The *benPK* operon, proposed to play a role in transport, is part of a regulon for benzoate catabolism in *Acinetobacter* sp. strain ADP1

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BenM and CatM are distinct, but similar, LysR-type transcriptional regulators of the soil bacterium *Acinetobacter* sp. strain ADP1. Together, the two regulators control the expression of at least 14 genes involved in the degradation of aromatic compounds via the catechol branch of the β-ketoadipate pathway. In these studies, BenM and CatM were each purified to homogeneity to test the possibility that they regulate the expression of two additional genes, *benP* and *benK*, that are adjacent to *benM* on the chromosome. Each regulator bound to a DNA fragment containing the *benP* promoter region. Additional transcriptional studies suggested that *benP* and *benK* are co-transcribed as an operon, and a site of transcription initiation was identified. Alignment of this initiation site with those of several CatM- and BenM-regulated genes revealed common regulatory motifs. Mutants lacking both CatM and BenM failed to activate *benP* transcription. The ability of each protein to regulate gene expression was inferred from strains lacking either CatM or BenM that were still capable of increasing *benP* expression in response to cis,cis-muconate. This compound has previously been shown to induce all enzymes of the catechol branch of the β-ketoadipate pathway through a complex transcriptional circuit involving CatM and BenM. Thus, the regulated expression of the *benPK* operon in concert with other genes of the regulon is consistent with the model that BenP, a putative outer-membrane porin, and BenK, an inner-membrane permease, transport aromatic compounds in strain ADP1.

Keywords: porin, BenM, CatM, LTTR (LysR-type transcriptional regulators)

INTRODUCTION

The adjacent *benP* and *benK* genes of the Gram-negative soil bacterium *Acinetobacter* sp. strain ADP1 are in a chromosomal region associated with aromatic compound degradation. A wide variety of aromatic compounds can serve as carbon and energy sources for ADP1 and are degraded through the β-ketoadipate pathway. This multi-step catabolic route feeds into the tricarboxylic acid cycle and is encoded by genes that are grouped together in large supraoperonic clusters (Harwood & Parales, 1996; Young et al., 2001). The location and sequence of *benP* and *benK* suggest that they participate in the uptake of compounds, such as benzoate, that are subsequently converted to catechol, a key metabolite of the β-ketoadipate pathway. BenK, an inner-membrane permease, plays a role in the cellular entry of benzoate and other aromatic compounds, although it is not essential for growth on benzoate as a sole carbon source (Collier et al., 1997). As described in this report, we tested the effect of inactivating *benP*, which encodes a putative outer-membrane porin. Moreover, the expression of *benP* and *benK* was investigated to determine whether transcriptional regulation of these genes is coordinated with that of other genes of the associated pathway.

Upstream of *benP* are genes involved in the degradation of alkyl salicylates (*salA*, -R, -E, -D) and the degradation of alkanote esters of benzyl alcohols (*areA*, -B, -C, -R) (Jones et al., 1999, 2000; Jones & Williams, 2001). The SalE and AreABC enzymes convert various aromatic compounds to salicylate (2-hydroxybenzoate) or benzoate. Salicylate is the substrate of SalA, a hydroxylase that mediates the production of catechol. Genes for the degradation of catechol and those that enable benzoate to be converted to catechol are immediately downstream.

Abbreviation: CCM, cis,cis-muconate.
of benK (Fig. 1) (Collier et al., 1998). Thus, benP and benK are sandwiched between genes that help funnel a wide array of aromatic compounds into catechol, the substrate of a ring-cleaving dioxygenase (Fig. 1). This genetic arrangement may reflect the participation of BenP and BenK in aromatic compound transport.

BenK is required for wild-type rates of benzoate uptake and for the unimpaired use of benzoate or benzaldehyde as a sole carbon source. In addition, the expression of a chromosomal benK::lacZ fusion increases in response to cis,cis-muconate (CCM), an inducer that regulates all genes known to participate in catechol degradation (Collier et al., 1997). CCM, which is produced from catechol ring cleavage, interacts with CatM, a LysR-type transcriptional regulator, to activate expression of the cateA gene and the catBCIJFD operon (Fig. 1) (Romero-Arroyo et al., 1995). One goal of the current studies was to determine whether CatM is responsible for the CCM-inducibility of benP and benK in aromatic compound transport.

This regulator, encoded by a gene adjacent to benK, is similar to CatM in sequence and in its ability to respond to CCM and activate cateA expression. Therefore, a possible role for BenM in benK expression was also investigated.

The function and expression of benP have not previously been investigated. Porin-like genes similar to benP have been identified near several genetic regions involved in bacterial aromatic compound degradation (Cowles et al., 2000; Segura et al., 1999). For example, phaK, essential for the assimilation of phenylacetate in a Pseudomonas putida strain, appears to encode a specific channel-forming protein (Olivera et al., 1998). The presence of genes likely to encode porins in different catabolic regions suggests that protein channels can facilitate the entry of aromatic compounds into Gram-negative bacteria. In ADP1, the location of the benP gene immediately upstream of benK suggested that both might be co-expressed. It also seemed likely that CatM and/or BenM would control gene expression. To test these possibilities, RT-PCR and primer-extension methods were used to study the transcriptional regulation of benP and benK. In addition, the CatM and BenM proteins were purified to enable further investigation of their roles in regulating the β-ketoadipate pathway for aromatic compound dissimilation.

