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

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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*-UL-^{14}C]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
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lesser degree by 3-CB and 3,5-dichlorobenzoate, but not by any of another 12 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.

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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 pK_a 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

Abbreviations: 3-CB, 3-chlorobenzoate; 4-CB, 4-chlorobenzoate; 2,4-D, 2,4-dichlorophenoxyacetate; 4-HB, 4-hydroxybenzoate; TRAP, tripartite ATP-independent periplasmic.

2 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.*, 1998), 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 *fcbT1T2T3* 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.jgi-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-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 (Kröckel & 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 OD_{600} 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 on Luria–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 pBBRCMS-2 (pBBRB3938) (Kovach *et al.*, 1995) were mobilized from *E. coli* DH5α to *C. necator* JMP134-B1 by triparental mating with the helper strain *E. coli* 3
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′-ACGAGTACCTGTGGGACGAC-3′) and RBENA (5′-GTCGTTGTTGTTCGGGATCT-3′), and primers FBENP (5′-GTTGTTCGGCATGATGTTTG-3′) and RBENP (5′-ATGGAGTCAGGCAGTTTGCT-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′-CCCCGACACTACCAGACAAT-3′) and R2BENA (5′-GGTACACGTTCGGGTACAGG-3′), and F2BENP 5′-CGCCACCTTCAACCGCTTCC-3′) and

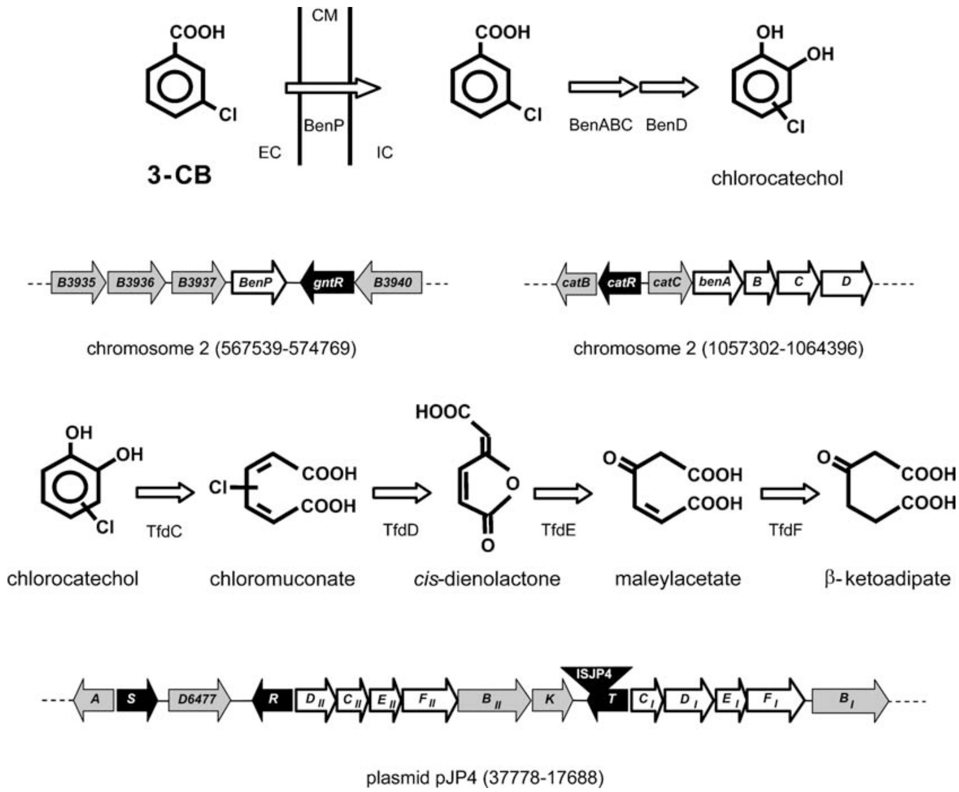

Fig. 1. 3-CB degradation pathway and the genes involved in *C. necator* JMP134. 3-CB is taken up into the cell by a putative transporter (upper left). Once in the cytoplasm, it is initially transformed by the BenABC and BenD enzymes into a mixture of 3- and 4-chlorocatechol (upper right). These compounds are substrates of the specialized *ortho* cleavage pathway encoded by the *tfd* genes, resulting in the production of β-ketoadipate, 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, extra- and intracellular space, respectively; CM, cytoplasmic membrane.

