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Uptake of Benzoic Acid and Chloro-Substituted Benzoic Acids by *Alcaligenes denitrificans* BRI 3010 and BRI 6011

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The mechanism of uptake of benzoic and 2,4-dichlorobenzoic acid (2,4-DCBA) by *Alcaligenes denitrificans* BRI 3010 and BRI 6011 and *Pseudomonas* sp. strain B13, three organisms capable of degrading various isomers of chlorinated benzoic acids, was investigated. In all three organisms, uptake of benzoic acid was inducible. For benzoic acid uptake into BRI 3010, monophasic saturation kinetics with apparent K_m and V_{max} values of 1.4 μM and 3.2 nmol/min/mg of cell dry weight, respectively, were obtained. For BRI 6011, biphasic saturation kinetics were observed, suggesting the presence of two uptake systems for benzoic acid with distinct K_m (0.72 and 5.3 μM) and V_{max} (3.3 and 4.6 nmol/min/mg of cell dry weight) values. BRI 3010 and BRI 6011 accumulated benzoic acid against a concentration gradient by a factor of 8 and 10, respectively. A wide range of structural analogs, at 50-fold excess concentrations, inhibited benzoic acid uptake by BRI 3010 and BRI 6011, whereas with B13, only 3-chlorobenzoic acid was an effective inhibitor. For BRI 3010 and BRI 6011, the inhibition by the structural analogs was not of a competitive nature. Uptake of benzoic acid by BRI 3010 and BRI 6011 was inhibited by KCN, by the protonophore 3,5,3′, 4′-tetrachlorosalicylanilide (TCS), and, for BRI 6011, by anaerobiosis unless nitrate was present, thus indicating that energy was required for the uptake process. Uptake of 2,4-DCBA by BRI 6011 was constitutive and saturation uptake kinetics were not observed. Uptake of 2,4-DCBA by BRI 6011 was inhibited by KCN, TCS, and anaerobiosis even if nitrate was present, but the compound was not accumulated intracellularly against a concentration gradient. Uptake of 2,4-DCBA by BRI 6011 appears to occur by passive diffusion into the cell down its concentration gradient, which is maintained by the intracellular metabolism of the compound. This process could play an important role in the degradation of xenobiotic compounds by microorganisms.

The first step in the degradation of most compounds by a microorganism is their transfer across the cell membrane(s) into the cytoplasm. Uptake of aromatic compounds has been studied from two different perspectives. For toxic compounds, control of their permeation into microorganisms unable to degrade them entails the evolution of physiological and structural modifications leading to restricted permeation. The capacity of cells to develop these may be an important survival mechanism (24, 25). For metabolizable aromatic compounds, uptake can occur by diffusion (17, 18, 31) or via the evolution of highly specific permeases (15, 43, 46). Aromatic acids, being lipophilic weak acids, are generally assumed to traverse cell membranes by passive diffusion. Indeed, such compounds have been used for measuring pH gradients across membranes (22, 25). Little is known, however, about the uptake mechanisms of chlorinated aromatic compounds.

Microbial metabolism of polychlorinated biphenyls (PCBs) by pure cultures of PCB-degrading microorganisms is generally incomplete and results in the formation of isomeric mixtures of chlorinated benzoic acids (CBAs) as dead-end metabolites (2, 13, 30a). More complete degradation of PCBs by cocultures consisting of both PCB- and CBA-degrading microorganisms, in which the rate of CBA removal from the growth medium dictated the rate of PCB degradation by the cells, has been reported (1, 27, 34, 38–40).

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Organisms capable of degrading 2-CBA (4, 9, 11, 12, 41, 42, 47), 3-CBA (5, 7, 16, 21, 35), and 4-CBA (23, 26, 30, 29, 44) have been isolated. Less is known about microorganisms with dichlorobenzoic acid (DCBA)-degradative capabilities (19, 20). Two strains of *Alcaligenes denitrificans*, BRI 3010 and BRI 6011, which can metabolize benzoic acid, 2-CBA, 2,3-DCBA, and 2,5-DCBA, have been isolated. In addition, strain BRI 6011 can degrade 2,4-DCBA (32). Degradation of benzoic acid and the CBAs by both organisms has been shown to proceed via intradiol cleavage of the aromatic ring. *Pseudomonas* sp. strain B13 also degrades benzoic acid via intradiol cleavage of the aromatic ring (7). Interestingly, a chlorocatechol 1,2-dioxygenase isolated from *Pseudomonas* sp. strain B13 was very similar, on the basis of biochemical and genetic characterization, to the purified enzyme from *A. denitrificans* BRI 6011 (14, 33). The only chlorinated benzoic acid isomer degraded by *Pseudomonas* sp. strain B13 is 3-CBA, a substrate not utilized by either BRI 3010 or BRI 6011, although the expected intermediates, 3- and 4-chlorocatechol, are effectively metabolized (32). A less-specific dioxygenation of the CBAs or a different uptake mechanism, or both, may be factors which influence the increased versatility for CBA degradation by BRI 3010 and BRI 6011.

In this study, the characteristics of the processes of benzoic acid uptake into *A. denitrificans* BRI 3010 and BRI 6011 and of 2,4-DCBA uptake into BRI 6011 were examined. These and associated findings have provided insight into the role of uptake in influencing substrate specificity in BRI 3010, BRI 6011, and *Pseudomonas* sp. strain B13.

MATERIALS AND METHODS

Organisms. The isolation and characterization of *A. denitrificans* BRI 3010 and BRI 6011 and *Pseudomonas* sp. strain B13 have been described previously (7, 32).

