3-Chlorobenzoate is taken up by a chromosomally encoded transport system in *Cupriavidus necator* JMP134

T. Ledger, F. Aceituno and B. González

*Correspondence*
Bernardo González
bernardo.gonzalez@uai.cl

1Departamento de Genética Molecular y Microbiología and Millennium Nucleus on Microbial Ecology and Environmental Microbiology and Biotechnology, and Center for Advanced Studies in Ecology and Biodiversity, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

2Facultad de Ingeniería y Ciencia, Universidad Adolfo Ibáñez, 7941169 Santiago, Chile

*Cupriavidus necator* JMP134(pJP4) is able to grow on 3-chlorobenzoate (3-CB), a model chloroaromatic pollutant. Catabolism of 3-CB is achieved via the expression of the chromosomally encoded *benABCD* genes and the *tfd* genes from plasmid pJP4. Since passive diffusion of benzoic acid derivatives at physiological pH is negligible, the uptake of this compound should be facilitated by a transport system. However, no transporter has so far been described to perform this function, and identification of chloroaromatic compound transporters has been limited. In this work, uptake experiments using 3-[ring-U-14C]CB showed an inducible transport system in strain JMP134, whose expression is activated by 3-CB and benzoate. A similar level of 3-CB uptake was found for a mutant strain of JMP134, defective in chlorobenzoate degradation, indicating that metabolic drag is not an important component of the measured uptake rate. Competitive inhibitor assays showed that uptake of 3-CB was inhibited by benzoate and, to a lesser degree, by 3-CB and 3,5-dichlorobenzoate, but not by any of 12 other substituted benzoates tested. The expression of several gene candidates for this transport function was analysed by RT-PCR, including both permease-type and ABC-type ATP-dependent transporters. Induction of a chromosomally encoded putative permease transporter (*benP* gene) was found specifically in the presence of 3-CB or benzoate. A *benP* knockout mutant of strain JMP134 displayed an almost complete loss of 3-CB transport activity. This is to our knowledge the first report of a 3-CB transporter.

**INTRODUCTION**

Degradation of aromatic and chloroaromatic pollutants is a well-documented feature of some environmental bacteria. Several aerobic pathways have been characterized for the degradation of compounds of this kind (Reineke, 1998). These biochemical pathways have been elucidated under laboratory conditions, when bacteria grow in a homogeneous medium of defined composition and are provided with relatively high initial concentrations of substrate (usually >1 mM). However, when these compounds are found in the environment, they usually appear in an uneven distribution, as many contaminants tend to be adsorbed by organic particles, resulting in low amounts of available compound in the aqueous phase. For efficient use of these compounds as carbon sources, chemotactic and transport functions must be present in bacteria with catabolic potential.

Chloroaromatic acids can be produced as intermediates of the degradation of several chlorinated molecules of higher molecular mass, and may accumulate as end products (Harwood & Parales, 1996). These polar compounds do not diffuse readily through membranes at physiological pH, i.e. when their carboxyl group is in the ionic form, and therefore the cell requires a strategy for uptake [the pKₐ of 3-chlorobenzoic acid (3-CB) is 3.8, and therefore <0.1% of the compound is in its protonated form at physiological pH]. Uptake of aromatic acids by bacteria can be mediated by transporters or driven by diffusion forced across intracellular/extracellular gradients of pH and substrate concentration (Kashket, 1985). Establishment and maintenance of concentration gradients requires the intracellular substrate concentration to be kept low relative to that of the external environment, which may be achieved by rapid transformation of the imported compound to...
metabolic intermediates (Harwood & Gibson, 1986; Merkel et al., 1989; Wong et al., 1994). In this case, uptake is effectively driven by the activity of catabolic enzymes, and this ‘metabolic drag’ mechanism (Wong et al., 1994) has been proposed for the uptake of benzoate (Harwood & Gibson, 1986) and 4-hydroxybenzoate (4-HB) (Merkel et al., 1989) in Rhodopseudomonas palustris, and for the uptake of 4-HB by Rhizobium leguminosarum (Wong et al., 1994). Transporter-mediated uptake has been reported for some non-chlorinated aromatic acids, such as benzoate (Collier et al., 1997; Thayer & Wheelis, 1982), 4-HB (Allende et al., 1993; Harwood et al., 1994), protocatechuate (Nichols & Harwood, 1997), mandelate (Higgins & Mandelstam, 1972), phenylacetate (Schleissner et al., 1994), 4-hydroxyphenylacetate (Prieto & García, 1997) and phthalate (Chang & Zylstra, 1999). Only a few of these permease-type transport proteins have been biochemically characterized, and the corresponding genes described. In most cases, identification of specific genes has been aided by the fact that candidate transport genes are located near to or within a gene cluster encoding the catabolic enzymes responsible for the degradation of aromatic compounds (Harwood et al., 1994; Collier et al., 1997; Chae & Zylstra, 2006).

