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Energy-Dependent Uptake of 4-Chlorobenzoate in the Coryneform Bacterium NTB-1

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The uptake of 4-chlorobenzoate (4-CBA) in intact cells of the coryneform bacterium NTB-1 was investigated. Uptake and metabolism of 4-CBA were observed in cells grown in 4-CBA but not in glucose-grown cells. Under aerobic conditions, uptake of 4-CBA occurred with a high apparent affinity (apparent K_t, 1.7 µM) and a maximal velocity (V_{max}) of 5.1 nmol min^{-1} mg of protein^{-1}. At pH values below 7, the rate of 4-CBA uptake was greatly reduced by nigericin, an ionophore which dissipates the pH gradient across the membrane (ΔpH). At higher pH values, inhibition was observed only with valinomycin, an ionophore which collapses the electrical potential across the membrane ($\Delta\psi$). Under anaerobic conditions, no uptake of 4-CBA was observed unless an alternative electron acceptor was present. With nitrate as the terminal electron acceptor, 4-CBA was rapidly accumulated by the cells to a steady-state level, at which uptake of 4-CBA was balanced by excretion of 4-hydroxybenzoate. The mechanism of energy coupling to 4-CBA transport under anaerobic conditions was further examined by the imposition of an artificial $\Delta\psi$, ΔpH, or both. Uptake of 4-CBA was shown to be coupled to the proton motive force, suggesting a proton symport mechanism. Competition studies with various substrate analogs revealed a very narrow specificity of the 4-CBA uptake system. This is the first report of carrier-mediated transport of halogenated aromatic compounds in bacteria.

The coryneform bacterium NTB-1 contains a hydrolytic dehalogenase that catalyzes the initial step in the degradation of 4-chlorobenzoate (4-CBA) and various other halobenzoates by forming 4-hydroxybenzoate (4-HBA) and halide ions (19, 20). In this dehalogenation reaction, water is used instead of molecular oxygen as the hydroxyl donor (10, 13). Under aerobic conditions, 4-HBA is rapidly oxidized to 3,4-dihydroxybenzoate by an NADPH-dependent 4-HBA hydroxylase, and further degradation proceeds via ring cleavage (19). The finding that no dehalogenation of 4-CBA takes place under anaerobic conditions is unexpected (20); cell extracts of NTB-1 readily dehalogenate 4-CBA in the absence of molecular oxygen ((P. E. J. Groenewegen et al., manuscript in preparation). When the cells are supplied with an alternative terminal electron acceptor, such as nitrate or ferricyanide, 4-CBA is dehalogenated and 4-HBA accumulates in the suspending medium, since further hydroxylation cannot take place. The lack of dehalogenase activity in intact cells under anaerobic conditions and in the absence of alternative electron acceptors suggests that an energy-dependent transport system is involved in the uptake of 4-CBA by NTB-1 cells.

Energy-dependent transport systems for haloaromatic compounds have been implied in some systems (16, 19, 20), but so far no solid evidence for carrier-mediated transport has been supplied. Uptake of these compounds as the first step in the degradative pathway is often ignored or considered to proceed by passive diffusion. The uptake of (2,4-dichlorophenoxy)acetic acid in a strain of *Pseudomonas fluorescens* which is unable to degrade this compound was studied to some extent (21). Accumulation of (2,4-dichlorophenoxy)acetic acid proceeded only up to the equilibration level, and a facilitated-diffusion type of uptake mechanism was suggested, mainly on the basis of the inhibition of uptake by azide and fluorodinitrobenzene (21). The uptake of

