

## Active Transport of Benzoate in *Pseudomonas putida*

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Benzoate uptake in *Pseudomonas putida* is mediated by an active transport system capable of accumulating benzoate against a 150-fold concentration gradient when subsequent metabolism is blocked by mutation. Initial benzoate transport rates are inhibited by CCCP, sodium azide, arsenate and DCCD. Uptake is stimulated by including a respirable carbon source during preincubation of the bacteria. The initial uptake rate and the ATP pool levels are not correlated and no periplasmic components were found to bind benzoate. These observations indicate that benzoate uptake is energized by the membrane potential, rather than by ATP hydrolysis.

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### INTRODUCTION

*Pseudomonas putida* dissimilates benzoic acid through the catechol branch of the  $\beta$ -ketoacid pathway (Ornston & Stanier, 1966; Reiner, 1971). Mandelate catabolism also occurs through this pathway by conversion to benzoate (Hegeman, 1966). These compounds are weak, relatively hydrophobic acids and were initially presumed to gain entry into cells by diffusion (Rottenberg, 1975). We reported (Thayer & Wheelis, 1976) that benzoate entry into *P. putida* is mediated by a saturatable uptake system whose synthesis (or function) is governed by a gene designated *benP*. Mutation in this gene drastically reduced the ability to accumulate benzoate intracellularly, and prevented growth when benzoate, but not mandelate, was the carbon source. Mutation in genes encoding proteins catalysing subsequent steps in benzoate catabolism did not appear to limit intracellular benzoate accumulation (Thayer & Wheelis, 1976).

While varying assay conditions for possible improvements, we noticed that addition of a respirable carbon source improved uptake rates and substantially decreased the observed  $K_m$  for benzoate. This indicated that active transport of benzoate occurred. We now report that benzoate transport is active and most probably energized by the membrane potential rather than by ATP hydrolysis.

### METHODS

*Organisms.* *Pseudomonas putida* PRS1, the type strain of this species (Stanier *et al.*, 1966), was the parent organism. PRS2003, a mutant derived from PRS1, bears a large deletion in the *catR-B* region of the *P. putida* chromosome and does not grow on benzoate [the *cat* genes are required for its catabolism (Wheelis & Ornston, 1972)]. The *benP* mutant PRS2017 is deficient in benzoate transport and hence does not grow on benzoate (Thayer & Wheelis, 1976).

*Cultural conditions.* Cultures were inoculated from L agar slants (Lennox, 1955) into Hutner's mineral base (Cohen-Bazire *et al.*, 1957) containing succinate, quinate, DL-mandelate or benzoate as a carbon source. Induction

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*Abbreviations:* CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide.

of benzoate transport activity required inclusion of mandelate or benzoate if neither was serving as the carbon source.

**Uptake assays.** Organisms were harvested and washed as previously described (Thayer & Wheelis, 1976) except that the phosphate buffer pH was 6.8. The washed cell suspension was diluted to 0.1 mg dry wt ml<sup>-1</sup> with prewarmed (30 °C) media containing succinate (35 µM) or catechol (54 µM) as a respirable carbon source, and preincubated for 17 min. Inhibitors of energy metabolism, when present, were added 60 s prior to addition of [<sup>14</sup>C]benzoate. Labeled benzoate was added to the final concentrations indicated. Samples (1 ml) were filtered through 25 mm diameter HAWP Millipore filters 0.3, 0.6, 0.9 and 1.2 min after adding the label. Filtered cells were washed with 7 ml prewarmed (30 °C) PAB buffer (Thayer & Wheelis, 1976) and the filters removed from the vacuum 60 s after adding the wash buffer. The filters were dried under IR light and counted in a toluene-based scintillation fluid (Patterson & Green, 1965). Counts bound to uninduced (succinate-grown) cells were subtracted from those bound to induced cells to correct for non-specific binding and diffusion.

**Oxygen uptake assays.** Cells grown to mid-exponential phase with 15 mM-quininate (control) or 15 mM-quininate plus 5 mM-benzoate were harvested by centrifugation, washed twice and resuspended in the growth medium containing chloramphenicol (200 µg ml<sup>-1</sup>), but lacking a carbon source. Two ml of this suspension (0.25 mg dry wt ml<sup>-1</sup>) was placed in 20 ml volume respirometer flasks. The centre well contained 2 M-KOH and a filter paper wick to trap CO<sub>2</sub> and the side-arm held 4.1 µmol sodium benzoate in 50 µl of resuspension medium. Oxygen uptake was measured manometrically in a Gilson differential respirometer at 30 °C for 60 min after mixing benzoate with the bacteria.