For chloroaromatic compounds, however, identification of genes encoding transport functions has proved more difficult, since putative uptake genes are not necessarily found near gene clusters encoding catabolic functions (Yuroff et al., 2003). So far, chlorinated aromatic compounds for which energy-dependent transport has been demonstrated are only 4-chlorobenzoate (4-CB) (Groenewegen et al., 1990), dichlorprop (Zipper et al., 1995), 2-chlorobenzoate (Yuroff et al., 2003) and 2,4-dichlorophenoxyacetate (2,4-D) (Leveau et al., 1998). Among these, the only known transport proteins specialized in the uptake of chloroaromatic compounds are the TfdK permease for 2,4-D (Leveau et al., 1998) and a TRAP (tripartite ATP-independent periplasmic) system transporter for 4-CB, encoded by the fcbTITT2T3 genes in Comamonas sp. strain DJ-12 (Chae & Zylstra, 2006). Evidence supporting the involvement of ABC-type transporters in uptake of chloroaromatics has been found for 2-chlorobenzoate, dichlorprop and 4-CB (Groenewegen et al., 1990; Yuroff et al., 2003; Zipper et al., 1998). Cupriavidus necator JMP134[pJP4] ex Ralstonia eutropha (Vandamme & Coenye, 2004) is a soil bacterium widely used as a model for the study of degradation of aromatic and chloroaromatic compounds (its complete genomic sequence is available at http://genomeportal.cgi-psf.org/raleu/raleu.home.html). Its most representative degradation pathway is encoded by the tfd genes in the catabolic plasmid pJP4, which are essential for the degradation of 2,4,5-D (Plumeier et al., 2002) and 3-CB (Pérez-Pantoja et al., 2000). Two complementary pathways are required for the degradation of 3-CB. The first steps of its degradation are encoded in the chromosome by the benABCD genes, which are clustered together with the rest of the classical ortho ring cleavage pathway for benzoate degradation (Pérez-Pantoja et al., 2008). The chlorocatechol produced by transformation of 3-CB by the BenABCD enzymes is then cleaved and degraded to β-ketoadipate by the Tfd enzymes encoded in plasmid pJP4 (Fig. 1). In contrast to 2,4-D, whose transporter gene, tfdK, is encoded on the pJP4 plasmid (Leveau et al., 1998), no putative transporter gene for 3-CB, or even benzoate, has so far been identified in strain JMP134. This is unusual, because in most of the catabolic gene clusters related to catabolism of non-chlorinated aromatic acids in C. necator, a putative transporter is found (Pérez-Pantoja et al., 2008). In spite of the relevance of intracellular uptake for the degradation of aromatic and chloroaromatic acids in environmental conditions, knowledge of transport systems for these compounds in bacteria is scarce. This paper reports the uptake of 3-CB and a gene encoding a putative permease active in the uptake of this compound in C. necator.

METHODS

Bacterial strains, plasmids and growth conditions. C. necator JMP134[pJP4] was grown at 30 °C in liquid minimal medium (Kroc& Focht, 1987), with 0.5–10 mM 3-CB, 2 mM benzoate or 10 mM fructose as the sole carbon source. Growth on 3-CB was determined by measuring the increase in OD600 in an HP 8452-A spectrophotometer (Hewlett Packard) equipped with a 1 cm path length cell. At least three replicates were used for each growth measurement. For induction experiments, C. necator derivatives unable to proliferate on 3-CB were grown on 2 mM benzoate plus kanamycin, or on 10 mM fructose. Escherichia coli strain DH5α (Promega) was maintained onuria–Bertani (LB) agar plates plus nalidixic acid. Antibiotics were used at the following concentration: 50 μg kanamycin ml⁻¹, 25 μg nalidixic acid ml⁻¹.