Growth conditions. The cells were grown in minimal salts medium (32) supplemented with yeast extract (5 g/liter) and 5 mM benzoic acid, 1 mM 2,4-DCBA, or 1 mM 2,5-DCBA. Cells were grown in Erlenmeyer flasks on a rotary shaker at 30°C.

Preparation of cells. Cells grown to late log phase were harvested by centrifugation (7,700 × *g* for 10 min at 4°C) and washed three times with volumes of 50 mM potassium phosphate (pH 7.2) equal to the volume of the original medium. The washed pellet was resuspended in the same buffer to yield a final concentrated stock suspension of 7 mg of cell dry weight per ml and kept on ice for the duration of the experiment.

Measurement of transport. Transport experiments were conducted by procedures reported previously (8). Washed cells were added to a final density of 200 μg of cell dry weight per ml to a reaction chamber containing phosphate buffer (pH 7.2) and chloramphenicol at final concentrations of 50 mM and 150 μg/ml, respectively. The reaction mixture, whose final volume was 3.5 ml, was kept homogeneous and aerated by magnetic stirring while the temperature was maintained at 30°C by circulating water. The cell suspensions were allowed to equilibrate for 15 min before the addition of ^{14}C-labeled substrates. The reaction was started by adding [*ring*-U-^{14}C]benzoic acid or 2,4-[*ring*-U-^{14}C]DCBA at a final concentration of 20 μM and at a specific activity of 5 μCi/μmol unless otherwise stated. To determine the effect of benzoic acid and 2,4-DCBA concentrations on initial rates of uptake of the respective compounds, concentrations were varied from 0.1 to 20 μM with specific activities varying appropriately. At no time did the amount of radioactivity taken up by the cells exceed 0.2% of that present in the incubation medium.

Uptake experiments under anaerobic conditions in the presence or absence of nitrate (KNO_3; final concentration, 60 mM) were performed essentially as for the aerobic uptake experiments except that the incubation mixture was flushed continuously with oxygen-free nitrogen gas. Cells were incubated for 30 min under these conditions prior to the addition of the labeled substrates.

After the addition of the labeled substrate at time zero, 0.5-ml samples were removed from the reaction mixture at 15-s intervals for 1 min and then at 1-min intervals for 4 min. The samples were filtered through membrane filters (Millipore type HA; pore size, 0.45 μm). The cells on the filters were washed immediately with 10 ml of phosphate buffer, placed in preheated scintillation vials, and then exposed to an infrared lamp to stop metabolic activity in the cells. For each experiment, a reaction mixture (i) with boiled cells, (ii) with live cells in the presence of the protonophore 3,3′,4′,5-tetrachlorosalicylanilide (TCS) (10 μM), which was predetermined to inhibit uptake, or (iii) without cells was filtered in the same manner to determine any nonspecific binding of radioactivity to the cells and/or filters. Initial rates of uptake were determined from the slopes of the tangents to the curves for uptake, passing in each case through the point at zero time.

Measurement of radioactivity. A 7-ml volume of Universal (ICN Biochemicals, Irvine, Calif.) was added to the cooled dried filters in the scintillation vials. Radioactivity was determined with an LS-7500 liquid scintillation counter (Beckman Instruments Inc., Irvine, Calif.) (8).

Extraction of intracellular substrate pools after transport. The procedure used was based on that described by Droniuk et al. (8). The uptake of benzoic acid was as described above, except that the volume of the reaction mixture was reduced to 1.4 ml. Sampling was done only once at 4 min for benzoic acid and at 10 min for 2,4-DCBA. After each sample was filtered and washed, the filter was transferred to a centrifuge tube containing 10 ml of water and kept at 90°C in a water bath. The uptake procedure was performed six times, and the filters were pooled. In this manner, six filters with a combined cell dry weight of 600 μg were collected. After the last filter was added, the tubes were kept at 90°C for 15 min, cooled, and centrifuged (40,000 × *g* for 15 min at 4°C). A second 10-ml portion of water was added to the filters, which were then held at 90°C for 15 min. After centrifugation, the second supernatant was pooled with the first. The pooled supernatants were acidified to pH 2 and extracted twice with equal volumes of ethyl acetate. The organic phase was separated and evaporated to dryness with a stream of nitrogen. The extracted residue was then dissolved in a known volume of ethyl acetate.

Analysis of cell extracts by thin-layer chromatography. A known volume (5 to 10 μl) of concentrated radioactive cell extract was spotted on a silica gel plate (Baker-Flex 1B2; J. T. Baker Chemical Co., Phillipsburg, N.J.). The plate was developed by the method of Reiner (37) with a benzene-methanol-acetic acid (4:6:1) solvent system. After being dried with warm air, the plate was autoradiographed against X-ray film (X-Omat AR; Kodak, Rochester, N.Y.) for ~96 h. Spots representing benzoic acid or 2,4-DCBA were identified by comparing their migration on the plate with that of authentic samples. The spot representing benzoic acid or 2,4-DCBA was scraped into a scintillation vial for measurement of radioactivity. The intracellular concentration of the unchanged metabolite was calculated by assuming an intracellular fluid volume of 1.5 μl/mg of cell dry weight. This value was based on an average of 1.46 ± 0.125 μl/mg of cell dry weight determined from figures reported for eight other gram-negative rod forms of similar dimensions (28).

TABLE 1. Effect of growth phase on initial rates of benzoic acid uptake by *A. denitrificans* BRI 3010 and BRI 6011 and *Pseudomonas* sp. strain B13

Organism[a]	Rate of uptake[b] (nmol/min/mg of cell dry wt) at:		
	Mid-log phase	Late log phase	Stationary phase
BRI 3010	0.33 ± 0.12	2.02 ± 0.18	0.59 ± 0.11
BRI 6011	0.63 ± 0.14	2.20 ± 0.13	1.02 ± 0.20
Pseudomonas strain B13	2.39 ± 0.19	2.43 ± 0.15	2.41 ± 0.18

[a] Cells were grown on minimal salts yeast extract medium supplemented with 5 mM benzoic acid. *Pseudomonas* strain B13 gave similar results when cells were also grown on minimal salts medium to which 10 mM benzoic acid but no yeast extract was added.