**Determination of ATP pools.** Cells grown under the same conditions as used for transport studies were harvested and washed as before, then resuspended in 30 °C growth medium lacking a nitrogen source. Metabolic inhibitors, when present, were included at the indicated concentrations for 1 min in the transport preincubation medium prior to Vortex extraction [2 ml cell suspension in 0.5 ml 30% (v/v) HClO<sub>4</sub> on ice]. The extracted cells were allowed to stand, on ice, for 10 min, neutralized with 1 M-KOH and the precipitated KClO<sub>4</sub> removed by centrifugation (1200 g, 5 min). Samples of the supernatant (0.2 ml) were assayed for ATP using the luciferin-luciferase reaction according to Collins (1974).

CCCP quenched the light emitted too severely to allow measurement of ATP pools in the presence of this inhibitor.

**Chemicals.** All chemicals obtained commercially were of the highest purity available. Uniformly labelled [<sup>14</sup>C]benzoate (108 mCi mmol<sup>-1</sup>; 4.0 GBq mmol<sup>-1</sup>) was obtained from Amersham-Searle. The metabolic inhibitors, CCCP and DCCD, were kindly provided by R. S. Criddle.

## RESULTS AND DISCUSSION

### *K<sub>m</sub> and V<sub>max</sub> determinations*

Values of *K<sub>m</sub>* and *V<sub>max</sub>* were determined with the modified assay using benzoate concentrations between 10- and 135-µM. Lineweaver-Burk plots of the data obtained gave a *K<sub>m</sub>* of 20 µM (compared to our previously published value of 100 µM) and a *V<sub>max</sub>* of 30 nmol min<sup>-1</sup> (mg dry wt)<sup>-1</sup>. Linear regression analysis gave a coefficient of determination (*r*<sup>2</sup>) greater than 0.99.

These kinetic parameters indicate that a *P. putida* cell can transport about 1.4 × 10<sup>5</sup> molecules of benzoate in 1 s. Hydroxyproline and succinate are transported into *P. putida* at a maximal rate of 2.9–3.0 × 10<sup>4</sup> molecules per cell s<sup>-1</sup> (Gryder & Adams, 1970; Dubler *et al.*, 1974). Thus, if transport were rate limiting for the dissimilation of these carbon sources, benzoate would be the best of these compounds. However, succinate yields the highest growth rate, so transport cannot be the rate-limiting factor for growth on benzoate in *P. putida*.

A previous comparison of benzoate uptake by the wild-type and *benP* mutant PRS2017 indicated that PRS2017 accumulated benzoate only to 9% of the level of the wild-type in 5 min (Thayer & Wheelis, 1976). In the present study, comparison of the initial rate of benzoate transport by these strains indicated that the mutant had less than 1% of the activity of the wild-type, [ $<100$  c.p.m. (mg dry wt)<sup>-1</sup> min<sup>-1</sup> for PRS2017 and 11300 c.p.m. (mg dry wt)<sup>-1</sup> min<sup>-1</sup> for PRS 1]. Thus the increase in ability to transport benzoate under the new conditions does not extend to the *benP* mutant. The increase in affinity and velocity of this transport system apparently resulting from the inclusion of a respirable carbon source during the preincubation indicates that this is probably an active transport system.

Table 1. Oxygen consumption due to benzoate oxidation by *P. putida* strains grown on quinate (15 mM) and benzoate (5 mM)

Strain	Genotype	O <sub>2</sub> consumption rate [ $\mu\text{l min}^{-1} (\text{mg dry wt})^{-1}$ ]*
PRS1	Wild-type	13.22
PRS2017	<i>benP1106</i>	0.212
PRS2003	<i>catB-R1111</i>	0.064

\* In excess of the endogenous rate [less than  $0.3 \mu\text{l min}^{-1} (\text{mg dry wt})^{-1}$ , in all cases].

Table 2. Accumulation of [<sup>14</sup>C]benzoate by *P. putida* after 20 min incubation

Organisms were grown on quinate plus benzoate.

Strain	[ <sup>14</sup> C]Benzoate <sub>(out)</sub>	[ <sup>14</sup> C]Benzoate <sub>(in)</sub>	<sup>14</sup> C <sub>(in)</sub> / <sup>14</sup> C <sub>(out)</sub>
PRS1	4.3 $\mu\text{M}$	1.75 mM	400
PRS2003	4.3 $\mu\text{M}$	0.65 mM	148

#### Accumulation of intracellular benzoate

If benzoate transport is active, cells having the permease should accumulate intracellular benzoate against a concentration gradient. To demonstrate such an accumulation, we used a mutant blocked in the subsequent metabolism of benzoate.