nonhalogenated aromatic compounds, such as aromatic carboxylic acids, has been studied in more detail (2, 6–8, 11, 12, 14, 17, 18). Conclusive evidence for the carrier-mediated transport of benzoate by *Pseudomonas putida* was obtained by Thayer and Wheelis (17, 18). Benzoate uptake is mediated by an inducible saturable transport system that can be eliminated by a mutation in a gene designated *benP*. A strain that was blocked in the subsequent metabolism of benzoate accumulated benzoate 150-fold against a concentration gradient. The mechanism of benzoate uptake is not sufficiently defined, although ample evidence has been presented for proton motive force (Δp)-driven rather than ATP-driven uptake (17, 18). The uptake of mandelate by *P. putida* has also been studied (7, 8). According to Hegeman (7), mandelate enters the cell by passive diffusion. Uptake occurred in both induced and uninduced cells, but no internal accumulation was observed. In contrast, a carrier-mediated mechanism for mandelate uptake was proposed by Higgins and Mandelstam (8). They suggested that induced cells accumulate mandelate against a concentration gradient, and uptake is blocked by the protonophore 2,4-dichlorophenol. The kinetics of benzoate (6) and 4-HBA (12) uptake in *Rhodopseudomonas palustris* grown photosynthetically on these compounds have been studied. These aromatic acids are immediately converted into their coenzyme A derivatives by an inducible benzoyl-coenzyme A ligase; no evidence of an active transport system was obtained. The kinetic properties of the benzoate- and 4-HBA-coenzyme A ligases correspond to the kinetics of benzoate (5, 6) and 4-HBA (12) uptake by intact cells, respectively, which confirms their role in catalyzing the first degradation steps.

The polar nature of aromatic acids such as benzoate is such that they are often used as rapidly diffusible acids (9) for measuring the pH gradient across membranes (ΔpH). Assuming that these compounds enter the cell by passive diffusion, uptake depends on the concentration gradient and ΔpH only. Nevertheless, as indicated above, bacteria have

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developed highly specific mechanisms for scavenging (ha-lo)aromatic acids from the external medium. In this report, evidence that the uptake of 4-CBA by the coryneform bacterium NTB-1 is mediated by an inducible transport system is presented. Some of the characteristics of the 4-CBA carrier are described.

MATERIALS AND METHODS

Media and culture conditions. The coryneform strain NTB-1 (NCIB 12617) was grown routinely in a chemostat (dilution rate, 0.04 h^{-1}) under carbon-limited conditions. Glucose or 4-CBA was added as the sole carbon source. The cells were grown aerobically at pH 7.0 on a mineral salts medium at 30°C, as described previously (17). In some experiments, cells capable of using nitrate as a terminal electron acceptor were used. For this purpose, cells were grown at low, controlled oxygen concentrations, with 60 mM KNO$_3$ added to the medium.

Assay of ^{14}C-labeled 4-CBA uptake by intact cells. Cells grown in a chemostat were harvested by centrifugation (10 min at 16,000 × g), washed twice in 50 mM potassium phosphate (pH 7.0), and resuspended in the same buffer. A sample (2 to 4 µl) of the concentrated cell suspension (20 mg of protein ml^{-1}) was added to 200 µl of potassium phosphate buffer at 30°C, and uptake was initiated by the addition of ^{14}C-labeled 4-CBA. Transport was terminated by dilution in 2 ml of ice-cold 100 mM LiCl followed by filtration through a 0.45-µm-pore-size cellulose-nitrate membrane filter. The filters were washed once with 2 ml of 100 mM LiCl and dried for 25 min at 120°C. The dried filters were transferred to a scintillation counter (Tri-Carb-460CD; Packard Instrument Co., Inc., Rockville, Md.). In some experiments, cells were preincubated with ionophores 15 min prior to the addition of ^{14}C-labeled 4-CBA. Nigericin and valinomycin were used at concentrations of 0.5 and 5.0 µM, respectively.

Uptake experiments under anaerobic conditions were performed essentially as described above. A sample (20 to 40 µl) of the concentrated cell suspension was diluted in 2 ml of 50 mM potassium phosphate, pH 7.0. This solution was magnetically stirred and flushed with oxygen-free nitrogen gas to ensure anaerobic conditions. Uptake was initiated by the addition of 20 µM ^{14}C-labeled 4-CBA. At the times indicated in the figures, 50-µl samples were removed with a Hamilton syringe (Bonaduz, Switzerland) and treated as described above.

