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NOTES

benK Encodes a Hydrophobic Permease-Like Protein Involved in Benzoate Degradation by *Acinetobacter* sp. Strain ADP1

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The chromosomal *benK* gene was identified within a supraoperonic gene cluster involved in benzoate degradation by *Acinetobacter* sp. strain ADP1, and *benK* was expressed in response to a benzoate metabolite, *cis,cis*-muconate. The disruption of *benK* reduced benzoate uptake and impaired the use of benzoate or benzaldehyde as the carbon source. BenK was homologous to several aromatic compound transporters.

The soil bacterium *Acinetobacter* sp. strain ADP1, formerly *Acinetobacter calcoaceticus* (10, 13), utilizes benzoate as a carbon source. The conversion of benzoate to tricarboxylic acid cycle intermediates has been studied (27), but the mechanism of benzoate transport has not been investigated. In its undissociated form, benzoic acid (pK_a, 4.19) diffuses across biological membranes. The release of protons upon entry into the cell contributes to benzoate being a toxic chemorepellent to *Escherichia coli* (5, 15, 25). Nevertheless, benzoate and other aromatic compounds such as 4-hydroxybenzoate can be chemoattractants to bacteria which metabolize them (12). The PcaK permease of *Pseudomonas putida*, required for chemotaxis, mediates the uptake of 4-hydroxybenzoate but not benzoate (11, 26). In nonmotile *Acinetobacter* sp. strain ADP1, a PcaK homolog mediates 4-hydroxybenzoate uptake but not chemotaxis (2, 17). The possibility that a novel gene, *benK*, from ADP1 encodes a benzoate transporter was raised by the similarity of its sequence to that of *pcaK*.

Sequence analysis of *benK*. The *benK* gene was found adjacent to *benM*, encoding a transcriptional regulator of benzoate degradation (3), and the *benABCDE* operon, encoding enzymes that convert benzoate to catechol (Fig. 1 and 2) (12, 27). DNA in the *benK* region was isolated by previously described methods (23), taking advantage of a drug resistance cartridge inserted in *benM* (3). The sequence of *benK* was determined by standard methods (30). Hydropathy plots of the deduced 466-amino-acid sequence indicated as many as 12 transmembrane-spanning helices in the hydrophobic BenK protein (data not shown) (1, 8, 18). Computer-assisted database searches (6) identified several homologs. Pairwise sequence comparisons between BenK and the 4-hydroxybenzoate transporter, PcaK, of *P. putida* (11, 26) and of *Acinetobacter* sp. strain ADP1 (2, 17) indicated 33 and 30% identity and 60 and 57% similarity, respectively, between aligned residues. BenK was 26% identical and 57% similar in sequence to MucK of ADP1. MucK was recently shown to confer the ability to utilize exogenous *cis,cis*-muconate, a metabolite generated during benzoate degradation (34) (Fig. 2). In addition, homology was detected to a putative protein encoded by an open reading frame located between 6 and 8 min on the *E. coli* chromosome (7) and TfdK from *Ralstonia eutropha* (33). The *tfd* genes are involved in the degradation of 2,4-dichlorophenoxyacetate (19). Phylogenetic analyses suggested that BenK, PcaK, MucK, and TfdK, all of which are involved in aromatic compound metabolism, form a cluster in the major facilitator superfamily of transport proteins (12, 20, 34).

