

## 4-Chlorobenzoate Uptake in *Comamonas* sp. Strain DJ-12 Is Mediated by a Tripartite ATP-Independent Periplasmic Transporter<sup>∇</sup>

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**The *fcB* gene cluster involved in the hydrolytic dehalogenation of 4-chlorobenzoate is organized in the order *fcB*-*fcBA*-*fcBT1*-*fcBT2*-*fcBT3*-*fcBC* in *Comamonas* sp. strain DJ-12. The genes are operonic and inducible with 4-chloro-, 4-iodo-, and 4-bromobenzoate. The *fcBT1*, *fcBT2*, and *fcBT3* genes encode a transporter in the secondary TRAP (tripartite ATP-independent periplasmic) family. An *fcBT1T2T3* knockout mutant shows a much slower growth rate on 4-chlorobenzoate compared to the wild type. 4-Chlorobenzoate is transported into the wild-type strain five times faster than into the *fcBT1T2T3* knockout mutant. Transport of 4-chlorobenzoate shows significant inhibition by 4-bromo-, 4-iodo-, and 4-fluorobenzoate and mild inhibition by 3-chlorobenzoate, 2-chlorobenzoate, 4-hydroxybenzoate, 3-hydroxybenzoate, and benzoate. Uptake of 4-chlorobenzoate is significantly inhibited by ionophores which collapse the proton motive force.**

Bacterial transport systems have traditionally been divided into four general classes based on energy coupling mechanisms, primary sequence, and mode of transport (7, 16, 25, 28). Primary and secondary transporters facilitate solute transport into the cell coupled with a source of energy (i.e., a chemical reaction, light absorption, or electron flow) or an ion electrochemical gradient, respectively (28). Third, group translocators modify their substrates during transport such as phosphorylation. The fourth group are channel-type proteins allowing energy-independent diffusion of the substrate. The first two families of solute transport systems occupy the majority among the known and predicted transporters encoded by microbial genomes (23).

Extracytoplasmic solute receptor-dependent uptake systems have been known as primary transporters for quite some time, which consist of a periplasmic binding protein, integral membrane proteins, and ABC (ATP-binding cassette) proteins (13, 16). This kind of system was once considered to be strictly limited to this ABC transporter family, with ATP hydrolysis as the mechanism of energy coupling, until the discovery of a new type of transporter designated TRAP (tripartite ATP-independent periplasmic) transporters (9, 14, 16, 25). This transporter was first delineated in the Dct system of *Rhodobacter capsulatus* as a type of secondary transporter which drives C<sub>4</sub>-dicarboxylate accumulation by an ion electrochemical gradient instead of ATP hydrolysis (9). From the available genome sequences, similar and hitherto-unrecognized TRAP transport systems are found to be widespread in bacteria and archaea, but not eukaryotes (16).

Uptake of aromatic compounds is a prerequisite for metabolic degradation in microorganisms and can occur via passive diffusion of neutral compounds or active transport for charged compounds (6, 12). Several transporters are known to facilitate

the movement of aromatic compounds across the membrane: BenK for benzoate (6), OphD for phthalate (5), PcaK for 4-hydroxybenzoate and protocatechuate (22), TfdK for 2,4-dichlorophenoxyacetate (18), StyE for styrene (21), and XylN for *m*-xylene (15). However, little is known about the transporter for 4-chlorobenzoate (4CBA), which is a metabolite in the microbial breakdown of certain chloroaromatic pollutants. Active transport of 4CBA has been suggested in the coryneform bacterium NTB-1 (11) and *Arthrobacter* sp. strain SB8 (31), and genes encoding putative transporters localized along with a 4CBA degradative gene cluster were found in *Arthrobacter* sp. strain SU (accession no. AF030397) (29), *Arthrobacter* sp. strain TM1 (accession no. AF042490) (10), *Arthrobacter* sp. strain FHP1 (accession no. AB041030), *Alcaligenes* sp. strain AL3007 (accession no. AF537222) (17), and *Pseudomonas* sp. strain DJ-12 (3).