Artificially imposed proton motive force-driven uptake of ^{14}C-labeled 4-CBA. For artificially imposed proton motive force-driven uptake of ^{14}C-labeled 4-CBA, cells were incubated for 1 h at 4°C in a solution containing 50 mM potassium phosphate (pH 7.0) and 100 mM potassium acetate in the presence of 5.0 µM valinomycin. Loaded cells were washed once in 100 mM potassium acetate and resuspended in the same buffer. The concentrated suspension was incubated in a sealed bottle and flushed with oxygen-free nitrogen gas. A gas-tight Hamilton syringe was filled anaerobically with the cell suspension (approximately 50 mg of protein ml^{-1}), and uptake of 4-CBA was initiated by diluting 20 µl of the cell suspension in 2 ml of a solution which contained, in addition to the indicated concentration of ^{14}C-labeled 4-CBA, 50 mM sodium phosphate (pH 7.0)–100 mM sodium 4-(2-hydroxy-ethyl)-1-piperazine-ethanesulfonic acid (HEPES) (Δp, interior negative and alkaline), 50 mM sodium phosphate (pH 7.0)–sodium acetate (Δψ, interior negative), 50 mM potassium phosphate (pH 7.0)–100 mM potassium HEPES (ΔpH, interior alkaline), or 50 mM potassium phosphate (pH 7.0)–
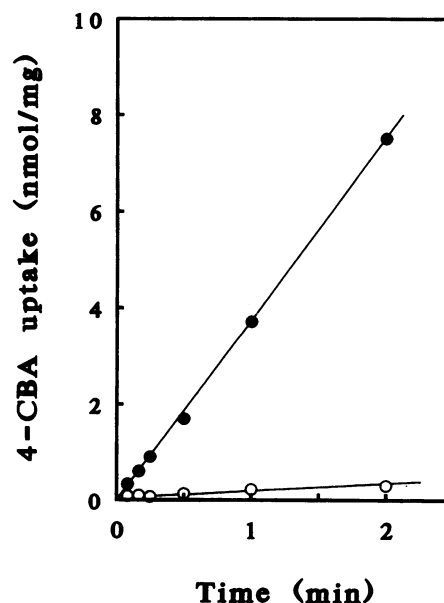
potassium acetate (no gradient) (3). The suspension was magnetically stirred and flushed with oxygen-free nitrogen gas. At the times indicated in the figures, 50-µl samples were removed and assayed for 4-CBA uptake.

Materials. ring-U-^{14}C-labeled 4-CBA (4.03 GBq mmol^{-1}) was obtained from the Radiochemical Centre, Amersham, England. The stock ^{14}C-labeled 4-CBA (3.1 mM) was diluted in the experiments as indicated in the figure legends. The halogenated aromatic compounds were obtained from Jans-sen Chimica (Beerse, Belgium). All other chemicals were of the highest purity commercially available.

RESULTS

Uptake of 4-CBA under aerobic conditions. The uptake of ^{14}C-labeled 4-CBA under aerobic conditions by a suspension of washed NTB-1 cells grown in the presence of 4-CBA is shown in Fig. 1. Under aerobic conditions, uptake and respiration of 4-CBA occurred at the same time. Uptake was linear for several minutes and ceased when most of the label was removed from the external medium (data not shown). A low rate of 4-CBA uptake was observed with glucose-grown cells, in either the absence (Fig. 1) or the presence of glucose (data not shown). It was shown previously that the hydro-lytic dehalogenase is present only in cells induced with 4-CBA (19). These results suggest that in NTB-1 cells, uptake of 4-CBA is mediated by an inducible transport system.