DNA manipulation. Restriction, ligation and dephosphorylation reactions, purification, and electroporation of DNA were performed by standard procedures (Ausubel et al., 1992). Derivatives of the broad-host-range plasmid vector pBBRCSM-2 (pBBBR3938) (Kovach et al., 1995) were mobilized from E. coli DH5α to C. necator JMP134 by triparental mating with the helper strain E. coli HB101(pRK600), as previously described (Pérez-Pantoja et al., 2000). Transconjugants were selected on minimal medium agar plates supplemented with 2 mM benzoate plus kanamycin.

Inactivation of the benA and benP genes. The benA and benP (corresponding to ORF B3938 in the genome of strain JMP134; see below) genes were independently inactivated in C. necator JMP134 by recombination with an inner fragment of each gene cloned in the pTOPO2.1 vector (Invitrogen). For this, PCR primers FBENA (5'-ACGGATCCGTGTGGGACGAC-3') and RBENA (5'-GCTGTTGTTGTTCCGAGC-3'), and primers FBENP (5'-GCTGTTGCTGCTGAGC-3') and RBENP (5'-ATGGAGTACCTGTGGGACGAC-3'), were synthesized, amplifying 509 and 402 bp within the benA and benP sequences, respectively. Amplified fragments were cloned in pTOPO, and the resulting plasmids, pTOPObenA and pTOPObenP, were purified and inserted by electroporation into competent cells of C. necator JMP134. Colonies of transformants were selected on LB plates plus kanamycin, and disruption of each ORF by the single recombinational insertion of the plasmid was verified by PCR amplification using primers F2BENA (5'-CCCAGCCTACGGAGCATT-3') and R2BENA (5'-GCTACACCTTCGGTCGCTGAGC-3'), and F2BENP (5'-CGCTACCTTCGGTCGCTGAGC-3') and
R2BENP (5’-GGCTCAACTACGGACACGA-3’), which anneal outside each cloned internal fragment, and combining them with the M13f and M13r primers (Invitrogen) annealing inside the pTOPO vector. PCR products obtained in this manner were then sequenced to confirm proper disruption of each gene and the region where recombination took place. For all PCRs, the following programme was used: 95 °C for 5 min, 32 cycles of 95 °C for 30 s, 54 °C for 30 s and 72 °C for 90 s, and then 72 °C for 10 min.

**3-CB uptake assay.** 3-[^14]C]CB [purity, 96%; specific activity, 29 mCi mmol⁻¹ (1.07 × 10⁶ Bq mmol⁻¹)] was obtained from Sigma. For 3-CB uptake assays, a hot-cold stock solution [specific activity, 2.9 mCi mmol⁻¹ (1.07 × 10⁶ Bq mmol⁻¹)] was prepared in sterile 50 mM Na₂HPO₄/KH₂PO₄ buffer with 100 μM 3-[^14]C]CB and 900 μM unlabelled 3-CB (purity, 98%; Aldrich). Cells were harvested by centrifugation (6000 g, 10 min), washed once with assay buffer (100 mM Na₂HPO₄/KH₂PO₄, pH 7.6), and resuspended in this buffer to an OD₆₀₀ corresponding to 0.01–0.05 mg total cellular protein ml⁻¹. For the assays, a cell suspension (1 ml) was incubated at 30 °C for 10 min on a shaker rotating at 120 r.p.m., and reactions were initiated by addition of the hot-cold stock solutions. For kinetic determinations, the concentration of 3-[^14]C]CB was varied from 1 to 200 μM; all other reactions contained 10 μM (29 mCi, 1.07 × 10⁶ Bq) 3-[^14]C]CB. Selected substituted benzoates were examined as potential competitors of 3-CB uptake. Test competitors were added to a final concentration of 10 μM at the initiation of the uptake assay. Aliquots (200 μl) were removed from the reaction mixture at 30 s intervals for 2.5 min. Cells were filtered onto nitrocellulose membranes (0.45 μm pore-size; Whatman International) on a vacuum manifold and rinsed with 2 ml stop buffer, consisting of cold 100 mM Na₂HPO₄/KH₂PO₄ with 20 mM HgCl₂, and 10 μM unlabelled 3-CB. Filters were then placed in a hybridization oven preheated to 65 °C and incubated for 15 min. Radioactivity on the filters was counted on a RackBeta liquid scintillation counter (LKB Wallac). Uptake rates for each reaction were calculated from the slope of the linear regression of total ^14C radioactivity in the cells versus time. For all assays, the linear regression extrapolated to time zero did not pass through the origin. Due to limitations of the sampling method, however, measurements before 0.5 min were not possible. Thus, unless otherwise indicated, the slope of the curve between 0.5 and 2.0 or 2.5 min was used to calculate uptake rates. All transport rates were normalized to total protein present in the reaction, which was determined by a modified Bradford method (Bradford, 1976). Values for \( V_{\text{max}} \) and \( K_m \) were estimated by nonlinear regression fitting to the Michaelis–Menten equation.