Phenotypic effect of *benK* disruption on growth. The chromosomal *benK* gene was disrupted by interposon mutagenesis (10), creating strain ACN71. A kanamycin resistance cassette introduced transcriptional and translational stop signals in the 5′ region of *benK* (Fig. 1) (9, 29). Although *benM* is downstream of *benK*, it is transcribed in the opposite direction (Fig. 1). The *benK* disruption, therefore, should not cause polar effects on the expression of other *ben* genes. Growth rates of ACN71 and ADP1 with a variety of carbon sources were compared. Cultures were grown to mid-log phase with 10 mM succinate or fumarate in minimal medium (MM) (32). Samples of these cultures, 100 to 200 μl, were used to inoculate 20 ml of MM, pH 7.0, supplemented with either 3 mM benzoate, 1

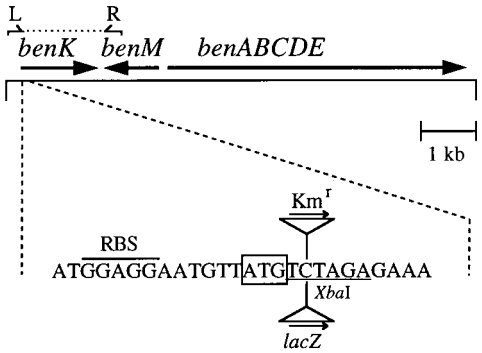

FIG. 1. ADP1 chromosomal *ben* gene region. Arrows indicate transcriptional directions. A possible ribosome binding site (RBS) and initiator codon (ATG) are indicated. In strain ACN71, *benK* was disrupted by the Ω cassette (Km^r) (9) in the *Xba*I site (TCTAGA). A *benK*::*lacZ* transcriptional fusion was constructed by introduction of the *lacZ* cassette from pKOK6 (16) into the *Xba*I site, generating strain ACN109. Half arrows (L and R) indicate positions of primers used for PCR amplification of *benK*. The resulting 1.7-kbp *benK* fragment was ligated into pRK415 (14), generating pBAC138.

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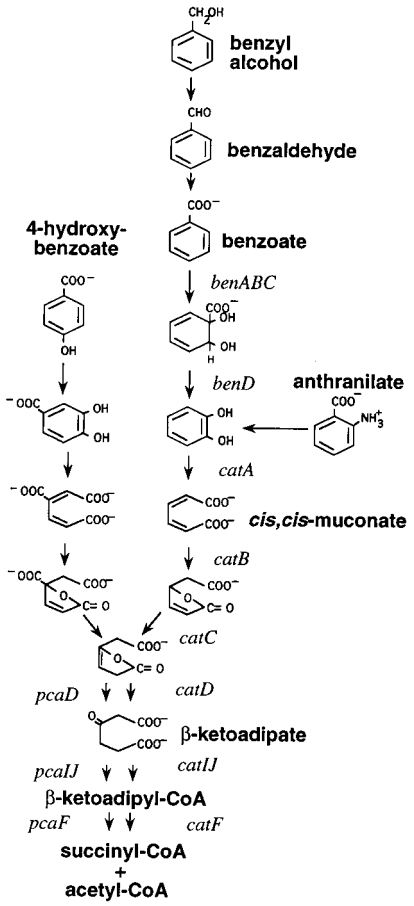

FIG. 2. The β-ketoadipate pathway of ADP1 for aromatic compound degradation. Metabolite names (boldface) and structural genes (italics) are indicated. CoA, coenzyme A.

mM benzaldehyde, 3 mM anthranilate, 3 mM 4-hydroxybenzoate, 3 mM *cis*,*cis*-muconate, or 10 mM benzyl alcohol. Growth was monitored turbidimetrically with a Klett-Summerson colorimeter, and generation times were determined (Fig. 3A).

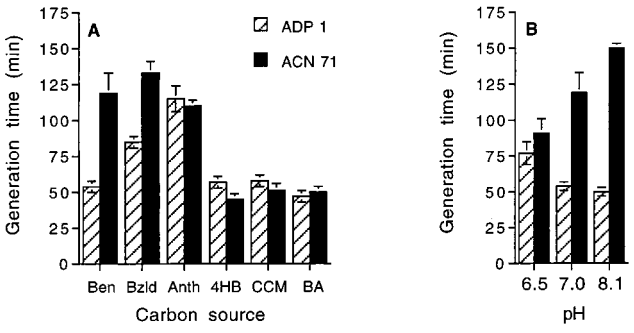

FIG. 3. Generation times of the wild-type (ADP1) and *benK* mutant (ACN71) strains. Error bars represent standard deviations. (A) Cells were grown at pH 7.0 with benzoate (Ben), benzaldehyde (Bzld), anthranilate (Anth), 4-hydroxybenzoate (4HB), *cis*,*cis*-muconate (CCM), or benzyl alcohol (BA) as the sole carbon source. (B) Cells were grown at pH 6.5, 7.0, or 8.1 with benzoate as the sole carbon source at 37°C with aeration. Each generation time represents the mean of at least three independent trials.