**METHODS**

**Strains and growth conditions.** Descriptions of strains and plasmids used in this study are listed in Table 1. Acinetobacter strains are derivatives of Acinetobacter sp. ADP1, originally designated *Acinetobacter calcoaceticus* BD413 (Juni & Janik, 1969). Plasmids were maintained in *Escherichia coli* DH5α (Gibco-BRL). All bacterial cultures were grown in Luria–Bertani (LB) broth or minimal medium (MM) at 37 °C as previously described (Sambrook et al., 1989; Shankley et al., 1986). Carbon sources were supplemented to LB or MM at the following final concentrations: anthranilate, 3 mM; benzoate, 3 mM; cis,cis-muconate (CCM), 2.5 mM; and succinate, 10 mM. Benzoaldehyde, benzyl acetate, benzyl alcohol, ethyl salicylate and salicylate were provided as sole carbon sources in a range of concentrations from 0.5 to 2.5 mM. Volatile
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; (φ80d lacZΔM15) Δ(lacZYA−argF)U169 deoR recA1 endA1 hsdR17 (r&lt;sub&gt;n&lt;/sub&gt; m&lt;sub&gt;6&lt;/sub&gt;) phoA supE44 λ&lt;sup&gt;−&lt;/sup&gt; thi-1 gyrA96 relA1</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>BL21-Gold (DE3)</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; dcm&lt;sup&gt;−&lt;/sup&gt; Tn5 hsdS&lt;sup&gt;−&lt;/sup&gt; (r&lt;sub&gt;K&lt;/sub&gt;−K&lt;sub&gt;m&lt;/sub&gt;+) gal (DE3) endA&lt;sup&gt;A&lt;/sup&gt; Te&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Acinetobacter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP1</td>
<td>Wild-type (BD413)</td>
<td>Juni &amp; Janik (1969)</td>
</tr>
<tr>
<td>ISA13</td>
<td>catM::Ω&lt;sub&gt;S&lt;/sub&gt;4013</td>
<td>Romero-Arroyo et al. (1995)</td>
</tr>
<tr>
<td>ISA25</td>
<td>Δ(catBCIJF)−4025</td>
<td>Gaines et al. (1996)</td>
</tr>
<tr>
<td>ISA36</td>
<td>benM::Ω&lt;sub&gt;S&lt;/sub&gt;4036</td>
<td>Collier et al. (1998)</td>
</tr>
<tr>
<td>ACN9</td>
<td>benM::Ω&lt;sub&gt;S&lt;/sub&gt;4013</td>
<td>Collier et al. (1998)</td>
</tr>
<tr>
<td>ACN32</td>
<td>benA::lacZ-Km&lt;sup&gt;+&lt;/sup&gt;5032</td>
<td>Collier et al. (1998)</td>
</tr>
<tr>
<td>ACN5400</td>
<td>benP::Ω&lt;sub&gt;S&lt;/sub&gt;450</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
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<tr>
<td>pUC19</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; cloning vector</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pRK415</td>
<td>Tc&lt;sup&gt;R&lt;/sup&gt;; cloning vector</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pET-21b</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; T7 expression vector</td>
<td></td>
</tr>
<tr>
<td>pCR-Blunt II-TOPO</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; PCR product cloning vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pH145</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; Sm&lt;sup&gt;R&lt;/sup&gt;; Sp&lt;sup&gt;R&lt;/sup&gt;; source of Ω&lt;sub&gt;S&lt;/sub&gt;</td>
<td>Frentki &amp; Krisch (1984)</td>
</tr>
<tr>
<td>pBAC14</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; 2.4 kb EcoRI−PstI fragment containing benM in pRK415</td>
<td>Collier et al. (1998)</td>
</tr>
<tr>
<td>pBAC68</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; 7.0 kb BglII−KpnI fragment containing the areR−benK region in pUC19</td>
<td>Jones et al. (1999)</td>
</tr>
<tr>
<td>pBAC84</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; 3.4 kb Smal−EcoRI fragment containing benP from pBAC68, inserted into HinII−EcoRI of pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pBAC370</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; Sm&lt;sup&gt;R&lt;/sup&gt;; Ω&lt;sub&gt;S&lt;/sub&gt; in ben&lt;sup&gt;P&lt;/sup&gt; of pBAC84</td>
<td>This study</td>
</tr>
<tr>
<td>pBAC381</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; PCR amplified fragment containing catM inserted into NdeI&lt;sup&gt;†&lt;/sup&gt;/BamHI&lt;sup&gt;†&lt;/sup&gt; sites of pET-21b</td>
<td>This study</td>
</tr>
<tr>
<td>pBAC382</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;; PCR amplified fragment containing ben&lt;sup&gt;M&lt;/sup&gt; inserted into NdeI&lt;sup&gt;†&lt;/sup&gt;/EcoRI&lt;sup&gt;†&lt;/sup&gt; sites of pET-21b</td>
<td>This study</td>
</tr>
<tr>
<td>pBAC539</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; 0.3 kb fragment containing pobRAO/P region in pCR-Blunt II-TOPO</td>
<td>Brzostowicz (1997)</td>
</tr>
<tr>
<td>pIB25</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;; 1.3 kb HinIII fragment containing catM in pRK415</td>
<td>Neidle et al. (1989)</td>
</tr>
</tbody>
</table>