*Comamonas* sp. strain DJ-12 (formerly *Pseudomonas*) has previously been reported as a 4CBA degrader containing an *fcB* gene cluster encoding enzymes responsible for hydrolytic dechlorination of 4CBA to 4-hydroxybenzoate (3). The genes encoding a coenzyme A (CoA) ligase (*fcBA*), a hydrolytic dehalogenase (*fcBB*), and a thioesterase (*fcBC*) are organized in the order *fcBB*-*fcBA*-*fcBT1*-*fcBT2*-*fcBT3*-*fcBC*. The *fcBT1*, *fcBT2*, and *fcBT3* genes were previously postulated by us to encode substrate binding protein and small and large membrane proteins, respectively, of a TRAP transport family 4CBA transporter by homology to TRAP transport family proteins known to transport other polar compounds into the cell (3). The current work was performed to prove that the *fcBT1T2T3* genes actually do encode a 4CBA transporter and to functionally characterize the ability to transport 4CBA and related compounds into the cell.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and cultivation.** *Escherichia coli* JM109 (35) and *E. coli* S17-1 (*Apr*) (24) were used as the recipient strains in the cloning experiments. Mineral salts basal (MSB) medium (32) was used as minimal medium, and LB broth (Difco) was used as complete medium. Ampicillin and kanamycin were added at 100 and 75 µg/ml, respectively, when necessary. *Comamonas* and *E. coli* transformants were cultivated at 30°C and 37°C, respectively.

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The pBluescript SKII(+) (Stratagene) and pJP5603 suicide vector (24) were used for cloning and knockout mutations. The promoter probe vector pKRZ-1 (27) was employed for the promoter assay. *E. coli* HB101 harboring pRK2013 was used as a helper strain in mating experiments (8). Plasmids were isolated with the NucleoSpin plasmid Miniprep kit (BD Biosciences).

**Isolation of total RNA and RT-PCR.** *Comamonas* sp. strain DJ-12 was grown in MSB with either 1 mM 4CBA or 5 mM succinate. To induce the corresponding genes, the cells were incubated at 30°C for at least 12 h. Total RNA was extracted with the Trizol reagent (Invitrogen) according to the manufacturer's recommended protocol. The extracted total RNA was further purified with the RNeasy mini kit (QIAGEN) and DNase I (QIAGEN) treatment according to the manufacturer's instructions. The reverse transcription-PCRs (RT-PCRs) were performed in 25- $\mu$ l reaction volumes with 0.5  $\mu$ g of the extracted RNA using the OneStep RT-PCR kit (QIAGEN). The thermocycler program was as follows: 50°C for 30 min, 95°C for 15 min, 30 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 3 min), and 72°C for 10 min. The following primers were designed to amplify the *fc* genes: for *fc*B*A*, 5'-ATGTACGAAGCCATCGGTC-3' (forward) and 5'-CTAACTTGACACCTGTCTG-3' (reverse); for *fc*B*AT1*, 5'-ATGTACGAA GCCATCGGTC-3' (forward) and 5'-TCCTCATGAGCCCTTCG-3' (reverse); and for *fc*B*TIT2T3C*, 5'-CCTGAAGCTGACGCTCTG-3' (forward) and 5'-TTAGCTACATAGGGCCAAAG-3' (reverse).

**Sequence analysis.** Deduced amino acid sequences were analyzed using the Lasergene software (DNASTAR, Inc.) and compared with the GenBank database using programs based on the BLAST algorithm (1).