**Fig. 1.** 3-CB degradation pathway and the genes involved in *C. necator* JMP134. 3-CB is taken up into the cell by a putative transporter (upper left). Once in the cytoplasm, it is initially transformed by the BenABC and BenD enzymes into a mixture of 3- and 4-chlorocatechol (upper right). These compounds are substrates of the specialized ortho cleavage pathway encoded by the *tfd* genes, resulting in the production of β-ketoacid, a compound that can be channelled into the central metabolism (lower panel). Genes encoding the enzymes involved in each step are highlighted in white. Additional genes whose functions are not shown in the pathways, as well as genes with unknown function, are shaded in grey. Regulator genes are shown as black arrows. Numbers in parentheses indicate the position of each cluster within chromosome 2, or plasmid pJP4. EC and IC, extracellular and intracellular space, respectively; CM, cytoplasmic membrane.
RESULTS AND DISCUSSION

3-Chlorobenzoate is actively taken up by C. necator JMP134(pJP4)

The aromatic degradation pathways in C. necator JMP134 have been intensively studied (Pérez-Pantoja et al., 2008). However, additional functions, such as transport and chemotaxis, have rarely been addressed. Active transport of aromatic acids increases the efficiency and rate of substrate acquisition, and may provide a growth advantage in natural environments where these compounds are present at low (micromolar) concentrations (Whitehead, 1964). In order to assess if there is an active transport system specialized in the uptake of 3-CB by C. necator JMP134, uniformly labelled 3-[14C]CB was added to resting cells of this strain. For these experiments, cells were grown with 3-CB or fructose as the carbon source. 3-CB-grown C. necator JMP134 cells were able to take up 3-CB at a rate of $1.10 \pm 0.06$ nmol 3-[14C]CB min$^{-1}$ (mg protein)$^{-1}$, while heat-inactivated cells did not accumulate 3-CB at all, indicating that uptake is not produced by adsorption of the labelled substrate to the cell surface (Fig. 2a). When cells were grown on fructose, no uptake of the compound occurred, even after 3.5 min, suggesting that simple diffusion does not account for the measured uptake rate, but rather, an inducible transport system is responsible for taking up 3-CB into the cells. The rate of uptake measured for this transport system is similar to uptake rates measured for haloaromatic acid transporter systems where energy-dependent transport has been proposed: 4.9 nmol compound min$^{-1}$ (mg protein)$^{-1}$ for 2-chlorobenzoate in Pseudomonas huttiensis D1 (Yuroff et al., 2003), 2.2 for 2,4-dichlorobenzoate in Alcaligenes denitrificans BRI 6011 (Miguez et al., 1995) and 2.0 for 4-CB in the coryneform bacterium NTB-1 (Groenewegen et al., 1990). Uptake rates were measured for different initial 3-[14C]CB concentrations, from 1 µM up to 200 µM (Fig. 2b). A typical hyperbolic curve was obtained, showing a saturation kinetics that reached a maximum value of 3 nmol 3-[14C]CB min$^{-1}$ (mg protein)$^{-1}$, at approximately 100 µM 3-CB. An apparent $K_m$ of 28.3 µM and a $V_{max}$ of 3.45 nmol 3-CB min$^{-1}$ (mg protein)$^{-1}$ for 3-CB uptake was calculated from double reciprocal plots (Fig. 2b). Partial double reciprocal plots calculated from the high and low 3-CB concentration data gave very similar apparent kinetic values, suggesting that activity of only one 3-CB uptake system is measured in this assay (Fig. 2b). Saturation of uptake at concentrations above 75 µM provided further confirmation of the nature of 3-CB transport in C. necator JMP134. The shape of the saturation curve strongly suggests once again that activity of only one transport system for 3-CB is being measured, but does not eliminate the possibility that other transport system(s) with lower substrate affinity can contribute to 3-CB uptake.