[b] Results represent the mean ± average deviation of at least two determinations.

Cell dry weight determinations. Dry weights of cell suspensions were determined turbidimetrically at 660 nm (microsample spectrophotometer; model 300-N; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). A curve relating cell dry weight to turbidity was established for each organism. From the linear portion of each curve, an optical density at 660 nm of 1.0 (1-cm light path) was calculated. This was found to be equivalent to 0.459, 0.496, and 0.530 mg (dry weight) of cells per ml of *A. denitrificans* BRI 3010 and BRI 6011 and *Pseudomonas* sp. strain B13, respectively. These values were determined by washing fractions of cell suspensions three times in distilled water followed each time by centrifugation. The cell pellets were dried to constant weight by lyophilization. To measure the dry weight of the cells in all other suspensions, dilutions of the suspensions were made into the optical density range corresponding to the linear portion of the curves.

Chemicals. The [*ring*-U-^{14}C]benzoic acid (specific activity, 15 mCi/mmol) was obtained from Sigma Chemical Co., St. Louis, Mo. The 2,4-[*ring*-U-^{14}C]DCBA (specific activity, 54 mCi/mmol) was obtained from Amersham, Oakville, Ontario, Canada. Nonradioactive aromatic substrates were obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis. All other chemicals used were reagent grade.

RESULTS

Effect of culture age on uptake rates of benzoic acid. Previous studies with *A. denitrificans* BRI 6011 have shown that the maximal specific activity of chlorocatechol-1,2-dioxygenase, a crucial enzyme involved in the degradation of chlorobenzoic acids, was obtained with cells in the early logarithmic phase of growth (33). Initial rates of uptake of benzoic acid into *A. denitrificans* BRI 3010 and BRI 6011 were found to be highest in cells harvested in the late logarithmic phase of growth, whereas in *Pseudomonas* strain B13 the uptake rate was unaffected by the age of the culture (Table 1). A similar study established that the uptake of 2,4-DCBA by *A. denitrificans* BRI 6011 was not affected by the age of the culture (results not shown).

Inducibility of benzoic acid and 2,4-DCBA uptake. Cells of *A. denitrificans* BRI 6011 grown on minimal salts yeast extract medium failed to take up benzoic acid at an appreciable rate unless the growth medium was supplemented with benzoic acid (Table 2). Similar results were obtained with strain BRI 3010 and *Pseudomonas* sp. strain B13 (results not shown). Table 2 also shows that the presence of 2,4-DCBA or benzoic acid in the growth medium for *A. denitrificans* BRI 6011 reduced the apparent rate of uptake of radioactivity into the subsequently harvested cells.

Kinetics of uptake of benzoic acid and 2,4-DCBA. When the initial rate of uptake of benzoic acid by *A. denitrificans* BRI 3010 was plotted against substrate concentration, evidence for saturation kinetics was obtained (Fig. 1). A double-reciprocal plot of the data gave a K_m for benzoic acid of 1.4 μM and a V_{max} of 3.2 nmol/min/mg of cell dry weight (inset in Fig. 1).

TABLE 2. Effect of benzoic acid and 2,4-DCBA in the growth medium on the subsequent apparent capacity of *A. denitrificans* BRI 6011 to transport the compounds into the cells

Supplement[a]	Concn (mM)	Rate of uptake[b] (nmol/min/mg of cell dry wt) of:	
		BA	2,4-DCBA
None	0	0.26 ± 0.03	0.63 ± 0.03
BA	5	2.51 ± 0.12	0.28 ± 0.02
2,4-DCBA	1	0.20 ± 0.01	0.41 ± 0.04
	2	0.21 ± 0.01	0.23 ± 0.02
	5	0.20 ± 0.02	0.12 ± 0.01

[a] To the minimal salts yeast extract medium. Cells were grown to late log phase. BA, benzoic acid.
[b] Mean ± average deviation of at least two determinations.

Uptake of benzoic acid by *A. denitrificans* BRI 6011 displayed evidence of biphasic saturation kinetics even when all the points obtained were plotted on the same scale (Fig. 2A). When the reciprocals of the initial rates of uptake were plotted against the reciprocals of the benzoic acid concentrations over the two concentration ranges, 0.1 to 1.67 μM and 5.0 to 20 μM, biphasic saturation kinetics was confirmed and two K_m and V_{max} values were obtained (Fig. 2B and C). This provides evidence for both high- and low-affinity benzoic acid transport systems in this organism with K_m and V_{max} values of 0.72 μM and 3.3 nmol/min/mg of cell dry weight and 5.3 μM and 4.6 nmol/min/mg of cell dry weight, respectively.

When the rate of uptake of 2,4-DCBA by *A. denitrificans* BRI 6011 was plotted against substrate concentration, it was found to be almost directly proportional to the concentration of substrate in the incubation medium (Fig. 3). A double-reciprocal plot of the data gave rise to a line passing through the origin, thus confirming the absence of saturation kinetics (Fig. 3, inset).

Effect of CBAs and other structurally related compounds on benzoic acid and 2,4-DCBA uptake. The effect of various CBAs and other compounds structurally related to benzoic acid on benzoic acid uptake was examined. With the exception of phenoxyacetic acid, all the compounds tested including those which are not metabolizable (32), when added at a concentration 50-fold greater than that of the benzoic acid, significantly inhibited benzoic acid uptake by *A. denitrificans* BRI 3010 and BRI 6011 (Table 3). Benzoic acid uptake by *Pseudomonas* sp. strain B13, however, was inhibited only by 3-CBA, the only CBA isomer that has been found to be metabolized by the organism.