* Ap<sup>R</sup>, ampicillin resistant; Sm<sup>R</sup>/Sp<sup>R</sup>, streptomycin and spectinomycin-resistant; Km<sup>R</sup>, kanamycin-resistant; Tc<sup>R</sup>, tetracycline resistant; Ω<sub>S</sub>, omega cassette conferring Sm<sup>R</sup>/Sp<sup>R</sup>; Ω<sub>K</sub>, omega cassette conferring Km<sup>R</sup>.
† Indicates restriction sites added by PCR (See Table 2).

Table 2. Oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′−3′)*</th>
<th>Relative position†</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>BENP-FOR</td>
<td>GACTTTAAGACCGCAACAGGTCTG</td>
<td>3047−3070</td>
<td></td>
</tr>
<tr>
<td>BENP2-FOR</td>
<td>GGCCGTTAGCCCTAATTCCGAACCT</td>
<td>2569−2592</td>
<td></td>
</tr>
<tr>
<td>BENK-REV</td>
<td>CATCATCTGACTAAACAGCAGCTG</td>
<td>4038−4062</td>
<td></td>
</tr>
<tr>
<td>BENP-PE</td>
<td>ATAAACTGATAAAAGACGGCTT</td>
<td>2102−2121</td>
<td></td>
</tr>
<tr>
<td>CATM-NDEI</td>
<td>TCAATTCTATGGAATGAGCAACCTCAGA</td>
<td>6127−61296</td>
<td>NdeI</td>
</tr>
<tr>
<td>CATM-BAMHI</td>
<td>ATAGAGTGCCATATTCCGATGATGGCTGAT</td>
<td>15385−15405</td>
<td>BamHI</td>
</tr>
<tr>
<td>BENM-NDEI</td>
<td>TCAATTCTATGGAATGAGCAACCTCAGC</td>
<td>5617−5637</td>
<td>NdeI</td>
</tr>
<tr>
<td>BENM-ECORI</td>
<td>ATAGAAGTTCTACAGTGGTTGGCCGCTAG</td>
<td>4723−47432</td>
<td>EcoRI</td>
</tr>
<tr>
<td>BENPO/P-FOR</td>
<td>TATGGCATAGGTCCCTTCCCAAAAAG</td>
<td>2071−2094</td>
<td></td>
</tr>
<tr>
<td>BENPO/P-REV</td>
<td>CCAGTCACAGCTTGATGAAATTTG</td>
<td>1777−1800</td>
<td></td>
</tr>
</tbody>
</table>

* Bold letters indicate restriction sites.
† Relative to complementary sequence of areR. Nucleotide 1 corresponds to the last position of the stop codon as depicted in Fig. 2.
compounds, such as the esters, were spotted onto filter paper in the lids of inverted Petri dishes containing minimal medium. Antibiotics were added as necessary at the following final concentrations: ampicillin, 150 \( \mu \text{g ml}^{-1} \); kanamycin, 25 \( \mu \text{g ml}^{-1} \); streptomycin, 25 \( \mu \text{g ml}^{-1} \); and spectinomycin, 25 \( \mu \text{g ml}^{-1} \). Bacterial growth was monitored by spectrophotometric measurement of the OD_{600} of cultures (Sambrook et al., 1989).

**DNA manipulations, plasmid construction, and interposon mutagenesis.** Standard methods were used for plasmid DNA purifications, restriction enzyme digestions, PCR, electrophoresis, ligations and Escherichia coli transformations (Sambrook et al., 1989). Oligonucleotides were purchased from Genosys (Table 2). Plasmid pBAC84, carrying benP, was constructed by ligating a 3.4-kbp SalI–EcoRI fragment from pBAC88 into HincII/EcoRI-digested pUC19. Plasmid pBAC370, used for interposon mutagenesis of benP, was constructed by inserting an omega cassette conferring resistance to streptomycin and spectinomycin (QS), into the centre of benP. A 2.0-kbp PstI fragment from pHP45, with QS followed by translational and transcriptional stop signals, was ligated into a 500 bp deletion in pBAC84 created by CiaI digestion at benP sites, at nucleotide positions 2323 and 2719 (numbering system of Fig. 2).

The CatM expression plasmid, pBAC381, was constructed after PCR-amplifying catM from plasmid pB25 with the primers CatM-NDEI and CatM-BAMHI (Table 2). The PCR product was electrophoresed on a 10% agarose gel, excised, and purified with the QIAquick gel extraction kit (Qiagen). At sites introduced by the amplification primers, the PCR product was digested with NdeI and BamHI and ligated into the similarly digested pET-21b expression vector (Novagen). The BenM expression plasmid pBAC382 was similarly constructed with primers BenM-NDEI and BenM-ECORI (Table 2) and plasmid pBAC14 as the template for PCR amplification. The DNA sequences of the entire catM and benM genes of pBAC381 and pBAC382, respectively, were verified.