Fig. 2. (a) 3-CB uptake by C. necator JMP134. 3-[14C]CB uptake was measured at 0.5 min intervals up to 3 min for strain JMP134 grown on 3-CB (●) or on fructose (○). Uptake was also measured in 3-CB-grown cells previously incubated for 10 min at 95 °C (heat-killed cells, ▲). All measurements were carried out at a cell density corresponding to 0.09 mg total protein. Results show the values obtained for at least three independent experiments. (b) Effect of 3-CB concentration on the rate of uptake by C. necator JMP134. 3-[14C]CB uptake was measured at substrate concentrations ranging from 1 to 200 µM in cells of strain JMP134 grown on 3-CB. Inset: double reciprocal plot including the entire concentration range (1–200 µM). Calculated apparent kinetic constants are conserved between high (50–200 µM) and low (1–25 µM) concentration ranges (not shown), suggesting that only one transport system is being measured. Representative values are shown for at least two independent measurements for each substrate concentration.
Induction of 3-CB uptake was investigated after growing strain JMP134 on different carbon sources, including chlorinated and non-chlorinated aromatic substrates. Absolute uptake values were measured as in Fig. 2, in *C. necator* cells using 2,4-D, fructose, p-hydroxybenzoate, salicylate, benzoate or muconate as sole growth substrate. Uptake values were calculated from three independent determinations. These experiments showed that uptake of 3-CB is induced by growth on 3-CB, reaching an uptake value of 1.11 ± 0.08 nmol 3-[^14]CB min⁻¹ (mg protein)⁻¹, and by benzoate [0.88 ± 0.04 nmol 3-[^14]CB min⁻¹ (mg protein)⁻¹], while 2,4-D, p-hydroxybenzoate, salicylate and muconate do not induce 3-CB transport rates, <0.05 nmol 3-[^14]CB min⁻¹ (mg protein)⁻¹]. Unfortunately, because of the nature of the uptake assay, it is difficult to separate genuine transport rates from the contribution of diffusion facilitated by degradation of the substrate that accumulates in the inside the cell (metabolic drag). Some reports have addressed this issue by showing that transport occurs against a concentration gradient, which involves purification of the substrate taken up by the cells and quantification based on an approximate estimate of cellular volume (Yuroff et al., 2003). In this work, the metabolic component was estimated by inactivation of the benzoate 1,2-dioxygenase, BenA, catalysing the first step in 3-CB catabolism. A benA mutant of *C. necator* JMP134 was obtained by the recombinational insertion of a suicide vector. This mutant (strain JMP134-benA) was unable to metabolize 3-CB (data not shown), and was therefore grown on fructose and induced with 1 mM 3-CB to compare its ability to take up 3-CB with that of the wild-type. Strain JMP134-benA exhibited only a 10% reduction of the 3-CB uptake rate compared to the wild-type strain (Fig. 3a), indicating that the influence of metabolic drag in 3-CB uptake values was negligible. In order to gain insight into the specificity of the 3-CB uptake system in *C. necator*, several chloro-, methyl- and hydroxybenzoates, with substitutions in the ortho, meta and para positions, were tested as competitive inhibitors of 3-CB transport in *C. necator* JMP134. Several of them were growth substrates for this bacterium (Table 1; Pérez-Pantoja et al., 2008). All these compounds were assayed at equimolar concentrations with the labelled substrate. The presence of benzoate strongly inhibited 3-CB uptake, suggesting that the non-chlorinated derivative is a better substrate for the putative transport system (Table 1). In addition to benzoate, only 3,5-dichlorobenzoate acted as an effective competitor of 3-CB uptake.