Uptake of 4-CBA under anaerobic conditions. The accumulation of 4-CBA under aerobic conditions may be the result of passive diffusion in conjunction with the conversion of this haloaromatic compound to a less lipophilic derivative. The degradation of 4-CBA involves a dehalogenation step yielding 4-HBA, which is further oxidized via protocatechu-ate. As shown previously, 4-CBA is not metabolized under anaerobic conditions (20). When 4-CBA-grown cells were incubated anaerobically, no 4-CBA entered the cell (Fig. 2). Restoring aerobic conditions caused a rapid uptake of the labeled substrate. These results demonstrate that 4-CBA is not accumulated under anaerobic conditions.
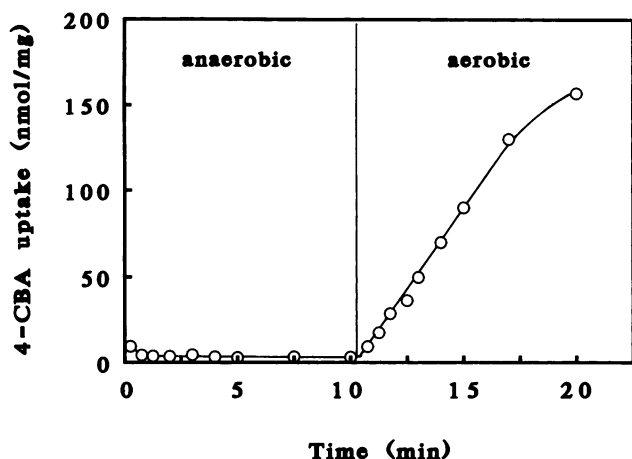
FIG. 2. Uptake of ^{14}C-labeled 4-CBA (final concentration, 78 μM) by NTB-1 cells in the absence and presence of oxygen.

Cells grown on 4-CBA in the presence of nitrate at low oxygen tension express a nitrate reductase system (Groenewegen et al., in preparation). These cells are able to use nitrate as the terminal electron acceptor under anaerobic conditions. The addition of nitrate to these cells during anaerobic transport experiments resulted in an immediate uptake of 4-CBA (Fig. 3). Unlike the uptake observed under aerobic conditions, the uptake under anaerobic conditions with nitrate as the electron acceptor reached a steady-state corresponding to a 30-fold accumulation of the label. Under these conditions, 4-HBA is excreted in the suspending medium (Groenewegen et al., in preparation). Therefore, the steady state most likely represents an equilibrium between uptake of 4-CBA and excretion of 4-HBA. The introduction of molecular oxygen in the system instantly resulted in a further uptake of label (Fig. 3). These results show that under anaerobic conditions, uptake of 4-CBA is impeded because of the lack of a suitable electron acceptor. With nitrate as the terminal electron acceptor, the accumulation of 4-CBA against a concentration gradient is evident, although a passive diffusion mechanism cannot definitely be ruled out.
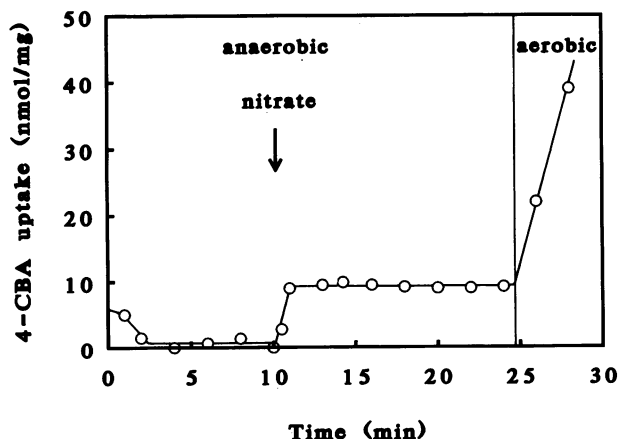

FIG. 3. Uptake of ^{14}C-labeled 4-CBA (final concentration, 78 μM) by NTB-1 cells under anaerobic conditions in the absence and presence of nitrate. The addition of KNO$_3$ to a final concentration of 60 mM is indicated by the arrow. At 25 min, molecular oxygen was introduced into the uptake vessel.
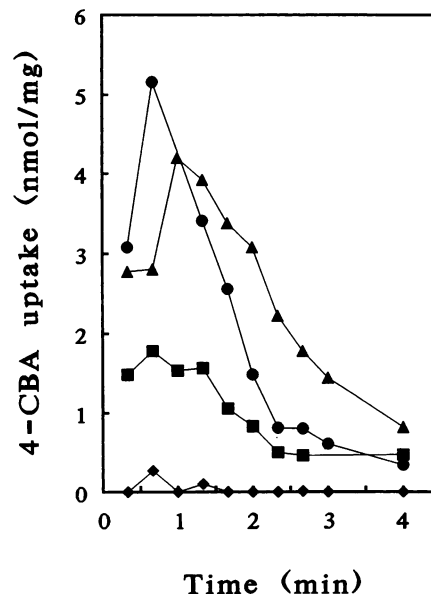