**$\beta$ -Galactosidase assays.** A 510-bp PCR product upstream of *fc*B operon harboring the putative promoter region was amplified using the primers *fc*B-P (CGTCTGACACAGCAGCTGCATTC) and *fc*B-P-R (GTACTCTAGAAAT GCTCCTTGACG) containing *S*alI and *X*baI restriction sites (underlined), respectively. The PCR product was obtained in a 50- $\mu$ l reaction mixture using Premix *Taq* polymerase (Sigma). The reactions were initiated at 94°C (2 min); followed by 30 cycles of denaturing at 94°C (30 s), annealing at 55°C (30 s), and extension at 72°C (30 s); and ended with incubation at 72°C for 10 min. The resultant PCR product was purified from an agarose gel with the QIAEXII gel extraction kit (QIAGEN). Purified DNA fragments were digested with the two corresponding restriction enzymes and then ligated with pKRZ-1 treated with the same enzymes to construct pFCBP1. The latter construct was transferred into *Comamonas* sp. strain DJ-12 by triparental conjugation (8) with selection on LB agar (Difco) containing ampicillin and kanamycin.

DJ-12(pFCBP1) was cultivated overnight in MSB containing 10 mM succinate as a carbon source and kanamycin as selective pressure. The cells were subcultured into the same growth medium supplemented with 0.5 mM benzoate derivatives and cultivated for 8 h. The optical density at 600 nm (OD<sub>600</sub>) was determined, and 200  $\mu$ l of the culture was used for a modified  $\beta$ -galactosidase assay (20). The cells were mixed with 800  $\mu$ l of buffer Z (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 40 mM 2-mercaptoethanol) following permeabilization by adding 20  $\mu$ l of chloroform and 30  $\mu$ l of 0.1% sodium dodecyl sulfate. *o*-Nitrophenyl- $\beta$ -D-galactopyranoside (100  $\mu$ l of a 4-mg/ml stock) was added to initiate the reaction, and the mixture was incubated at 30°C for 30 min. The reaction was stopped by adding 250  $\mu$ l of 1 M sodium carbonate to the mixture. Reported  $\beta$ -galactosidase activity values are presented in units as specified by Miller (20). The results given in this study were determined from three independent cultures.

**Construction of a knockout mutant.** An *fc*B*T1*, *-T2*, and *-T3* mutant was constructed by homologous recombination. A BamHI-NotI DNA fragment from pKC158 (3) containing the *fc*B gene cluster was subcloned into the pBluescript SKII(+) vector. Using the *S*acI recognition site originating from the vector, a BamHI-*S*acI insert fragment was ligated into the pJP5603 suicide vector and transformed into *E. coli* S17-1 ( $\lambda$ pir). This construct (designated pMUT01) was digested with *C*laI and self-ligated to remove the *fc*B*T1T2T3* genes. The resultant pMUT02 was transformed into *E. coli* S17-1 ( $\lambda$ pir) and transferred into *Comamonas* sp. strain DJ-12 by triparental conjugation. Single-crossover mutants were screened in solid LB medium with ampicillin and kanamycin. The recombinant strain was cultivated in liquid LB medium without selective pressure overnight. After continuous cultivation in the same medium for three subcultures, the cells were plated on solid LB medium with ampicillin. Double-crossover mutants were selected for the inability to grow on medium with kanamycin. The knockout strain was designated MUT1. Deletion of the *C*laI fragment by double-crossover recombination was verified by colony PCR and Southern blotting. The same primers were used in PCR and to make a probe for the Southern blot: forward, 5'-ATGCGTATTCATCGTCGCCAG-3'; and reverse, 5'-TTAA CGTCCACGAACATCG-3'. PCR amplification using intact cells was performed in a 10- $\mu$ l reaction mixture. The PCR conditions were the same as those described above except for initiation at 94°C for 10 min to disrupt the cells and

extension of the PCR cycle at 72°C for 2 min. BamHI-NotI-digested genomic DNA was used for the Southern blotting, which was performed as described previously (4).

**Chemical analysis.** A cell culture collected in the incubation was filtered and analyzed with a high-performance liquid chromatography apparatus (Agilent 1100 series, German). 4-Chlorobenzoate was separated on a C<sub>18</sub> column (4.6 mm in diameter; 250 mm in length; Spherisorb) at a flow rate of 1 ml/min with a mobile phase of methanol-water-acetic acid mixture (55:45:2) and monitored by absorbance at a wavelength of 254 nm.