### 3-CB is taken up by a chromosomally encoded transport system in *C. necator* JMP134(pJP4)

Transporter-mediated uptake of aromatic acids has been reported in several bacteria (Allende et al., 1992, 1993, 2000, 2002; Chang & Zylstra, 1999; Collier et al., 1997; Harwood et al., 1994; Higgins & Mandelstam, 1972; Nichols & Harwood, 1997; Prieto & Garcia, 1997; Saint & Romas, 1996; Schleissner et al., 1994; Thayer & Wheelis, 1992). 3-Chlorobenzoate transport in *C. necator* JMP134 is mediated by a chromosomally encoded transport system. A benP mutant (strain JMP134-benP) was unable to metabolize 3-CB (data not shown), and was therefore grown on fructose and induced with 1 mM 3-CB to compare its ability to take up 3-CB with that of the wild-type. Strain JMP134-benP exhibited only a 10% reduction of the 3-CB uptake rate compared to the wild-type strain (Fig. 3a), indicating that the influence of metabolic drag in 3-CB uptake values was negligible. In order to gain insight into the specificity of the 3-CB uptake system in *C. necator*, several chloro-, methyl- and hydroxybenzoates, with substitutions in the ortho, meta and para positions, were tested as competitive inhibitors of 3-CB transport in *C. necator* JMP134. Several of them were growth substrates for this bacterium (Table 1; Pérez-Pantoja et al., 2008). All these compounds were assayed at equimolar concentrations with the labelled substrate. The presence of benzoate strongly inhibited 3-CB uptake, suggesting that the non-chlorinated derivative is a better substrate for the putative transport system (Table 1). In addition to benzoate, only 3,5-dichlorobenzoate acted as an effective competitor of 3-CB uptake.

![Figure 3](http://mic.sgmjournals.org)
Energy-dependent transport of chlorinated aromatics has been demonstrated in a few cases (Groenewegen et al., 1990; Leveau et al., 1998; Yuroff et al., 2003; Zipper et al., 1998). Among these, a transporter gene has been characterized only for 2,4-D (Leveau et al., 1998). In contrast to the non-chlorinated compounds described above, permease-type transporters are not necessarily the most common kind of transporter involved in chloroaromatic acid uptake. Although the TfdK permease is the only chloroaromatic transport protein described so far, evidence of ABC-type transporters has been found for 2-chlorobenzoate, dichlorprop and 4-CB (Groenewegen et al., 1990; Yuroff et al., 2003; Zipper et al., 1998). Evidence was recently obtained for the involvement of a third type of transport system, a TRAP transporter for 4-CB, encoded by the fcbTIT2T3 genes in Comamonas sp. strain DJ-12 (Chae & Zylstra, 2006).

In order to find the 3-CB transport system in the C. necator JMP134 genome, a BLAST search was performed for different types of transporters. For permease-type transporters, the benK sequence from A. baylyi ADP-1 was selected, since it is the only benzoate transporter gene with a biochemically confirmed function. The proteins VanK, MucK and PcaK from strain ADP-1 were also selected as representatives of biochemically confirmed transport functions, and genomic sequence searches were performed in C. necator JMP134 for these proteins as well. Transporter proteins with a proposed function were also searched for in the genome of strain JMP134, including BenK from P. putida PRS2000, PcaK from Azoarcus sp. EbN1, BenK from Rhodococcus jostii RHA1 and a putative A. baylyi ADP-1 transport gene. For ABC-type transporters, the sequences from Azoarcus evansii were selected, along with a putative ABC transporter of unknown function from plasmid pJP4 (Trefault et al., 2004). As an additional type of transporter gene, the TRAP family transporter (Chae & Zylstra, 2006) was also included in the search. However, no member of this family could be found by homology search in the C. necator JMP134 genome. A homology search for permease-type transporters yielded some 30 possible 3-CB transporters, a group of five putative permease sequences (ORFs Reut_A1616, Reut_A1823, Reut_B3938, Reut_C5941 and Reut_C6046) from the C. necator JMP134 genome could be related to benzoate, and possibly 3-CB, transport (see branch at the top of Fig. 4). In order to investigate their participation in 3-CB transport, the expression of each of these ORFs was explored qualitatively by RT-PCR, with RNA obtained from C. necator cells grown on 3-CB or fructose. The results of this experiment showed that only ORF Reut_B3938 increased its expression when strain JMP134 grew on 3-CB relative to fructose, and so this ORF (hereinafter named benP) was selected as a