To generate an Acinetobacter strain with an inactivated chromosomal benP gene, plasmid pBAC370 was digested with NdeI to yield a linear fragment with the disrupted benP::ΩS5450 allele. This fragment was purified from a 1% agarose gel using the Qiaquick purification kit (Qiagen). As described previously (Neidle et al., 1989), the linearized DNA fragment was used to transform and to replace the corresponding chromosomal region of strain ADP1, generating ACN450. The correct chromosomal configuration of ACN450 was confirmed by Southern hybridization analysis as previously described (Gregg-Jolly & Ornston, 1990).

**Expression and purification of CatM and BenM.** E. coli strain BL21-Gold (DE3) (Novagen) was transformed with pBAC381 or pBAC382 and plated on solid medium with ampicillin. An isolated colony was used to inoculate 5 ml LB broth with ampicillin, and the culture was grown at 37 °C with agitation until reaching an OD_{600} of 0.4—0.8. This 5 ml culture was used to inoculate 1 litre of fresh medium and allowed to grow to an OD_{600} of 0.4—0.8, at which point the culture was put on ice. After 10 min on ice, the culture was transferred to a 16 °C incubator, and protein expression was induced with the addition of IPTG to a final concentration of 1 mM. After overnight incubation (10—14 h), each 1 litre culture was divided into 100 ml batches that were harvested by centrifugation at 7000 × g for 10 min at 4 °C. After removal of the supernatant fluid, cell pellets were immediately stored at −70 °C.

Cell pellets containing CatM or BenM were suspended in ice in 10 ml buffer A1 (50 mM Tris/HCl pH 6.0, 50 mM NaCl, 5%, v/v, glycerol, 0.5 mM EDTA and 0.5 mM DTT) containing PMSF at a final concentration of 100 μg ml^{-1}. Cells were lysed by two passages through a 4 °C chilled French pressure cell at 15000 p.s.i. (103-5 MPa). The resulting lysate was centrifuged at 15000 × g for 15 min at 4 °C. All column purification steps were done with an FPLC system from Amersham-Pharmacia. A 10 ml sample of the supernate was loaded onto a 5 ml HITRAP SP cation-exchange column that had been equilibrated with 5 ml buffer A1 containing 25 ml buffer A2. Protein was eluted from the column at a flow rate of 1 ml min^{-1} over a linear gradient of buffer B1 (buffer A1 with 1 M NaCl) and immediately placed on ice. Fractions containing CatM (300—350 mM NaCl) or BenM (250—350 mM NaCl) were pooled following analysis by 12% SDS-PAGE (Sambrook et al., 1989).

To lower the NaCl concentration and allow subsequent binding of CatM or BenM to additional columns, pooled samples were diluted fivefold with buffer A2 (50 mM Tris/HCl pH 7.0, 50 mM NaCl, 5%, v/v, glycerol, 0.5 mM EDTA and 0.5 mM DTT). The mixture was concentrated to a 10 ml volume with an Ultrafree S-10 centrifuge concentrator (Millipore). Samples were loaded onto a 5 ml HITRAP heparinagarose affinity column that had been equilibrated with 25 ml buffer A2. Protein was eluted over a linear gradient of buffer
B2 (Buffer A2 with 1 M NaCl) at 1 ml min⁻¹. Fractions containing CatM (500–600 mM NaCl) or BenM (300–400 mM NaCl) were identified by SDS-PAGE. Samples containing pure protein were pooled, diluted fivefold with buffer A2, and concentrated to 1–3 ml using an Ultrafree S-10 centrifuge concentrator. Samples were stored in 20 µl aliquots at −70 °C, at a final concentration of 0.5–10 mg ml⁻¹.

Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as the standard. The molecular mass of CatM was estimated by gel-filtration chromatography. A 5 mg sample of purified CatM (1.0 mg ml⁻¹) was loaded onto an HiPrep Sephacryl S-200 gel-filtration column that had been equilibrated with buffer A2. Protein was eluted and fractions analysed by SDS-PAGE to determine the elution volume for CatM. Known proteins served as standards according to the directions of the Molecular mass-Gel Filtration-200 kit (Sigma).