Time (min)

FIG. 4. Uptake of ^{14}C-labeled 4-CBA (final concentration, 12 μM) by NTB-1 cells under anaerobic conditions (pH 7.0) in the absence of a Δp (◆) or with an artificially imposed Δψ (■), ΔpH (▲), and Δp (●).

Artificially imposed proton motive force-driven uptake of 4-CBA under anaerobic conditions. The inability of NTB-1 cells to accumulate 4-CBA under anaerobic conditions may be caused by a low Δp. To test this hypothesis, the effect of an artificially imposed Δp on 4-CBA transport under anaerobic conditions was studied. A Δψ, inside negative, can be created by diluting K$^+$-loaded cells in a K$^+$-free (i.e., Na$^+$) buffer in the presence of valinomycin. A ΔpH, inside alkaline, can be generated by diluting acetate-loaded cells into a solution containing a less permeable anion, such as HEPES. A Δp, inside negative and alkaline, can be established by the simultaneous imposition of outwardly directed diffusion gradients of K$^+$ ions and acetate. Transient accumulation of 4-CBA was observed with a ΔpH or Δψ (Fig. 4). No accumulation of 4-CBA was observed in the absence of a gradient, while the highest level of accumulation was observed when both a ΔpH and a Δψ were imposed simultaneously (Fig. 4). In contrast, the imposition of an artificial Δp in glucose-grown cells failed to promote uptake of 4-CBA (data not shown). These results unequivocally demonstrate that uptake of 4-CBA is dependent on the Δp and can only be explained if the uptake is carrier mediated.

Effect of ionophores on 4-CBA transport. To define the role of the Δp in more detail, the effects of the ionophores nigericin and valinomycin on the initial rate of 4-CBA uptake under aerobic conditions were investigated. Nigericin mediates the electroneutral H$^+$-K$^+$ exchange, thereby collapsing the ΔpH. At pH 5.0, the rate of 4-CBA uptake was dramatically reduced by this ionophore (Fig. 5). Nigericin was ineffective at more alkaline pH values. An opposite effect was obtained with valinomycin, which dissipates the Δψ in the presence of K$^+$ ions. At pH 5.0, the rate of 4-CBA uptake was stimulated by valinomycin at least twofold. With increasing pHs, the stimulating effect of valinomycin was gradually changed to an inhibitory effect (Fig. 5). The simultaneous addition of both ionophores completely prevented 4-CBA uptake at low pHs, although at pH 7.0 and above, some residual uptake remained (Fig. 5). The data
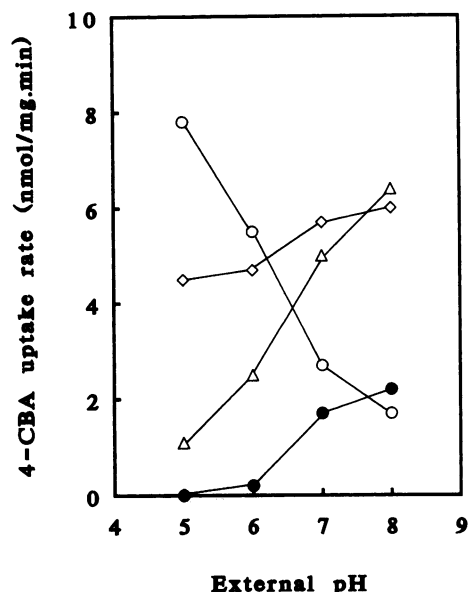
FIG. 5. Effects of ionophores on ^{14}C-labeled 4-CBA (5.9 μM) uptake by NTB-1 cells at different pH values. Cells were incubated under aerobic conditions, and the initial rate of uptake was determined in the absence (◇) or presence of 0.5 μM nigericin (△), 5 μM valinomycin (○), or both (●).