A 50-fold concentration of each of the same structurally related compounds had a greater inhibitory influence on the uptake of 2,4-DCBA by *A. denitrificans* BRI 6011 than on benzoic acid uptake by the same strain. In addition, phenoxyacetic acid partially inhibited 2,4-DCBA uptake.

When the effect of 2-hydroxybenzoic acid on benzoic acid uptake by *A. denitrificans* BRI 6011 was examined in more detail, it was observed that at low concentration the compound actually stimulated benzoic acid uptake (Fig. 4). Only when the concentration of 2-hydroxybenzoic acid exceeded that of benzoic acid was inhibition obtained. Two other compounds, 2,4- and 2,5-DCBA, which are metabolized by the organism and which also inhibit benzoic acid uptake when added at a concentration 50-fold that of benzoic acid, failed to stimulate benzoic acid uptake when added at lower concentrations (results not shown).

The addition of the metabolizable substrate potassium citrate (1 mM) to cell suspensions 10 min prior to the addition of the labeled substrate resulted in a 30 to 40% increase in the

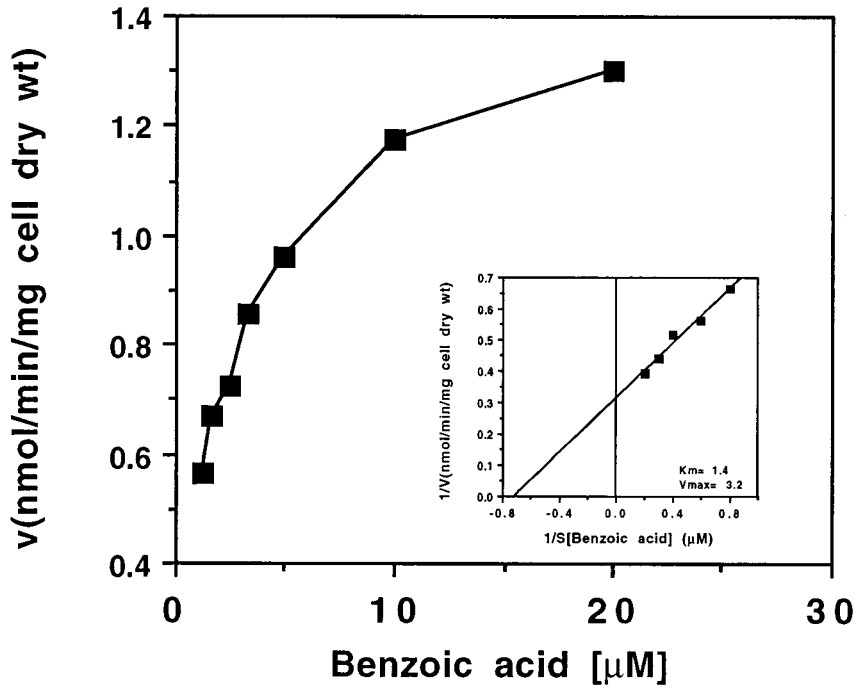

FIG. 1. Effect of benzoic acid concentration on the initial rates of benzoic acid uptake by *A. denitrificans* BRI 3010 under aerobic conditions. The inset shows a double-reciprocal plot of the data and the K_m and V_{max} values derived from it.

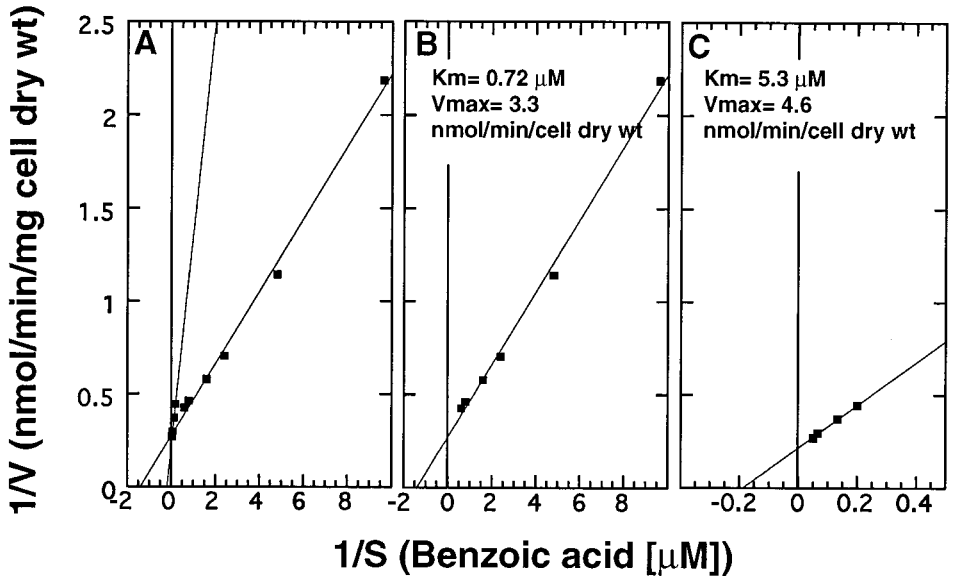

FIG. 2. Double-reciprocal plots of initial rates of benzoic acid uptake at various concentrations of benzoic acid by *A. denitrificans* BRI 6011 under aerobic conditions over the concentration ranges 0.1 to 20 μM (A), 0.1 to 1.67 μM (B), and 5 to 20 μM (C) benzoic acid. Shown are the K_m and V_{max} values derived from plots B and C.

rate of uptake of benzoic acid by *A. denitrificans* BRI 3010 and BRI 6011 and *Pseudomonas* sp. strain B13 (results not shown).