RNA extraction, RT-PCR, and primer-extension analysis. Total RNA was extracted from mid-exponential-phase (OD₆₀₀ 0.5–0.5) Acinetobacter cultures by a hot phenol extraction method as described by Williams & Rogers (1987). RNA samples were treated with DNase I (Promega). The Dnase I method as described by Williams & Rogers (1987). RNA was later removed from the RNA with RNeasy columns (Qiagen). For primer-merase (Fisherbrand) and RNasin ribonuclease inhibitor instructions. G-25 spin columns (Amersham-Pharmacia) were used to remove free [γ-³²P]ATP from the labelling reactions. One microlitre of the end-labelled primer was combined with 2 µg total RNA in a final volume of 6 µl and incubated at 65 °C for 5 min to allow primer annealing. A DNA sequencing ladder was generated with the fmol DNA Cycle Sequencing kit for 5 min to allow primer annealing. A DNA sequencing Taq for both RT-PCR and primer-extension methods. The primer used for these experiments (BENP-PE, Table 2) was end-labelled with T4 polynucleotide kinase (Promega) and [γ-³²P]ATP (ICN Biomedicals) according to the manufacturer’s instructions. G-25 spin columns (Amersham-Pharmacia) were used to remove free [γ-³²P]ATP from the labelling reactions. One microlitre of the end-labelled primer was combined with 2 µg total RNA in a final volume of 6 µl and incubated at 65 °C for 5 min to allow primer annealing. A DNA sequencing ladder was generated with the fmol DNA Cycle Sequencing kit (Promega), [γ-³²P] end-labelled BENP-PE primer, and plasmid pBAC84 containing the wild-type benP region as template. Primer-extension products and sequencing reactions were analysed by 6% denaturing PAGE and visualized by autoradiography (Sambrook et al., 1989).

Gel-retardation assays. A double-stranded DNA fragment with the benP operator–promoter region (P₃₅) was radio-labelled as previously described (Parsek et al., 1994). The BENPO/P-FOR and BENPO/P-REV primers, after being 5’ end-labelled with [γ-³²P]ATP and T4 polynucleotide kinase (Promega), were used to PCR-amplify a 317 bp DNA fragment from pBAC84. This labelled fragment was purified following separation by 5% PAGE. The conditions for DNA-binding reactions were previously described (Romero-Arroyo et al., 1995). Binding reactions were initiated by mixing 5–1000 ng CatM or BenM with 1000 c.p.m. of the [³²P]-labelled benP fragment (approx. 10 nM) in the presence or absence of 50 mM CCM. The DNA–protein complexes were analysed as described by Parsek et al. (1994).

Unlabelled DNA fragments were used in competition studies with the labelled fragment to verify the specificity of CatM and BenM binding. For use as a specific competitor, a DNA fragment with P₃₅ was generated by PCR as described above except that the BENPO/P-FOR and BENPO/P-REV primers were not labelled with [³²P]. To generate a fragment for non-specific DNA competition studies, the M13 forward and M13 reverse universal primers (Promega) were used in PCR to amplify a 656 bp fragment from pBAC539 (Table 1), which contains the pobRA operator–promoter region. This region of the ADP1 chromosome regulates the catabolism of p-hydroxybenzoate (DiMarco et al., 1993) and does not contain a recognizable binding sequence for CatM or BenM. Competition studies were done by adding an increasing range of competitor DNA, from 1- to 50-fold molar excess, to the reaction as previously described (Tobiason et al., 1999).

RESULTS

Co-expression of benP and benK

The orientation of other genes in the vicinity suggested that benP and benK would be transcribed either individually or as a bicistronic operon (Fig. 1). To understand the regulation of gene expression, we first explored the possibility that the two adjacent genes were co-transcribed. Total RNA was isolated from the wild-type strain, ADP1, grown with sole carbon sources that are degraded via the catechol branch of the β-ketoadipate pathway (benzoate or CCM). In addition, RNA was isolated from ADP1 grown with succinate as the sole carbon source, a growth condition that does not induce expression of genes associated with the β-ketoadipate pathway.

RT-PCR analysis was carried out with the BENP-FOR primer in benP and the BENK-REV primer in benK (Fig. 2, Table 2). With RNA from ADP1 grown on benzoate or CCM, but not succinate, a 1015 bp product of the size expected for these promoters was obtained (Fig. 2b). Control reactions lacking reverse transcriptase did not yield a product, indicating no significant DNA contamination in the RNA samples. RT-PCR products were isolated and digested with XbaI to confirm the presence of a known recognition sequence in benK. Although complete digestion was not always achieved, the sizes of the cleavage products were consistent with the correct fragments having been amplified, as shown for CCM-grown cells in Fig. 2(b), lane 7. Additional RT-PCR experiments with the same BENK-REV primer and a primer closer to the 5’ end of benP (BENP2-FOR, Table 2) yielded a product of the expected size with RNA from cells grown on CCM (data not shown). Thus, there were benK transcripts that initiate more than 700 nt upstream of its translational start codon. These results indicate that benP and benK are co-transcribed and that expression of the benPK operon occurs during growth on CCM or benzoate, but not succinate.

Transcript initiation and inducible expression of benPK

Expression of benP was further explored with primer-extension methods. The primer used for these experiments (BENP-PE, Table 2) annealed to a region downstream of the predicted benP AUG start codon. In
initiating 49 and 40 nucleotides upstream of the predicted
mark major products of sizes corresponding to transcripts
and G indicate the DNA sequencing ladder. Arrows A and B
benzoate (BEN) or CCM as a sole carbon source. Lanes C, T, A
ences among the RNA samples from cells grown on
phoretic detection of rRNA revealed no obvious differ-
samples. Spectral analysis of total RNA and electro-
multiple repetitions with independently isolated RNA
40 nt upstream of the start codon, labelled A and B in
correspond to transcripts that initiate at sites 49 and
products (A and B) are of identical sizes in panels (a) and (b).