FIG. 6. Kinetic analysis of ^{14}C-labeled 4-CBA uptake by NTB-1 cells. Cells were incubated under aerobic conditions in 50 mM potassium phosphate (pH 7.0), and the initial rate of ^{14}C-labeled 4-CBA uptake was determined from times of label uptake taken within 1 min. The 4-CBA concentration varied between 0.9 and 12 μM.

indicate that uptake of 4-CBA at low pHs is predominately coupled to the ΔpH. At high pHs, uptake is an electrogenic process, which suggests that transport occurs in symport with more than one proton.

Kinetics and specificity of 4-CBA transport. The kinetic parameters of 4-CBA uptake were estimated from the initial rate of uptake under aerobic conditions at concentrations from 0.9 to 12 μM. At pH 7.0, uptake of 4-CBA displayed monophasic saturation kinetics (Fig. 6), with an apparent affinity constant (K_t) of 1.7 μM. The maximal velocity (V_{max}) was 5.1 nmol min^{-1} mg of cell protein^{-1} but varied in different experiments. The rate of 4-CBA uptake decreased slowly upon prolonged storage of the NTB-1 cells at 4°C.

The specificity of the 4-CBA transport system was assessed from the extent of inhibition of ^{14}C-labeled 4-CBA transport by a 30-fold excess of a wide range of substrate analogs. The transport system appeared to be specific for parasubstituted mono- and dihalobenzoates (Table 1). 4-CBA uptake is blocked by other halobenzoates in the following order of efficiency: 4-CBA ≈ 4-bromobenzoate > 4-iodobenzoate > 3,4-dichlorobenzoate > 4-fluorobenzoate (4-FBA) > 2,4-dichlorobenzoate (Table 1). 4-HBA did not inhibit uptake of 4-CBA. The carboxyl group on the benzene nucleus seems to be essential for recognition by the 4-CBA uptake system.

DISCUSSION

The main objective of this study was to obtain information about the mechanism of 4-CBA uptake by the coryneform bacterium NTB-1 and the mechanism of energy coupling. Several lines of evidence are consistent with the involvement of a Δp-driven uptake system for this haloaromatic compound and argue against a passive diffusion mechanism. (i) The imposition of Δp under anaerobic conditions resulted in a transient accumulation of 4-CBA. (ii) The uptake of 4-CBA under anaerobic conditions was dependent solely on
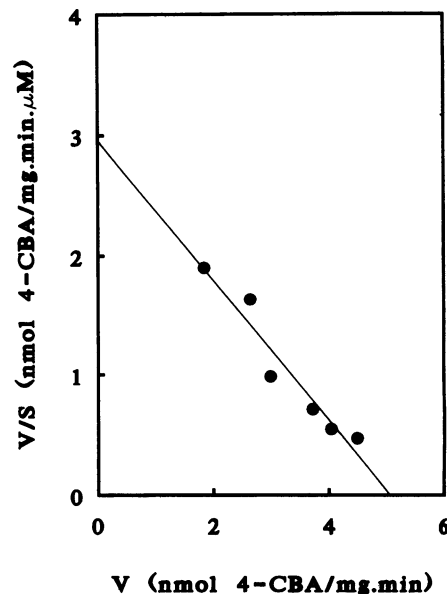
the presence of a suitable electron acceptor. (iii) The uptake of 4-CBA under aerobic conditions was sensitive to ionophores and electrogenic at alkaline pH values. (iv) The rate of 4-CBA uptake showed saturation kinetics with a high apparent affinity. (v) The substrate specificity of the transport system was rather narrow. To our knowledge, this is the first report of a specific transport system for halogenated aromatic compounds in bacteria.