### Table 1. Competitors of 3-CB uptake by 3-CB-grown cells of strain JMP134

<table>
<thead>
<tr>
<th>Test competitor</th>
<th>Metabolism of competitor</th>
<th>Uptake rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NA</td>
<td>100 ± 8*</td>
</tr>
<tr>
<td>Benzoate analogues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoate</td>
<td>+</td>
<td>26 ± 7</td>
</tr>
<tr>
<td>3-Chloro</td>
<td>+</td>
<td>58 ± 8</td>
</tr>
<tr>
<td>3-Methyl</td>
<td>+</td>
<td>83 ± 11</td>
</tr>
<tr>
<td>3-Hydroxy</td>
<td>+</td>
<td>96 ± 6</td>
</tr>
<tr>
<td>4-Chloro</td>
<td>–</td>
<td>94 ± 6</td>
</tr>
<tr>
<td>4-Fluoro</td>
<td>+</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>4-Methyl</td>
<td>–</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>4-Hydroxy</td>
<td>+</td>
<td>97 ± 8</td>
</tr>
<tr>
<td>2-Chloro</td>
<td>–</td>
<td>101 ± 6</td>
</tr>
<tr>
<td>2-Methyl</td>
<td>–</td>
<td>98 ± 5</td>
</tr>
<tr>
<td>2-Hydroxy</td>
<td>+</td>
<td>102 ± 4</td>
</tr>
<tr>
<td>2,3-Dichloro</td>
<td>–</td>
<td>111 ± 9</td>
</tr>
<tr>
<td>2,4-Dichloro</td>
<td>–</td>
<td>110 ± 5</td>
</tr>
<tr>
<td>3,4-Dichloro</td>
<td>–</td>
<td>83 ± 8</td>
</tr>
<tr>
<td>3,5-Dichloro</td>
<td>–</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>Phenoxyacetates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4-D</td>
<td>+</td>
<td>99 ± 6</td>
</tr>
<tr>
<td>Phenoxyacetate</td>
<td>–</td>
<td>103 ± 3</td>
</tr>
</tbody>
</table>

*An uptake of 1.16 ± 0.09 nmol 3-[14]C]CB min<sup>-1</sup> (mg protein)<sup>-1</sup> was measured in the absence of competitors.

1982). In most cases, however, the description of transport is limited to assessment of uptake of the radiolabelled substrate and the proposition of the type of transporter involved. For two of the compounds and organisms described above, uptake was proposed to be mediated by an ABC-type primary transporter (energized by ATP hydrolysis): 4-hydroxyphenylacetate in K. pneumoniae strain M5a1 (Allende et al., 1992) and 4-HB in Acinetobacter sp. strain BEM2 (Allende et al., 2000). Most of the remaining transport systems are proposed to be secondary transporters, which utilize energy stored in electrochemical gradients of the cytoplasmic membrane to drive substrate movement. A few of these permease-type transport proteins have been biochemically characterized, and the corresponding gene has been described. This category includes benK for benzoate transport, vanK for vanillate, haaK for hydroxycinnamate, and mucK for mucronate, all found in Acinetobacter baylyi ADP-1 (Collier et al., 1997; D’Argenio et al., 1999; Parke & Ornston, 2003; Williams & Shaw, 1997), and pcaK for 4-HB and protocatechuate in P. putida PRS2000 (Harwood et al., 1994).

Energy-dependent transport of chlorinated aromatics has been demonstrated in a few cases (Groenewegen et al., 1990; Leveau et al., 1998; Yuroff et al., 2003; Zipper et al., 1998). Among these, a transporter gene has been characterized only for 2,4-D (Leveau et al., 1998). In contrast to the non-chlorinated compounds described above, permease-type transporters are not necessarily the most common kind of transporter involved in chloroaromatic acid uptake. Although the TfdK permease is the only chloroaromatic transport protein described so far, evidence of ABC-type transporters has been found for 2-chlorobenzoate, dichlorprop and 4-CB (Groenewegen et al., 1990; Yuroff et al., 2003; Zipper et al., 1998). Evidence was recently obtained for the involvement of a third type of transport system, a TRAP transporter for 4-CB, encoded by the fcbTIT2T3 genes in Comamonas sp. strain DJ-12 (Chae & Zylstra, 2006).