(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-[*ring*-UL-^{14}C]CB [purity, 96 %; specific activity, 29 mCi mmol^{-1} (1.07×10^{9} Bq mmol^{-1})] was obtained from Sigma. For 3-CB uptake assays, a hot-cold stock solution [specific activity, 2.9 mCi mmol^{-1} (1.07×10^{8} Bq mmol^{-1})] was
4 prepared in sterile 50 mM Na_2HPO_4/KH_2PO_4 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 Na_2HPO_4/KH_2PO_4, pH 7.6), and resuspended in this buffer to an OD_{600} 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 nCi,1.07×10^{6} 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 Na_2HPO_4/KH_2PO_4 with 20 mM $HgCl_2$, 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 ^{14}C 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_{max} and K_m were estimated by nonlinear regression fitting to the Michaelis–Menten equation.

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-[^{14}C]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±0.06 nmol 3-[^{14}C]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-[^{14}C]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-[^{14}C]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 5
substrate affinity can contribute to 3-CB uptake.

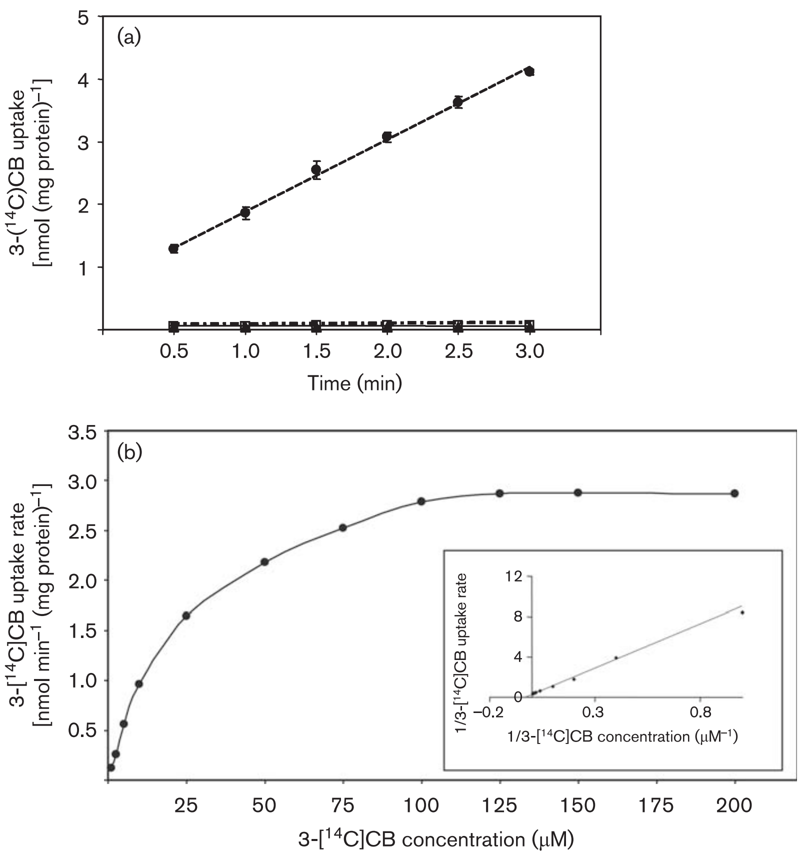

Fig. 2. (a) 3-CB uptake by *C. necator* JMP134. 3-[^{14}C]CB uptake was measured at 0.5 min intervals up to 3 min for strain JMP134 grown on 3-CB (●) or on fructose (□). Uptake was also measured in 3-CB-grown cells previously incubated for 10 min at 95 °C (heat-killed cells, ▲). All measurements were 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-[^{14}C]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}C]CB min^{-1} (mg protein)$^{-1}$, and by benzoate [0.88 ± 0.04 nmol 3-[^{14}C]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}C]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. 6

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, Allende *et al.*, 2002; Chang & Zylstra, 1999; Collier *et al.*, 1997; Harwood *et al.*, 1994; Higgins & Mandelstam, 1972; Nichols & Harwood, 1997; Prieto & García, 1997; Saint & Romas, 1996; Schleissner *et al.*, 1994; Thayer & 7