ACN71 grew with benzoate as the sole carbon source despite the *benK* mutation. Its generation time, however, was more than twice that of the wild type (Fig. 3A). Growth of ACN71 with benzaldehyde (Fig. 2) was also slow relative to the wild type (Fig. 3A). In contrast, ADP1 and ACN71 grew at similar rates when the growth substrate was benzyl alcohol, anthranilate, 4-hydroxybenzoate, or *cis*,*cis*-muconate (Fig. 3A). In ACN71(pBAC138) with *benK* in *trans* (Fig. 1), growth with benzoate or benzaldehyde was comparable to that of ADP1(pRK415), the wild-type strain carrying the cloning vector. Although BenK was not required for growth, slower growth in its absence is consistent with BenK mediating the uptake of benzoate and benzaldehyde.

The effect of pH on growth was tested, since the higher the pH, and thus the less benzoic acid in its undissociated form, the less diffusion there is of the acid. A 100-μl sample of a mid-log-phase culture was used to inoculate 20 ml of MM, at pH 6.5, 7.0, or 8.1, containing 3 mM benzoate. ACN71, but not ADP1, grew more slowly with benzoate the higher the pH (Fig. 3B). The pH-dependent generation time of ACN71 was consistent with simple diffusion governing the entry of benzoate in this strain. In ADP1, BenK may have mediated benzoate transport, so simple diffusion at high pH did not limit the growth rate. The generation time of ACN71 did not increase at high pH with benzaldehyde or 4-hydroxybenzoate (data not shown), probably because diffusion of the former compound is not pH dependent and because uptake of the latter compound is not catalyzed by BenK. In similar studies, a *P. putida pcaK* mutant grew more slowly the higher the pH, with its substrate, 4-hydroxybenzoate, as the carbon source (11).

Benzoate uptake by the wild-type and *benK* strains. The uptakes of [^{14}C]benzoate (13.3 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) by ADP1 and ACN71 were measured. Cultures, grown in pH 6.8 MM with succinate or benzoate, were prepared as previously described (11, 28). The benzoate-grown cultures were divided into thirds that were harvested, washed, and aerated in phosphate buffer at pH 6.8, 7.5, or 8.1. Individual cell suspensions (0.25 to 1 mg of protein/ml) were combined with 20 μM [^{14}C]benzoate and 10 mM glucose in phosphate buffer of the appropriate pH. Samples were removed at timed intervals and filtered through glass microfiber filters (GF/F; 0.2-μm pore diameter; Whatman International, Ltd., Maidstone, England). The filters were washed, and the [^{14}C]benzoate concentrations were determined as previously described (21).

Benzoate-grown ADP1 accumulated [^{14}C]benzoate at an initial linear rate of approximately 13 nmol of benzoate/min/mg of protein (Fig. 4A), regardless of assay pH. The benzoate-grown ACN71 also took up [^{14}C]benzoate; however, the rate was pH dependent. These initial rates, 12 (pH 6.8), 7 (pH 7.5), and 4 (pH 8.1) nmol of benzoate/min/mg of protein (Fig. 4B), were all lower than those of ADP1. The rate of uptake decreased with increased pH, consistent with uptake in ACN71 being mediated by benzoic acid diffusion followed by metabolism. The rapid conversion to metabolic intermediates might serve to maintain a downhill concentration gradient, thus allowing the continued diffusion of benzoic acid into the cells by mass action. In succinate-grown cells, which do not metabolize benzoate (12, 24), benzoic acid may rapidly enter and exit the cells, accounting for their lack of [^{14}C]benzoate accumulation (Fig. 4B). Moreover, in the wild-type cells, growth with succinate may not induce BenK.