When the kinetics of inhibition of benzoic acid uptake by 2-hydroxybenzoic acid into *A. denitrificans* BRI 6011 were examined by using a Cornish-Bowden plot (6), the lines obtained by linear regression were convergent rather than parallel and thus were not indicative of competitive inhibition (Fig. 5). The effects of 2,5-DCBA on benzoic acid uptake by *A. denitrificans* BRI 3010, when plotted in the same way, gave rise to lines which were similarly convergent and also not indicative of competitive inhibition (results not shown).

Capacity to accumulate substrates against a gradient. To establish whether active transport of benzoic acid or of 2,4-DCBA had occurred, the ability of the cells to accumulate each compound in an unchanged state against a gradient was determined. For this purpose, the amount of radioactivity extracted

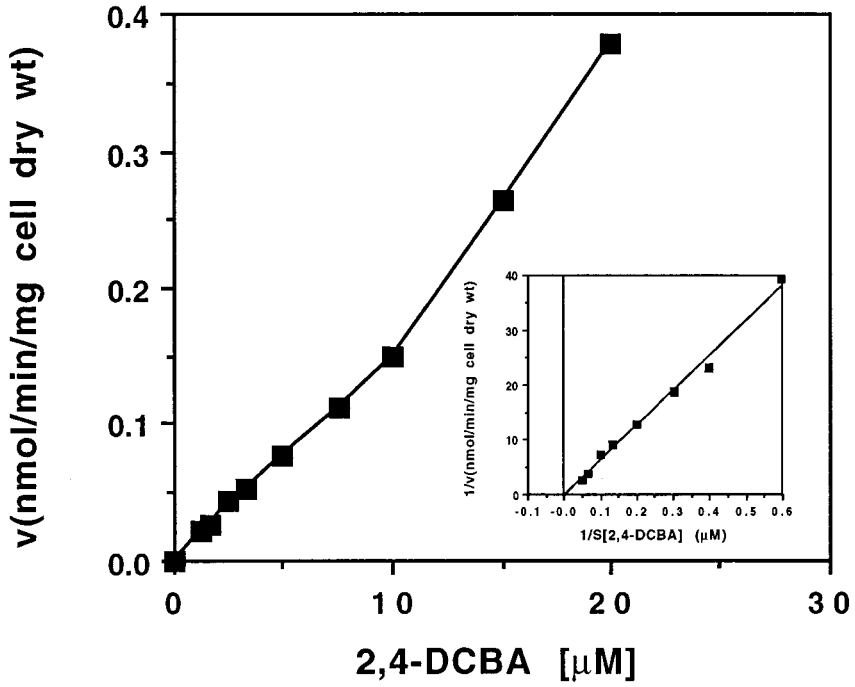

FIG. 3. Effect of 2,4-DCBA concentration on the initial rates of 2,4-DCBA uptake by *A. denitrificans* BRI 6011 under aerobic conditions. The inset shows a double-reciprocal plot of the data.

TABLE 3. Effect of various CBAs and other structurally related compounds on initial rates of benzoic acid and 2,4-DCBA uptake[a]

Compound added[b]	% Inhibition of uptake of:			
	BA			2,4-DCBA by BRI 6011
	BRI 3010	BRI 6011	B13	
BA[c,d]	95.1 ± 2.5	96.3 ± 1.1	94.9 ± 2.0	83.2 ± 4.1
2-CBA[c]	91.1 ± 1.1	71.8 ± 4.2	83.2 ± 3.3	98
3-CBA[d]	87.4 ± 1.3	65.4 ± 7.1	80.8 ± 1.8	99
4-CBA	78.7 ± 3.3	72.9 ± 3.5	11.5 ± 2.4	ND[e]
2,3-DCBA[c]	70.6 ± 4.1	62.8 ± 7.7	ND	ND
2,4-DCBA[c]	73.4 ± 6.3	50.4 ± 8.1	8.3 ± 3.3	92.1 ± 3.5
2,5-DCBA[c]	88.5 ± 3.2	75.4 ± 4.4	9.2 ± 2.9	100
2,5-DCBA	55.6 ± 10.0	41.7 ± 9.3	11.4 ± 2.2	100
3,4-DCBA	81.3 ± 3.4	76.6 ± 5.0	19.5 ± 3.7	100
3,5-DCBA	84.3 ± 2.9	82.3 ± 2.4	ND	100
2,4-DFBA[c]	71.3 ± 4.0	80.4 ± 2.9	ND	84.1 ± 3.6
2,5-DFBA[c]	90.5 ± 1.5	77.9 ± 4.2	10.7 ± 2.1	ND
2-HBA[c]	95.7 ± 1.2	96.1 ± 1.0	16.6 ± 2.3	93.8 ± 1.1
2,4-DHBA[c]	54.4 ± 11.1	40.9 ± 9.2	8.2 ± 1.9	78.2 ± 4.4
2,5-DHBA[c]	56.5 ± 9.9	ND	9.1 ± 1.2	ND
3,4-DHBA[c]	53.2 ± 9.2	ND	6.6 ± 2.0	91.6 ± 2.0
PA	0	0	7.2 ± 2.5	33.9 ± 6.7
2,4-D	47.8 ± 10.2	55.6 ± 8.9	0	95.3 ± 1.6

[a] The unlabeled compound (1 mM) was added to the incubation medium 1 min prior to the addition of [^{14}C]benzoic acid or 2,4-[^{14}C]DCBA (20 μM; 5 μCi/μmol). The percent inhibition of benzoic acid uptake was calculated from the initial rates of benzoic acid uptake in the presence and absence of each compound. Cells were grown to late log phase in minimal salts yeast extract medium supplemented with benzoic acid.