Fig. 3. Primer-extension analysis of benP. (a) RNA was extracted
from the wild-type strain ADP1 grown on succinate (SUC),
benzoate (BEN) or CCM as a sole carbon source. Lanes C, T, A
and G indicate the DNA sequencing ladder. Arrows A and B
mark major products of sizes corresponding to transcripts
initiating 49 and 40 nucleotides upstream of the predicted benP
translational start site. Nucleotides at these positions are
highlighted in bold and marked with an asterisk. (b) RNA was
analysed from LB-grown mutants. Strain ISA25 has a large
cat-region deletion (∆cat) that prevents CCM catabolism and strain
ACN32 has an insertion (in benA) that prevents benzoate
catabolism. CCM was added to the growth medium of the
former and benzoate (BEN) to the latter. Arrows showing major
products are of identical size to those labelled A and B in Fig. 3.

Primer-extension analysis indicated that benP expression of a chromosomal
- gene deletion that prevents CCM catabolism.

Roles of CatM and BenM in regulating \( P_{bp} \)

The identification of CCM as an inducer raised the possibility that CatM or BenM can activate transcription from \( P_{bp} \). To test this possibility, benP expression was studied in mutants (listed in Table 1) lacking CatM (strain ISA13), BenM (strain ISA36), or both of these transcriptional regulators (strain ACN9). Total RNA was isolated from the wild-type and from each of these mutants grown on rich medium supplemented with CCM. Primer-extension reactions with the benP primer (BENP-PE, Table 2) indicated that the loss of both transcriptional regulators significantly reduced transcription from \( P_{bp} \) (Fig. 4). In contrast, the absence of either CatM or BenM alone had little effect on transcription relative to that in the wild-type strain under the same growth conditions (Fig. 4). These results suggested that both BenM and CatM are individually able to regulate \( P_{bp} \) in response to CCM. The overlap of their regulatory capabilities may account for the requirement that both BenM and CatM be absent in order for the loss of regulated gene expression to observed. A similar overlap in regulation by BenM and CatM occurs for the

extension reactions with total RNA from ADP1 grown on
benzoate or CCM as the carbon source, two
prominent products were generated. Their sizes would
be corresponding to transcripts that initiate at sites 49
and 40 nt upstream of the start codon, labelled A and B
in Fig. 3(a). No extension products were detected in
reactions with RNA from succinate-grown cells, despite
multiple repetitions with independently isolated RNA
samples. Spectral analysis of total RNA and electrophoretic detection of rRNA revealed no obvious differences among the RNA samples from cells grown on different carbon sources. Consistent with the RT-PCR results, these studies indicated that benP expression is inducible.

Mutants were used to determine whether benzoate or
CCM, in the absence of catabolism, could induce gene
expression. RNA was isolated from ISA25, a strain with
a large cat-gene deletion that prevents CCM catabolism.
Primer-extension analysis indicated that benP is expressed when this strain is grown on rich medium in the

presence of CCM (Fig. 3b). Therefore, CCM itself, rather than a catabolite generated during growth on CCM, appears to cause increased benP expression. CCM similarly stimulated benP expression in ACN32, which has a large insertion in benA that prevents benzoate catabolism (data not shown). However, when ACN32 was grown on rich medium supplemented with benzoate, no benP expression was detected (Fig. 3b). Consistent with this result, benzoate does not increase expression of a chromosomal benK::lacZ transcriptional fusion in the absence of benzoate catabolism (Collier et al., 1997). Therefore, transcription from the benPK promoter, \( P_{bp} \), in benzoate-grown wild-type cells most likely results from the endogenous generation of CCM during benzoate degradation.

The identification of CCM as an inducer raised the possibility that CatM or BenM can activate transcription from \( P_{bp} \). To test this possibility, benP expression was studied in mutants (listed in Table 1) lacking CatM (strain ISA13), BenM (strain ISA36), or both of these transcriptional regulators (strain ACN9). Total RNA was isolated from the wild-type and from each of these mutants grown on rich medium supplemented with CCM. Primer-extension reactions with the benP primer (BENP-PE, Table 2) indicated that the loss of both transcriptional regulators significantly reduced transcription from \( P_{bp} \) (Fig. 4). In contrast, the absence of either CatM or BenM alone had little effect on transcription relative to that in the wild-type strain under the same growth conditions (Fig. 4). These results suggested that both BenM and CatM are individually able to regulate \( P_{bp} \) in response to CCM. The overlap of their regulatory capabilities may account for the requirement that both BenM and CatM be absent in order for the loss of regulated gene expression to observed. A similar overlap in regulation by BenM and CatM occurs for the
expression of catA from its promoter region P_{cat} (Romero-Arroyo et al., 1995).