Regulated expression of *benK*. To investigate *benK* expression, a *benK*::*lacZ* fusion was chromosomally integrated into strain ACN109 (Fig. 1). Methods for its construction have been described previously (10, 31). β-Galactosidase (LacZ) activity

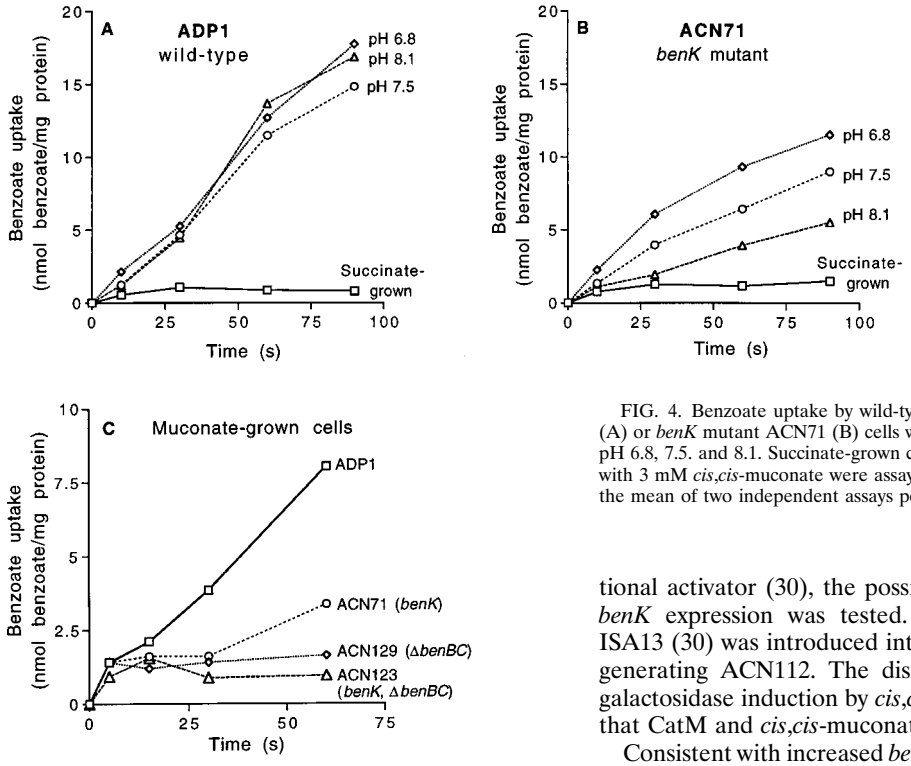

FIG. 4. Benzoate uptake by wild-type and mutant strains. Wild-type ADP1 (A) or *benK* mutant ACN71 (B) cells were grown with benzoate and assayed at pH 6.8, 7.5. and 8.1. Succinate-grown cells were assayed at pH 6.8. Cells grown with 3 mM *cis*,*cis*-muconate were assayed at pH 6.8 (C). Each point represents the mean of two independent assays performed in duplicate.

(22) in cells grown overnight in Luria-Bertani broth (31) with or without the addition of possible inducers was measured. Although benzoate increased the expression of the *benK*::*lacZ* fusion in ACN109 (Fig. 5), even higher β-galactosidase activity resulted from the addition of *cis*,*cis*-muconate, a metabolite derived from benzoate (Fig. 5). To prevent benzoate from being converted to *cis*,*cis*-muconate, strain ACN111 was made by introducing a chromosomal deletion (23) into ACN109, between *Cla*I recognition sites in *benB* and *benC* (24). In ACN111, *cis*,*cis*-muconate, but not benzoate, induced β-galactosidase (Fig. 5). Since *cis*,*cis*-muconate regulates benzoate degradation through interactions with the CatM transcriptional activator (30), the possibility that this regulator affects *benK* expression was tested. The *catM*-disrupted allele of ISA13 (30) was introduced into the chromosome of ACN109, generating ACN112. The disruption of *catM* eliminated β-galactosidase induction by *cis*,*cis*-muconate (Fig. 5), indicating that CatM and *cis*,*cis*-muconate regulate *benK* expression.