[b] BA, benzoic acid; PA, phenoxyacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2-HBA, 2-hydroxybenzoic acid; DHBA, dihydroxybenzoic acid; DFBA, difluorobenzoic acid.

[c] Metabolized by BRI 3010 and BRI 6011, with the exception of 2,4-DCBA which is not metabolized by BRI 3010.

[d] Metabolized by *Pseudomonas* sp. strain B13.

[e] ND, not determined.

by hot water as unchanged metabolite from a known weight of cells was determined. The intracellular concentration of unchanged metabolite was then calculated, assuming an intracellular fluid volume of 1.5 μl/mg of cell dry weight. The results reported in Table 4 show that both *A. denitrificans* BRI 3010 and BRI 6011 were able to concentrate benzoic acid inside the cells against a gradient to levels 8.5 and 10.4 times, respectively, the external concentration. The percentage of radioactivity in the cells that was recovered as unchanged metabolite after a 4-min exposure to [^{14}C]benzoic acid was low (3%), however, and the results indicate a rapid conversion of the benzoic acid into extractable intermediary metabolites and nonextractable cell components.

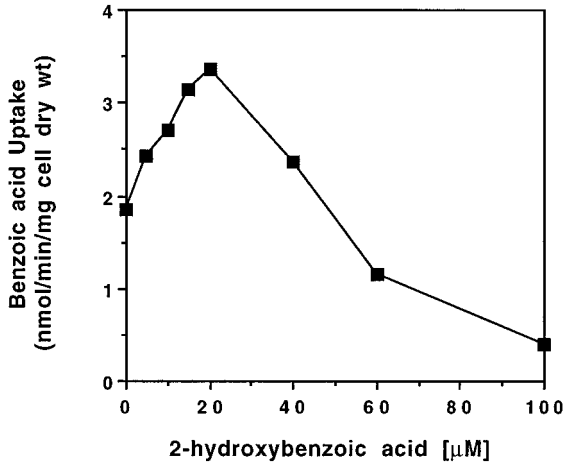

FIG. 4. Effect of 2-hydroxybenzoic acid concentration on the initial rates of benzoic acid uptake by *A. denitrificans* BRI 6011 under aerobic conditions.

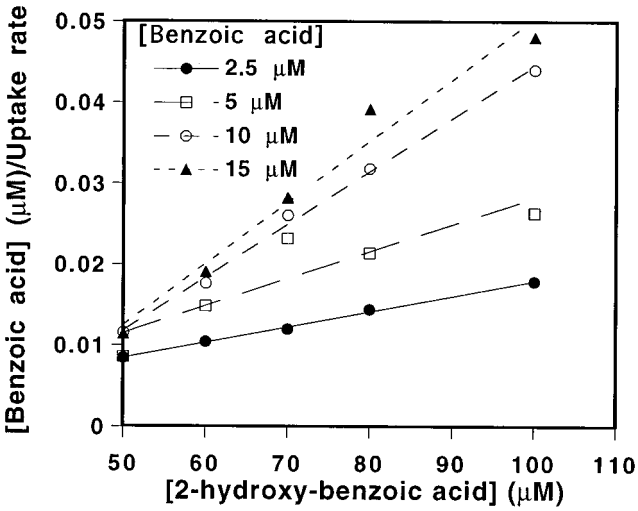

FIG. 5. Cornish-Bowden plot showing the kinetics of inhibition of benzoic acid uptake into *A. denitrificans* BRI 6011 by 2-hydroxybenzoic acid. Rates of uptake of benzoic acid (in nanomoles per minute per milligram of cell dry weight) were determined at the indicated concentrations of benzoic acid in the presence of increasing concentrations of 2-hydroxybenzoic acid.

Table 4 also shows that *A. denitrificans* BRI 6011 failed to concentrate 2,4-DCBA inside the cells against a gradient. After a 10-min exposure of the cells to 2,4-[^{14}C]DCBA, only 0.4% could be recovered as unchanged metabolite while the rest had been converted to other extractable metabolites and nonextractable cell material.

Effect of metabolic inhibitors on uptake. The protonophore TCS and the respiratory inhibitor KCN, which destroy and prevent, respectively, the formation of a proton motive force (Δp), inhibited benzoic acid uptake almost completely and 2,4-DCBA uptake to a somewhat lesser extent (Table 5).

Effect of anaerobic conditions on uptake. The ability of TCS and KCN to inhibit the uptake of benzoic acid and 2,4-DCBA by the organisms tested indicates that energy is involved in the uptake process in the organisms tested. The effects on uptake

TABLE 4. Ability of *A. denitrificans* BRI 3010 and BRI 6011 to concentrate benzoic acid inside the cells against a gradient and failure of BRI 6011 to do the same with 2,4-DCBA

Metabolite taken up	Organism	*I*/*E* ratio[a]	% Recovery[b] as:		
			Unchanged metabolite	Total extractable	Non-extractable
BA[c]	BRI 3010	8.5	3.0	58	42
	BRI 6011	10.4	3.1	54	46
2,4-DCBA	BRI 6011	0.6	0.4	30	70

[a] The *I*/*E* ratio is the ratio of intracellular concentration of unchanged metabolite (*I*) to initial extracellular concentration (*E*).

[b] Percent recovery in each category is expressed as the percentage of total radioactivity present in the cells after incubation for 4 min in the presence of [^{14}C]benzoic acid or 10 min in the presence of 2,4-[^{14}C]DCBA. The substrates were each added as their K^+ salt.

[c] BA, benzoic acid.