### Protein purification following expression of catM and benM in E. coli

To study the interactions of BenM and CatM with DNA in the vicinity of P_{bp} in vitro, the two regulators were purified. Expression vectors pBAC381 and pBAC382 (Table 1) were constructed for the production of CatM and BenM, respectively, in E. coli. When protein production was induced at 37 °C, problems were encountered with inclusion body formation. As described in Methods, conditions were identified for purification following protein induction at 16 °C. CatM was purified by cation-exchange and heparin-agarose affinity chromatography. From 1 g dry cell mass, CatM protein (approx. 2 mg total) was obtained with an estimated purity of > 95% (Fig. 5a). BenM was similarly purified, yielding approximately 1:5 mg (g dry cell mass)^{-1} (Fig. 5b). Gel-filtration analysis indicated that the sole oligomeric form of CatM was approximately 140 kDa. Since denaturing SDS-PAGE analysis was consistent with the prediction that the monomeric protein is 35-3 kDa, the CatM protein was inferred to be tetrameric in solution. A similar conclusion was drawn from gel-filtration analysis of BenM (Bundy, 2001).

### Binding of CatM and BenM to the benP regulatory region

Based on studies of other LysR-type transcriptional regulators (McFall et al., 1998), we predicted that if CatM and BenM activate benP expression, then they should bind to DNA in the region of P_{bp} in the presence or absence of the inducer, CCM. Gel-retardation assays were used to test this prediction. Samples of purified CatM and BenM were incubated with a radiolabelled fragment carrying P_{bp} (Fig. 6). Increasing amounts of either protein increased the proportion of DNA with retarded electrophoretic mobility. To confirm that the changes in mobility patterns resulted from BenM or CatM binding specifically to the P_{bp} region, competition experiments were done with unlabelled DNA (described...
in Methods). In the presence of 100 ng of either BenM or CatM, reactions containing the unlabelled \((5\text{P})\)-labelled fragment in 50-fold molar excess to the \((5\text{P})\)-labelled probe resulted in the absence of detectable fragments with retarded mobility (data not shown). In contrast, reactions containing the non-specific competitor DNA fragment in 50-fold molar excess did not reduce the ability of CatM or BenM to retard the mobility of the fragments with \((5\text{P})\) (data not shown).

The function of BenP

The regulation of \(\text{PbP}\) by BenM and CatM should coordinate the transcription of the \(\text{benPK}\) operon with the expression of additional \(\text{ben}\) and \(\text{cat}\) genes involved in benzoate degradation (Fig. 1). Therefore, the ability to degrade benzoate and related compounds was tested in ACN450, a strain in which the chromosomal copy of BenP was disrupted by insertional inactivation (Table 1, Methods). ACN450 was capable of growing on solid medium using benzoate, benzaldehyde, benzyl alcohol, benzyl acetate, ethyl salicylate, salicylate or anthranilate as the sole carbon source (data not shown). With these substrates, possible differences in growth rates between ACN450 and the wild-type strain were not characterized. The mutation in ACN450 should also prevent expression of \(\text{benK}\), a gene known to affect the rate of growth on some of these compounds (Collier et al., 1997). These results indicate that neither BenP nor BenK is essential for the cellular entry of this set of aromatic compounds under the laboratory conditions tested. Different experimental conditions, different substrates and/or different genetic backgrounds may be needed to reveal the phenotypic effects caused by the loss of BenK and BenP.

The function of BenP

The regulation of \(\text{PbP}\) by BenM and CatM should coordinate the transcription of the \(\text{benPK}\) operon with the expression of additional \(\text{ben}\) and \(\text{cat}\) genes involved
(TC) system of Saier, BenP and several other putative porins involved in aromatic compound transport form a family that includes the OprD porin of Pseudomonas aeruginosa (TC# 1.B.25; http://tcdb.ucsd.edu/tcdb/search2.php) (Saier, 2000). The substrates of OprD include cationic amino acids, peptides and an analogous compound, the antibiotic imipenem (Trias & Nikaido, 1990).

No protein structure has yet been determined for a BenP-like porin, although various bacterial porins have a β-barrel structure (Koebnik et al., 2000). A 16 β-strand topology model proposed for OprD has been tested by deletion mutagenesis (Huang et al., 1995; Ochs et al., 2000). The predicted OprD β-strands with sequences similar to those of BenP are underlined in Fig. 7. Furthermore, 23 amino acid residues that are conserved among all sequences in the alignment, marked by asterisks, are likely to have a role in protein structure or function. Proteins in the alignment that may be involved in aromatic compound catabolism include PhaK and BenF of Pseudomonas putida. PhaK was inferred to form an outer-membrane channel with narrow substrate specificity since inactivation of its gene prevents the use as a sole carbon source of phenylacetate but not 4-hydroxyphenylacetate or additional phenylacetate-related compounds (Olivera et al., 1998). A predicted role for BenF in aromatic compound catabolism is based on the co-expression of benF with several genes in an operon for benzoate dissimilation (Cowles et al., 2000).