In order to find the 3-CB transport system in the C. necator JMP134 genome, a BLAST search was performed for different types of transporters. For permease-type transporters, the benK sequence from A. baylyi ADP-1 was selected, since it is the only benzoate transporter gene with a biochemically confirmed function. The proteins VanK, MucK and PcaK from strain ADP-1 were also selected as representatives of biochemically confirmed transport functions, and genomic sequence searches were performed in C. necator JMP134 for these proteins as well. Transporter proteins with a proposed function were also searched for in the genome of strain JMP134, including BenK from P. putida PRS2000, PcaK from Azoarcus sp. EbN1, BenK from Rhodococcus jostii RHA1 and a putative A. baylyi ADP-1 transport gene. For ABC-type transporters, the sequences from Azoarcus evansii were selected, along with a putative ABC transporter of unknown function from plasmid pJP4 (Trefault et al., 2004). As an additional type of transporter gene, the TRAP family transporter (Chae & Zylstra, 2006) was also included in the search. However, no member of this family could be found by homology search in the C. necator JMP134 genome. A homology search for permease-type transporters yielded some 30 possible 3-CB transporters, a group of five putative permease sequences (ORFs Reut_A1616, Reut_A1823, Reut_B3938, Reut_C5941 and Reut_C6046) from the C. necator JMP134 genome could be related to benzoate, and possibly 3-CB, transport (see branch at the top of Fig. 4). In order to investigate their participation in 3-CB transport, the expression of each of these ORFs was explored qualitatively by RT-PCR, with RNA obtained from C. necator cells grown on 3-CB or fructose. The results of this experiment showed that only ORF Reut_B3938 increased its expression when strain JMP134 grew on 3-CB relative to fructose, and so this ORF (hereinafter named benP) was selected as a
candidate for inactivation. This was carried out by recombination with an internal fragment of \( \text{benP} \) cloned in the pTOPO plasmid. The \( \text{benP} \) mutant strain showed a significant reduction in 3-CB transport rate (Fig. 3a). Although these results seem to implicate \( \text{benP} \) in 3-CB transport, the genomic context surrounding the gene

**Fig. 4.** Permease-type transporters in the genome of *C. necator* JMP134. Dendrogram of 30 genes encoding proteins with over 30 % identity to permeases described for transport of benzoate or 4-hydroxybenzoate. Transporters implicated in the degradation of other aromatic compounds and/or intermediates of their degradation, such as vanillate (VanK), muconate (MucK) and hydroxycinnamate (HcaK), are included. Amino acid sequence alignments were performed by CLUSTAL W, and were analysed using the Mega3 software package. Proteins with an experimentally determined function are in bold. * Sequences used for homology searches in the complete genome sequence of strain JMP134.
appears unrelated to catabolic functions towards benzoic acid or any substituted derivative. As shown in Fig. 1, benP is flanked downstream by a gntR family putative regulator gene (26% amino acid identity with NorG from Staphylococcus aureus subsp. aureus USA300), while an ORF with no significant identity to an experimentally determined protein function is located upstream (Reut_B3937). Further upstream of this ORF, Reut_B3936 and Reut_B3935 are homologous to a glycosyltransferase CasB from Bacillus subtilis (25% amino acid identity) and a 3-oxoacyl reductase FabG from Synechocystis sp. PCC 6803 (49% amino acid identity), respectively. Downstream of the putative gntR gene, ORF Reut_B3940 has 26% identity with a NirA nitrite reductase from Synechococcus elongatus PCC 7942. Growth of the benP mutant on 3-CB was assessed in order to investigate the influence of transport on 3-CB degradation under standard laboratory conditions (Fig. 3b). The fact that growth of this C. necator JMP134 mutant was only slightly retarded suggests that at least one other transport protein is able to take up the function of 3-CB uptake, although probably at a slower rate than that exhibited by the benP gene product. It is also possible that 3-CB uptake in the benP gene mutant strain is carried out by diffusion facilitated by metabolic drag of the substrate, but this appears unlikely, since metabolic drag seems to contribute very little to transport of the chloroaromatic compound, as shown by the transport rate of the benA mutant strain (see above). However, contribution of other transport systems with overlapping specificity towards 3-CB could account for the remaining uptake rate that is measured in the absence of the BenP protein.

ACKNOWLEDGEMENTS

This work was financed by FONDECYT grants 1030493 and 1070343, and by the Millennium Scientific Initiative through the Millennium Nucleus in Microbial Ecology, Biotechnology and Applied Microbiology (EMBA) grant P/04-007-F.

REFERENCES


Pérez-Pantoja, D., Guzmán, L., Manzano, M., Pieper, D. H. & González, B. (2000). Role of tfd CIDIEF I and tfd CIDIEF II gene...


Edited by: D. J. Arp