The initial step in benzoate metabolism is the addition of molecular oxygen by a dioxygenase (Reiner & Hegeman, 1971; Stanier *et al.*, 1966). No mutants blocked in this step are available in *P. putida*. However, blocks later in the pathway can effectively prevent oxygenation of benzoate by an unknown mechanism, as shown in Table 1, in which oxygen consumption by strain PRS2003 is compared to that of the wild-type and that of the *benP* mutant. Negligible amounts of oxygen were consumed by either mutant, indicating that benzoate oxidation is completely blocked in both strains by the inability of PRS2017 to transport benzoate and the inability of PRS2003 to metabolize it.

The volume of an average *P. putida* cell growing with a generation time of 60 min (e.g. quinate or benzoate as carbon and energy source) has been determined by particle sizing techniques to be 1.45 fl (W. D. Rubenstein, personal communication). The intracellular concentration of [<sup>14</sup>C]benzoate or its metabolic derivatives can thus be estimated. When *P. putida* cultures grown on quinate in the presence of benzoate are permitted to take up [<sup>14</sup>C]benzoate for 20 min, an intracellular accumulation of radioactivity occurs. Table 2 shows the extent of this accumulation in strains PRS1 and PRS2003. PRS1 accumulates <sup>14</sup>C to 400-fold the initial extracellular level in 20 min when labelled benzoate is present at 4  $\mu\text{M}$ . PRS2003, which cannot dissimilate carbon from benzoate, accumulates <sup>14</sup>C to 150 times its extracellular concentration. These estimates are based on total cell volume rather than on the intracellular water volume and therefore represent minimum estimates, indicating that, when metabolism is blocked, *P. putida* is capable of accumulating benzoate against a large concentration gradient.

#### Effects of inhibitors of energy metabolism on benzoate transport and ATP pools

To gain further evidence that benzoate transport is active, we exposed cell suspensions to inhibitors of energy metabolism, then measured their ATP levels and their ability to take up benzoate. Table 3 shows the effect of CCCP, azide, arsenate and DCCD on the initial rates

Table 3. *Effect of 1 min exposure of cells to metabolic energy inhibitors on ATP pools and initial rates of benzoate transport*

Inhibitor	ATP [nmol (mg dry wt) <sup>-1</sup> ]	Percentage of control pool size	[ <sup>14</sup> C]Benzoate [c.p.m. (mg dry wt) <sup>-1</sup> ]*	Percentage of control value
None	1.43	100	17 550	100
48 μM-CCCP	†	†	1 500	9
4.9 mM-Azide	0.41	29	3 640	20
9.7 mM-AsO <sub>4</sub>	1.00	70	4 797	27
1.18 mM-DCCD	1.39	97	4 483	25

\* Average of two determinations.

† CCCP quenches scintillation too severely for ATP to be measured.

of benzoate transport and the formation of ATP pools when included in the preincubation mixture for 1 min. The proton ionophore, CCCP, which dissipates the proton gradient across membranes (Ramos *et al.*, 1976; Johnston *et al.*, 1977), depressed the initial transport rate to about 10% of the control value. Sodium azide, arsenate and DCCD have complex effects (Robertson & Boyer, 1955; Mitchell & Moyle, 1971; Gutowski & Rosenberg, 1976; Reynafarje *et al.*, 1976; Gerdes *et al.*, 1977; Rosenberg *et al.*, 1977). These inhibitors each reduced benzoate uptake by 70–80%, but reduced the ATP pools by 70% (azide), 30% (arsenate) and 3% (DCCD). Clearly the benzoate transport rate is not a specific correlate of the ATP concentration.

Attempts to obtain benzoate binding proteins from concentrated cold osmotic shock fluids of *P. putida* according to the method of Stinnet & Eagen (1975) and identify these by equilibrium dialysis (Furlong *et al.*, 1972) were unsuccessful (unpublished observations).

The stimulation of activity and affinity of the benzoate permease system by respiration, the marked inhibition of transport by transient exposure to the proton ionophore, CCCP, and by azide, arsenate and DCCD, the accumulation of labelled benzoate against a concentration gradient by mutants incapable of dissimilating transported benzoate, the failure of transport to correlate with ATP pool sizes, and our inability to demonstrate the presence of benzoate binding proteins in periplasmic shock fluid (Berger & Heppel, 1974), all indicate that benzoate transport in *P. putida* is active and probably energized by the membrane potential.

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