DISCUSSION

Regulation of transcription initiation

A region of transcription initiation for the benPK operon was identified (Figs 3 and 4). Alignment of this P_{bp} region with other CatM- and BenM-controlled promoters helped identify potential regulatory sequences (Fig. 1c). Recent studies with DNase I footprinting demonstrated that CatM and BenM bind strongly to the P_{ba} region, which controls expression of the divergently transcribed benM and benA genes. CatM and BenM each recognize a consensus LysR-type binding sequence in P_{ba} (T-11 nt-A, with a small region of dyad symmetry), boxed in Fig. 1(c) (Collier, 2000). An identical sequence to that in P_{bA}, ATAC-7 nt-GTAT, is recognized by CatM in the P_{sb} region, which controls expression of the divergently transcribed catM and catB genes (Romero-Arroyo et al., 1995). Similar sequences are recognized by a subfamily of LysR-type transcriptional regulators involved in bacterial aromatic compound catabolism, to which CatM and BenM belong (Coco et al., 1994). Therefore, the boxed regions of P_{bp} and P_{ca} are also likely to serve as BenM and CatM binding sites (Fig. 1c). Furthermore, six additional nucleotides, which are highlighted in Fig. 1(c), are identical in all these promoter regions and occur in locations shown to be important for the regulation of P_{ba} (Bundy, 2001).

This sequence alignment places the previously identified transcriptional start sites of the benA, catA and catB genes in an identical position (+1) (Fig. 1c) (Collier, 2000; Romero-Arroyo et al., 1995). This position of benP would correspond to a primer-extension product of the size denoted as ‘A’ in Figs 3 and 4. Thus, the placement of putative regulatory sequences and the observation that ‘A’ is the largest observed primer-extension product suggests that benP transcription initiates at a position corresponding to that of the other CatM- and BenM-regulated genes. The multiple products in the benP primer-extension reactions may result from RNA secondary structure, RNA processing and/or degradation, multiple transcription initiation sites, or some combination of these factors. The significance of the experimental variation in relative intensities of the products is not evident. Nevertheless, the presence of the primer-extension products clearly indicated the conditions under which benP is expressed.

The regulon controlled by CatM and BenM

CCM caused transcription from P_{bP} to increase in strains with CatM or BenM (Figs 3 and 4). The absence of both regulators significantly reduced benP expression, although the resultant expression was higher than that of the wild-type strain grown in the absence of CCM (Fig. 4). Similarly, in a mutant lacking both BenM and CatM, the expression of benA is approximately fourfold higher than in strains with the regulators when no inducer is present (Casper et al., 2000). This increased expression results from the ability of CatM or BenM, in the absence of inducers, to bind to a segment of P_{bA} and repress basal benA expression by preventing access to RNA polymerase (Bundy, 2001). BenM and CatM, which are able to bind to the P_{bp} region in the absence of CCM (Fig. 6), may similarly repress basal benP expression.

The gel-retardation studies indicate that CatM can form at least two different complexes with the P_{bp} region. The presence of CCM may alter the interactions between the regulatory proteins and one or more binding site in this region. Multiple binding sites for BenM and CatM have been demonstrated in the P_{ba} region (Bundy, 2001). Multiple binding sites in the target genes have also been identified in Pseudomonas putida for the CatR and CICR regulators, which control catechol and chlorocatechol degradation, respectively (McFall et al., 1998). Based on structural studies of transcriptional regulators, the putative CatM/BenM binding site in the P_{bp} region, boxed in Fig. 1(c), should bind a protein dimer (Branden & Tooze, 1999). However, BenM and CatM, like many LysR-type regulators, were tetrameric in solution (Schell, 1993). Additional studies are needed to determine if the presence of CCM affects the oligomeric structure of CatM or BenM and to characterize the specific binding of these regulators to the P_{bp} region.

While CatM and BenM both regulate transcription from P_{bP}, P_{bA}, P_{ca} and P_{cb} (depicted in Fig. 1), the specific regulation at each locus varies considerably. For example, benzoate interacts with BenM to activate transcription from P_{bA} and most likely from P_{ca}, as well
Transporters in aromatic compound catabolic pathways

The proposed porin functions have yet to be demonstrated for BenP or for similar proteins likely to be involved in aromatic compound uptake. However, open reading frames encoding hypothetical bacterial porins are frequently located in the vicinity of genes known to participate in aromatic compound degradation. For example, two different BenP/PhaK-like hypothetical proteins of Pseudomonas sp. strain CA10 are encoded in genetic regions involved in the degradation of the heterocyclic aromatic compound carbazole via the catechol branch of the β-ketoadipate pathway (Nojiri et al., 2001). Moreover, the putative porin-encoding genes are often located near genes encoding proteins resembling inner-membrane permeases such as BenK, PcaK and MucK that are involved in the uptake of compounds degraded via the β-ketoadipate pathway (Nichols & Harwood, 1997; Williams & Shaw, 1997). Taken collectively, the data support a model in which a porin, such as BenP, and a permease, such as BenK, function together to facilitate the cellular entry of some substrates of bacterial aromatic compound catabolic pathways.

ACKNOWLEDGEMENTS

We are grateful to Drs Lauren S. Collier, Becky M. Bundy and James E. Posey for their contributions to protein purification and primer-extension analysis. We also thank Drs Frank Gherardini, Timothy R. Hoover, Anna C. Glasgow Karls and Mark A. Schell for many helpful discussions. This research was supported by NSF grant MCB-9808784 (to E.L.N).

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Received 27 September 2001; revised 21 November 2001; accepted 23 November 2001.