TABLE 5. Effect of metabolic inhibitors on initial rates of uptake of benzoic acid and 2,4-DCBA

Inhibitor[a]	% Inhibition of uptake[b] of:		
	Benzoic acid		2,4-DCBA by BRI 6011
	BRI 3010	BRI 6011	
TCS (10 μM)	87.2 ± 1.6	89.7 ± 1.3	77.2 ± 1.3
KCN (10 mM)	92.1 ± 1.5	94.4 ± 1.1	80.3 ± 2.4

[a] Inhibitors were added to the suspension of cells in the incubation medium 15 min prior to the addition of the radioactive substrate.

[b] Cells were grown in minimal salts yeast extract medium supplemented with 5 mM benzoic acid or 1 mM 2,4-DCBA for benzoic acid and 2,4-DCBA uptake studies, respectively. Results are presented as the average ± average deviation of two determinations.

under anaerobic conditions confirm this (Fig. 6). *A. denitrificans* BRI 6011 failed to take up benzoic acid in the absence of O_2 unless nitrate was present. The rate of uptake with nitrate, however, was much lower than with O_2. Nitrate failed to substitute for O_2 for the uptake of 2,4-DCBA.

From the results in Fig. 6, it can be calculated that in the presence of O_2 the rate of uptake of benzoic acid into *A. denitrificans* BRI 6011 is some 13 times higher than the rate of uptake of 2,4-DCBA.

DISCUSSION

The uptake of benzoic acid into *A. denitrificans* BRI 3010 and BRI 6011 and *Pseudomonas* sp. strain B13 was inducible. The rates of uptake of benzoic acid into *A. denitrificans* BRI 3010 and BRI 6011 in response to benzoic acid concentration demonstrated saturation kinetics. These observations support the conclusion that the uptake of benzoic acid into *A. denitrificans* BRI 3010 and BRI 6011 is carrier mediated. Furthermore, in both *A. denitrificans* BRI 3010 and BRI 6011, benzoic acid was concentrated inside the cells against a gradient, indicating that the transport process was an active one. The results obtained also show that both organisms metabolize benzoic acid rapidly once it is inside the cells. This very probably accounts for the internal/external concentration ratio not exceeding 10. *Pseudomonas putida* was found to bring about the inducible energy-dependent uptake of benzoic acid and to be capable of accumulating the substrate against a 150-fold concentration gradient when the subsequent metabolism of benzoic acid was blocked by mutation (43).

The monophasic saturation kinetics observed when benzoic acid was taken up by *A. denitrificans* BRI 3010 indicates that there is one high-affinity transport system for benzoic acid in this organism. The biphasic saturation kinetics obtained with strain BRI 6011 is consistent with the presence of two systems, a low-affinity system and a high-affinity system, for the uptake of this compound. Dual transport systems are common, particularly for the transport of branched-chain amino acids (3, 10), but are less well known for aromatic compounds. *Rhizobium leguminosarum* has been shown to accumulate two closely related compounds, 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid, against a gradient via separate transport systems (46).

The ability of the protonophore TCS and the respiratory inhibitor KCN to inhibit transport of benzoic acid into *A. denitrificans* BRI 3010 and BRI 6011 argues for a proton symport system being involved in the uptake process. The inability of BRI 6011 to take up benzoic acid under anaerobic conditions unless nitrate was present or 2,4-DCBA unless oxygen was present is further support for the conclusion that energy is involved in the uptake of these compounds.

The rate of uptake of 2,4-DCBA by *A. denitrificans* BRI 6011 was some 13 times lower than the rate of uptake of benzoic acid by the same organism. Growth in the presence of 2,4-DCBA reduced rather than increased the rate of uptake of radioactivity; hence, induction of a carrier system appeared not to be involved. The initial rate of uptake of 2,4-DCBA into *A. denitrificans* BRI 6011 showed no evidence of saturation kinetics but instead was directly proportional to the extracellular 2,4-DCBA concentration over the range of concentrations tested. These observations are consistent with 2,4-DCBA entering strain BRI 6011 by diffusion through the inner and outer membranes of this organism down a concentration gradient into the cells. A lipophilic weak acid would be expected to diffuse through the lipid bilayers of this organism in an undissociated form with the downhill gradient maintained by a ΔpH inside alkaline and by the further conversion of the entering compound to other, less lipophilic, intermediates and end

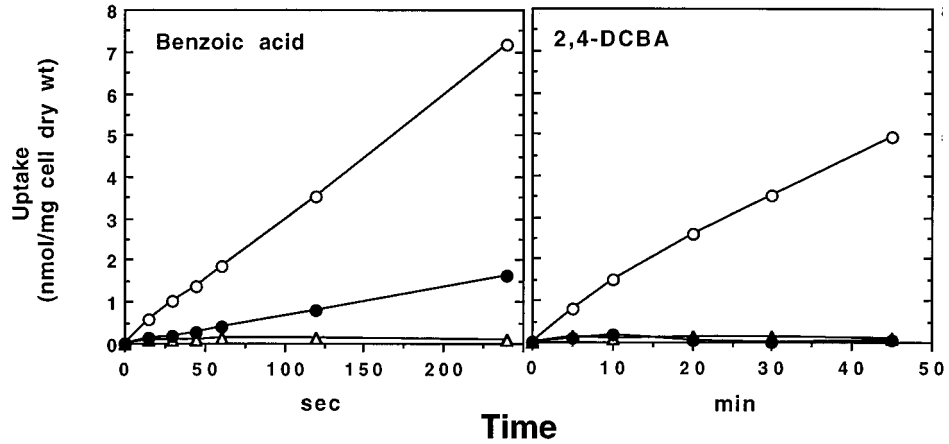

FIG. 6. Uptake of benzoic acid and 2,4-DCBA by *A. denitrificans* BRI 6011 cells under aerobic (○) and anaerobic conditions in the absence (△) and presence (●) of nitrate (added as KNO_3; final concentration, 60 mM). Cells were grown aerobically in the presence of either 5 mM benzoic acid or 1 mM 2,4-DCBA for benzoic acid and 2,4-DCBA uptake experiments, respectively. Uptake under anaerobic conditions was performed in an identical fashion to that for aerobic uptake experiments, except that the incubation mixture was flushed continuously with oxygen-free nitrogen gas. Cells were incubated for 30 min under these conditions prior to the addition of the ^{14}C-labeled substrate.