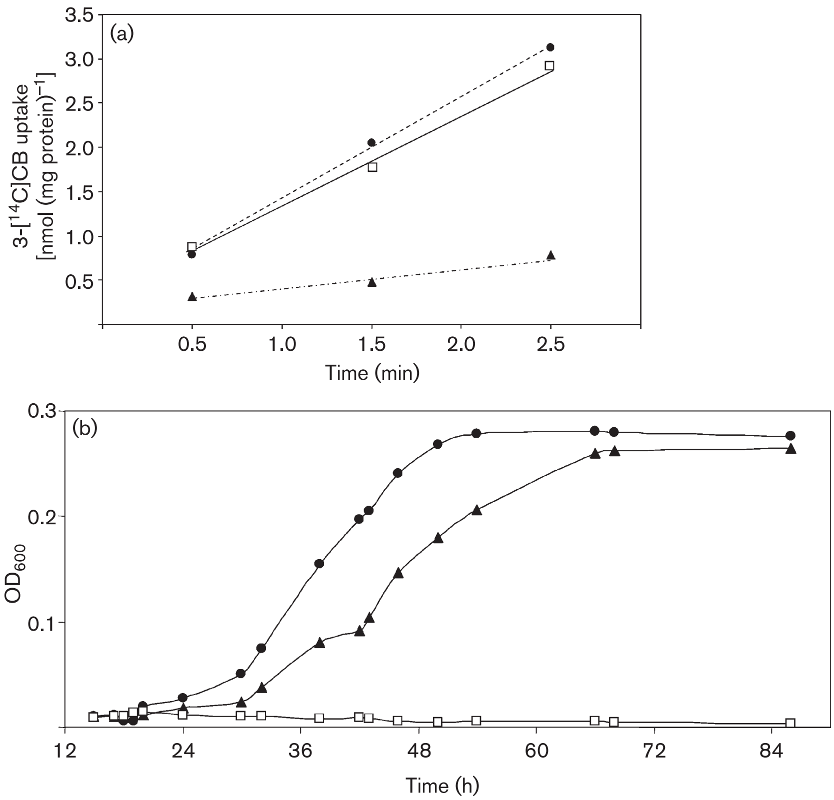

Fig. 3. *benP* (ORF Reut_B3938) encodes a 3-CB transporter in *C. necator* JMP134. (a) 3-[^{14}C]CB uptake by the *benP* mutant (▲) compared to the wild-type JMP134 (●) and the *benA* mutant (□). Cells were grown on fructose and induced with 1 mM 3-CB for 3 h in order to exclude differences in 3-CB growth between the wild-type and mutant strains. All measurements were carried out at a cell density corresponding to 0.09 mg total protein. Results show the values obtained for three independent experiments. (b) Growth of the wild-type (●), the *benP* mutant (▲) and the *benA* mutant (□) on 2 mM 3-CB. Results show representative values obtained in three independent experiments.

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

The values presented represent the mean $\pm$ SD of three independent determinations. Metabolism of competitor does not imply utilization for growth.

Test competitor	Metabolism of competitor	Uptake rate (%)
None	NA	100 $\pm$ 8*
Benzoate analogues		
Benzoate	+	26 $\pm$ 7
3-Chloro	+	58 $\pm$ 8
3-Methyl	+	83 $\pm$ 11
3-Hydroxy	+	96 $\pm$ 6
4-Chloro	−	94 $\pm$ 6
4-Fluoro	+	103 $\pm$ 5
4-Methyl	−	104 $\pm$ 4
4-Hydroxy	+	97 $\pm$ 8
2-Chloro	−	101 $\pm$ 6
2-Methyl	−	98 $\pm$ 5
2-Hydroxy	+	102 $\pm$ 4
2,3-Dichloro	−	111 $\pm$ 9
2,4-Dichloro	−	110 $\pm$ 5
3,4-Dichloro	−	83 $\pm$ 8
3,5-Dichloro	−	67 $\pm$ 3
Phenoxyacetates		
2,4-D	+	99 $\pm$ 6
Phenoxyacetate	−	103 $\pm$ 3

*An uptake of 1.16 $\pm$ 0.09 nmol 3-[^{14}C]CB min^{-1} (mg protein)$^{-1}$ was measured in the absence of competitors.

