVGDRLVVAEGQKGFVA	101
GNTR_BACSU	VAYELMRIVSGLIESGT IL SENTIAAE FS VS RSF VR EA LK IT ASEKIIRLE-RMGAVVI	81
	[SS S S S**S * ** SS* * SSSS S* ** * S* S]	
Orf0-KF707	PMSLGDMRDLAATRIAESEALRQSVLNGDAEWEARIVSSFHRLSLIEEPTMRDPARWFN	154
Orf0-B4	PMSLGDMRDLAATRIAESEALRQSVLNGDAEWEARIVSSFHRLSLIEEPTMRDPARWFN	154
Orf0-LB400	PMSLDDMRDLAATRIAECEALRQSVLNGDAEWEARVVSSYHRLSLDERTMRDPVHLFN	154
BphS-Tn4371	PMSLCDLDDLSARITLETTAIVESINLGGGDWEDQLVISFRRLARAQERVEANPAEAFD	161
GNTR_BACSU	GLTEKKIAEIYDVRLLETFVFERLVKIDIEPLVKDLSKILEMMKVSIKYEDAEFEFS---	138
	[*** * ** S* SS * * * ** * * ** * * *]	
Orf0-KF707	EWEPVNRGFHEALISACSSVWIRRFSLISILYVHMERYRRLTAMH--NPPTRN---VHEEHL	206
Orf0-B4	EWEPVNRGFHEALISACSSVWIRRFSLISILYVHMERYRRLTAMH--NPPTRN---VHEEHL	206
Orf0-LB400	QWEQANRDFHEALISACSSAWTORFLSILYLQMERYRRLTAMH--NRPARN---VHEEHL	206
Orf0-Tn4371	AWEARNQEFHNALMAASPSKWLANFREILFHNSEYRRLSGTQ--GPISVE---VHEEHL	213
GNTR_BACSU	---FQDVLFHETIIRAIDHSYIQMIWNNLKPVMSFILLSMRVRLKEKYEDFTIRILDNHE	195
	[** * SS ** S * * * *S S****S *****S]	
Orf0-KF707	ALRDSALARDAERCAALMAEHIESSISVVREFGLLR	245
Orf0-B4	ALRDSALARDAERCAALLAEHIESSISVVREFGLLR	245
Orf0-LB400	ALRDSALARDAERCTELLRMHIESSISVVRFGLLR	245
BphS-Tn4371	TIFEAAIARDANQAVSVLSQHIRRSAM	243
GNTR_BACSU	LYIQAIKTDRALMIQSLHQNFDDVDQKVEDLWLSQQMLAKGAEYNNND	243
	[* **S* ** *]	

Fig. 6. Sequence alignment of the two domains of the ORF0 protein from *Burkholderia* sp. strain LB400 with related transcriptional regulatory proteins from biphenyl-degrading bacteria and with the prototype GntR protein from *Bacillus subtilis*. Orf0-KF707, ORF0 protein from *P. pseudoalcaligenes* KF707 (Watanabe *et al.*, 2000); Orf0-B4, ORF0 protein from *Pseudomonas* sp. strain B4 (NCBI protein database accession no. CAB93964); BphS-Tn4371, transposon-encoded biphenyl regulatory protein (Mouz *et al.*, 1999). Domains according to the ProDom database (<http://protein.toulouse.inra.fr/prodom.html>) are indicated by square brackets. Residues identical in all sequences are indicated by \$, residues identical in all sequences from biphenyl-degrading strains are indicated by *. A consensus sequence for the helix-turn-helix (HTH) DNA-binding region, derived from 40 sequences, is shown in boldface; residues identical with the consensus sequence are highlighted in the individual sequences.

note that all of the different techniques employed by us, particularly the RNA blotting and primer extension analyses, indicated strongly enhanced transcription from this region in the presence of biphenyl.

Full activity of the *bphA1* promoter in biphenyl-grown cells required more than 700 bp upstream of the start of transcription. Regulatory regions that map hundreds of base-pairs distant from their cognate promoter have been described in other systems such as the *deo* promoters of *E. coli* (Valentin-Hansen *et al.*, 1986) or the *algD* promoter of *Pseudomonas aeruginosa* (Kato & Chakrabarty, 1991). With respect to the dependence of P_{bphA1} on the ORF0 protein (see below), we note that regions with 5/6 or 6/6 matches, respectively, with the binding motif for GntR-type transcriptional regulators are found approximately 180 bp upstream of the ORF0 stop codons in the *bph* loci of strains LB400 and KF707. If this sequence acts in concert with the motif upstream of ORF0 or if the latter acts alone, e.g. via a DNA looping mechanism, this gives a rationale for the observed dependence of P_{bphA1} on sequences between positions 364 and 724.

Five base-pairs upstream of the start codon of ORF0 a potential Shine-Dalgarno sequence is found. We demonstrated translation of ORF0 in the heterologous *E. coli* system. As mentioned previously (Hofer *et al.*, 1996; SWISS-PROT database entry P37335), the sequence of the gene product resembles those of transcriptional regulators, specifically of the GntR family (Haydon & Guest, 1991); an alignment is shown in Fig. 6. A strain LB400 mutant in which ORF0 was inactivated by a frameshift mutation showed a significant decrease in biphenyl-induced transcription of *bphA1A2* RNA which was compensated by the presence of a second, intact ORF0. This demonstrates that the ORF0 gene product exerts a positive effect on P_{bphA1} and provides a rationale for the biphenyl-dependent induction of BphC activity observed in biphenyl-utilization-deficient recipients after introduction of the *bph* locus of strain LB400 (Dowling & O'Gara, 1994; Hofer *et al.*, 1996). Negative regulation by GntR-like proteins has recently been reported for gene clusters encoding phenol (Arai *et al.*, 1999) and biphenyl (Mouz *et al.*, 1999) catabolism. However, also positive regulation has been described for members of the GntR family, such as GlcC (Pellicer *et al.*,

1996, 1999), LuxZ (GenPept database accession no. AAD00703) and MatR (GenPept database accession no. AAF28803). Recently, evidence has been obtained that the closely related protein encoded by the ORF0 of *P. pseudoalcaligenes* KF707 (82% sequence identity with the ORF0 protein of strain LB400) acts as a positive regulator for the transcription of its own gene and of all or most of the genes of the *bphX0X1X2X3D* region (Watanabe *et al.*, 2000). Our results show that although the ORF0 protein of strain LB400 is not absolutely required for the transcription from P_{bphAP} it significantly enhances the activity of this promoter.

Finally, we note that transcription of the *bph* gene clusters in strains LB400 and KF707, though similar, appears not to be identical. Thus, after inactivation of ORF0, only strain LB400 retained its ability to use biphenyl as sole carbon source. Furthermore, while the induction of ORF0 expression by growth on biphenyl relative to growth on succinate was two- to threefold in both bacteria, the induction of *bphD* in strain KF707 (no data are presently available for the other *bph* genes of KF707) was in the same range, but was significantly higher in strain LB400.

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