products by metabolism. Inhibition of the accumulation of radioactivity by cells incubated with 2,4-[^{14}C]DCBA by TCS, KCN, and anaerobiosis, compounds or conditions which interfere with the maintenance and generation of ΔpH and of the ATP needed for bioconversions, is consistent with this mechanism of entry. The fact that nitrate can replace O_2, although much less effectively for the accumulation of radioactivity by *A. denitrificans* BRI 6010 when [^{14}C]benzoic acid but not when 2,4-[^{14}C]DCBA is the substrate, suggests that the carrier-mediated transport process is more energy efficient than the combination of diffusion and metabolism.

When 2,4-DCBA (at 1 to 5 mM) and benzoic acid (at 5 mM) were present in the growth medium, the subsequent rate of uptake of 2,4-DCBA appeared to be reduced (Table 2). Unlabeled 2,4-DCBA and benzoic acid in the growth medium would be expected to enter the cell and give rise to common metabolites which would reduce the specific activity of metabolites derived from incoming 2,4-[^{14}C]DCBA (added to the cell suspension at 20 μM) in the subsequent transport experiment. The actual rate of uptake and metabolism of 2,4-DCBA should thus remain the same, but the rate of incorporation of radioactivity into the cells would be reduced.

The results (Table 4) show that 70% of the radioactivity in BRI 6011 after incubation with 2,4-[^{14}C]DCBA for 10 min was recovered in the nonextractable fraction. Since intradiol cleavage ultimately leads to intermediates of the tricarboxylic acid cycle (32), one would expect all the cell polymers as well as other compounds insoluble in hot water to become radioactive. The only exception expected would be protein, since the medium used to suspend the cells in the transport studies contained chloramphenicol.

A wide range of chlorobenzoic acids and other structurally related compounds both metabolizable and nonmetabolizable by the cells showed a marked capacity to reduce the rate of uptake of benzoic acid by *A. denitrificans* BRI 3010 and BRI 6011. Since induction of benzoic acid transport in strain BRI 6011 was shown not to induce an increased rate of 2,4-DCBA uptake by the organism, it is apparent that 2,4-DCBA is not a substrate for the benzoic acid transport systems in this organism. This is further confirmed by the fact that 2,4-DCBA appears not to enter the cells by a carrier-mediated system but, rather, by diffusion through the cytoplasmic membrane. Thus inhibition of benzoic acid uptake by 2,4-DCBA cannot be ascribed to competition between the two compounds for a binding site on a carrier protein. It also seems unlikely that 2,4-DCBA inhibits benzoic acid uptake by interfering with enzymes involved in the metabolism of benzoic acid inside the cells, since the blocking of metabolism should not prevent the accumulation of benzoic acid in the cells against a gradient. It seems likely that at high enough external concentrations, these lipophilic weak acids can provide a sufficient influx of protons to destroy the proton motive force in the cells. This would explain why 2-hydroxybenzoic acid, which, like citrate, is metabolizable and able to stimulate respiration, would be able to stimulate benzoic acid uptake at low concentrations but be inhibitory at high concentrations. Inhibition of the rate of uptake of 2,4-DCBA into BRI 6011 by the various CBAs was even more complete than that of benzoic acid, suggesting again that the diffusion metabolism system of uptake is less energy efficient than the carrier-mediated one.

Some of the inhibition of uptake of benzoic acid and 2,4-DCBA by the metabolizable but not the nonmetabolizable analogs of these compounds in BRI 3010 and BRI 6011 may be more apparent than real because, as indicated above, the metabolizable compounds can give rise to common metabolites in the cells which would reduce the specific activity of those arising from the radioactive substrate.

The evidence obtained from the benzoic acid uptake inhibition studies (Table 3) suggests that all of the mono- and dichlorobenzoic acid isomers tested are able to enter the cells of *A. denitrificans* BRI 3010 and BRI 6011 by passive diffusion. Which of these isomers can be metabolized thus appears to be determined by the degree of specificity of enzymes inside the cells involved in their metabolism.

Finally, of the chlorinated analogs tested, only 3-CBA inhibited benzoic acid uptake by *Pseudomonas* sp. strain B13, and of these compounds, only 3-CBA was metabolizable by the cells. *Pseudomonas* sp. strain B13 possesses a benzoate 1,2-dioxygenase capable of dioxygenating both benzoic acid and 3-CBA but not the other chlorobenzoic acids tested (36). Thus, there are two possible explanations for the inhibition of benzoic acid uptake by 3-CBA into *Pseudomonas* sp. strain B13, competition for a specific carrier in the membrane or competition for a key metabolic enzyme inside the cell. The fact that all of the analogs tested, both metabolizable and nonmetabolizable, inhibit benzoic acid uptake by BRI 3010 and BRI 6011 and only 3-CBA is effective for *Pseudomonas* sp. strain B13 argues for a less penetrable membrane(s) and a specific carrier for uptake in strain B13. Direct measurement of the kinetics of uptake of benzoic acid and 3-CBA into *Pseudomonas* sp. strain B13 is required to resolve this question definitively.

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