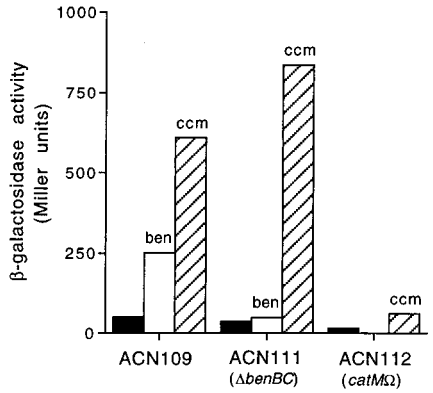

FIG. 5. β-Galactosidase (LacZ) activities of strains each carrying a chromosomal *benK*::*lacZ* fusion. ACN109, ACN111, and ACN112 were derived from wild-type, Δ*benBC* mutant, and *catM*Ω mutant strains, respectively. Cells were grown in Luria-Bertani broth without an inducer (solid bars) or with an inducer, 3 mM benzoate (ben, open bars) or 3 mM *cis*,*cis*-muconate (ccm, hatched bars). Activities are the averages of at least three independent trials, with standard deviations being <20% of average values.

Consistent with increased *benK* expression in the presence of *cis*,*cis*-muconate, *cis*,*cis*-muconate-grown ADP1 rapidly took up [^{14}C]benzoate (Fig. 4 C). In the absence of BenK, benzoate uptake by *cis*,*cis*-muconate-grown ACN71 may result from diffusion followed by benzoate metabolism. To measure benzoate uptake in the absence of its metabolism, mutants blocked in the first step of its degradation were constructed and grown with *cis*,*cis*-muconate to induce expression of *benK*. By previously described methods (10), the *benB-benC* deletion of ACN111 was introduced into the chromosomes of wild-type ADP1 and *benK* mutant ACN71, creating strains ACN129 and ACN123, respectively. In the absence of its metabolism, benzoate did not accumulate to high levels in either ACN129 or ACN123 (Fig. 4C). Although the BenK protein probably catalyzes the accumulation of benzoate against a concentration gradient, rapid diffusion of benzoic acid out of the cell might prevent high-level accumulation under conditions where intracellular benzoate is not being metabolized.

Genetic organization in ADP1. The dependence of *benK* expression on *cis*,*cis*-muconate and CatM connects this novel gene to a regulon involved in benzoate degradation (30). In ADP1, functionally related genes for aromatic compound degradation are found in supraoperonic clusters (10). The *benK* gene, at chromosomal map position 2257 (10), is near genes needed for the conversion of benzoate to tricarboxylic acid cycle intermediates as well as those encoding enzymes for the conversion of benzyl alcohol and benzaldehyde to benzoate (4). The *benK* homologs, *pcaK* and *mucK*, are both located in a chromosomal supraoperonic cluster distal to the *ben* genes, at chromosomal map positions near 2547 (10). Whereas inactivation of *benK* did not affect 4-hydroxybenzoate or *cis*,*cis*-muconate utilization, it not only impaired benzoate metabolism but did so in a pH-dependent fashion, consistent with BenK mediating benzoate transport. Its role as a permease is supported by the evidence presented above and by the sequence homology to better-characterized transporters of the major facilitator superfamily (20, 26).

Nucleotide sequence accession number. The DNA sequence of *benK* has been submitted to GenBank under accession no. AF009224.

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