Wheelis, 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, *hcaK* for hydroxycinnamate, and *mucK* for muconate, 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
9 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
10 transport system, a TRAP transporter for 4-CB, encoded by the *fcbT1T2T3* 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 with variable homology to described aromatic acid transporters. A dendrogram was constructed in order to select candidates for the 3-CB transporter (Fig. 4). Candidates of the ABC-type transporter group, on the other hand, were less abundant, mainly due to the smaller number of genes of this family associated with aromatic acid transport (data not shown). For permease-type 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

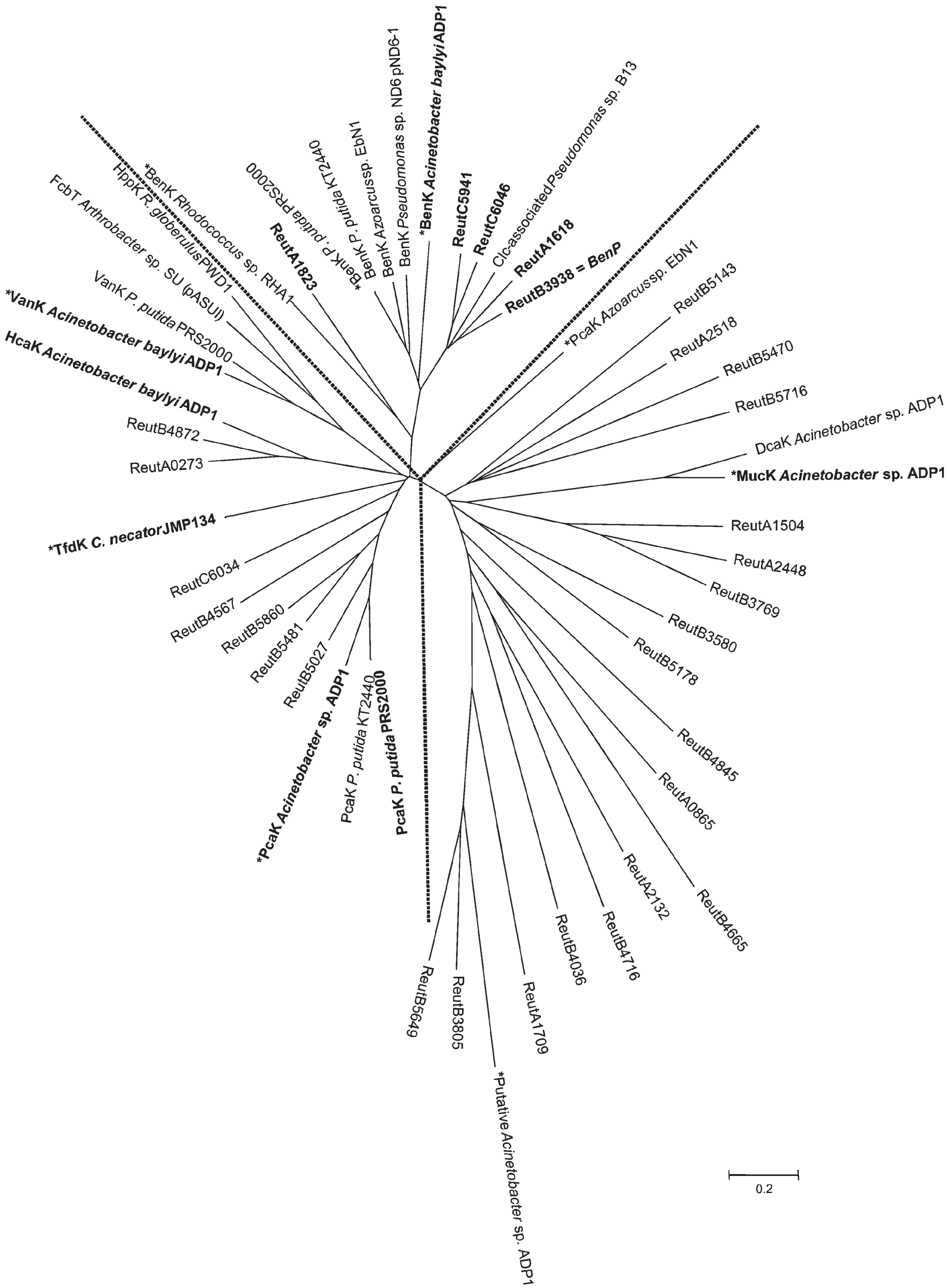

Fig. 4. Permease-type transporters in the genome of *C. necator* JMP134. Dendrogram of 30 genes encoding proteins with over 30 % identity to permeases described for transport of benzoate or 4-hydroxybenzoate. Transporters implicated in the degradation of other aromatic compounds and/or intermediates 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.

candidate for inactivation. This was carried out by recombination with an internal fragment of *benP* cloned in the pTOPO plasmid. The *benP* mutant strain showed a significant reduction in 3-CB transport rate (Fig. 3a). Although these results seem to implicate *benP* in 3-CB transport, the genomic context surrounding the gene

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
8 *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 CsbB 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. 4b). 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.

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