Lipophilic compounds usually enter the cytoplasmic membrane by passive diffusion. For a lipophilic weak acid, such as 4-CBA, with a high permeability coefficient (log P = 2.65) (4), passive diffusion is the most obvious mechanism for uptake. Uptake by such a mechanism would, however, be coupled solely to the ΔpH and the inwardly directed con-

TABLE 1. Inhibition of ^{14}C-labeled 4-CBA uptake by structural analogs[a]

Inhibitor	Residual ^{14}C-labeled 4-CBA uptake (%)
Benzoate	80
3-Chlorobenzoate	82
4-FBA	26
4-CBA	0
4-Bromobenzoate	0
4-Iodobenzoate	1
4-HBA	100
4-Nitrobenzoate	90
4-Methoxybenzoate	30
4-Aminobenzoate	100
4-Chloro-3,5-dinitrobenzoate	100
2,4-Dichlorobenzoate	39
3,4-Dichlorobenzoate	16
2,5-Dichlorobenzoate	82
4-Chlorophenylacetate	100

[a] Inhibitor and ^{14}C-labeled 4-CBA were present at concentrations of 100 and 3.1 μM, respectively.

centration gradient, which is sustained by metabolism. The dramatic effects of nigericin and valinomycin at low pH values are consistent with this model. However, at alkaline pHs, uptake is almost completely abolished by valinomycin, which indicates that uptake of 4-CBA is an electrogenic process. This implies that translocation takes place in symport with more than one proton. At this stage, it is not possible to determine the value of the 4-CBA–H$^+$ stoichiometry. It is possible that uptake of 4-CBA can be regulated by the intracellular pH, as has been demonstrated for uptake of solutes in lactococci (15). With the prevailing metabolism, a steady state which does not necessarily represent a thermodynamic equilibrium with the existing Δp is reached.

Intact cells require an electron acceptor for the dehalogenation of 4-CBA (16, 20), whereas studies with cell extracts indicate that the enzymatic reaction itself is independent of molecular oxygen or any other electron acceptor (16; Groenewegen et al., in preparation). Our results show that the inability of cells to dehalogenate 4-CBA under anaerobic conditions is due to a lack of activity of the transport system. Activity of 4-CBA uptake is restored when a Δp is generated by the addition of an alternative electron acceptor (i.e., nitrate) or the imposition of diffusion gradients. Similar phenomena have been observed for the degradation of pentachlorophenol by Rhodococcus chlorophenolicus. This organism was isolated on pentachlorophenol and requires a hydrolytic dehalogenase for the degradation of polychlorinated phenols (1). Intact cells were unable to dehalogenate any of the polychlorinated phenols during anaerobiosis, while cell extracts did not need molecular oxygen. The suggestion that oxygen is essential for generating energy or for restoring the activity of the enzymes or cofactors used in the dehalogenation-hydroxylation reaction has been made (1). The analogy of the oxygen requirement of dehalogenation of 4-CBA by the coryneform bacterium NTB-1 indicates that an active transport system may be involved in the uptake of polychlorinated aromatic compounds by R. chlorophenolicus.

NTB-1 cells are able to use 4-HBA as their sole carbon and energy source (19, 20). Cells grown on halobenzoates rapidly oxidize 4-HBA at a rate comparable to the rate of 4-CBA oxidation (20). 4-HBA is not a substrate for the halobenzoate carrier, which may indicate that a separate transport system for 4-HBA is present in NTB-1 cells. On the other hand, 4-FBA efficiently inhibits uptake of 4-CBA, and rapid counterexchanges between both substrates under anaerobic conditions can be observed (data not shown), indicating that 4-FBA is efficiently translocated. The growth of NTB-1 cells is not supported by this compound (19, 20). 4-FBA is not oxidized or dehalogenated by a washed-cell suspension or a cell extract (Groenewegen et al., in preparation).

The results presented here demonstrate that the first enzymatic step of the degradation of 4-CBA, and other halobenzoates, is the carrier-mediated uptake of these compounds. Similar experiments with other microorganisms capable of converting haloaromatic compounds are essential for understanding the kinetics and regulatory mechanisms involved in these degradative processes.

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