**4CBA transport assay.** Mid-log-phase cells of *Comamonas* sp. strain DJ-12 and MUT1 grown on LB broth were transferred into MSB minimal medium containing 5 mM succinate and 1 mM 4CBA. The cells were incubated for 12 h, harvested by centrifugation at 3,000  $\times$  g, washed three times with 50 mM sodium/potassium phosphate buffer (pH 6.8), and resuspended in the same buffer to an optical density of 1.0 at 600 nm. All assays were done at room temperature. Uptake was initiated by adding the cells to an equal volume of phosphate buffer containing 100  $\mu$ M [ring-UL-<sup>14</sup>C]4CBA (130 mCi/mmol; Amersham Pharmacia Biotech.). Samples (0.1 ml) were removed from the reaction mixture at various times (10 s, 1 min, 2 min, 3 min, and 4 min) and filtered through a Nuclepore track-etched polycarbonate membrane (0.2- $\mu$ m pore size; Whatman). The filters were washed before and after addition of the sample with 2 ml of phosphate buffer. Accumulated 4CBA inside the cells was determined by scintillation counting of the cells retained on the filters. Cell protein was determined by the method of Bradford (2).

Kinetic parameters for 4CBA uptake were determined by performing an uptake assay using different concentrations of 4CBA (0.05 to 150  $\mu$ M). Reaction mixtures were prepared in triplicate, and samples were taken at 1 min and 2 min. The kinetic parameters  $V_{max}$  and  $K_m$  were obtained from nonlinear fit of data to the Michaelis-Menten equation using PRIZM software (version 3.02; GraphPad Software).

The effect of aromatic acid inhibition of 4CBA uptake was investigated using 50  $\mu$ M of radiolabeled 4CBA and 1 mM of competing substrate. The transport assay mentioned above was initiated by adding an equal volume of cells to the reaction mixture containing 100  $\mu$ M of 4CBA and 2 mM of competing substrate.

Sodium orthovanadate, 2,4-dinitrophenol (DNP), 1,3-dicyclohexylcarbodiimide (DCCD), and *m*-chlorophenylhydrazine (CCCP) were used for the energetic inhibition study (19, 26). DNP, CCCP, and DCCD were added to the cells at final concentrations of 2 mM, 0.2 mM, and 2 mM, respectively, 10 min prior to starting a transport assay. The cells were pretreated with 1 mM sodium orthovanadate for 15 min.

## RESULTS AND DISCUSSION

**Transcription of the *fc*B gene operon.** The nucleotide sequence of the *fc*B gene cluster encoding enzymes for the hydrolytic dehalogenation of 4CBA was previously reported by us from *Comamonas* sp. strain DJ-12 (3). In between the *fc*B*A* and *fc*B*C* genes are three genes recognizable as encoding a potential TRAP-type transporter (Fig. 1). These genes, designated *fc*B*T1*, *fc*B*T2*, and *fc*B*T3*, are not present in hydrolytic dehalogenation gene clusters found in other microorganisms (10, 17, 29, 30). Transcriptional analysis was accomplished to investigate the expression of the *fc*B gene cluster. When RT-PCR was performed with the same amount of total RNA extracted from *Comamonas* sp. strain DJ-12 grown on 4CBA or succinate, the *fc*B*BA* genes encoding 4CBA-CoA dehalogenase and 4CBA-CoA ligase were amplified only from the RNA extracted from cells induced by 4CBA (Fig. 1A). To examine whether the six open reading frames in the *fc*B gene cluster are transcribed as an operon, primer sets were designed to amplify the region in two overlapping PCR products. The expected RT-PCR products were as follows: *fc*B*BAT1* and *fc*B*TIT2T3C* (3.2 kb and 3.0 kb, respectively). As shown in Fig. 1B, the expected two PCR products were amplified from total RNA extracted from *Comamonas* sp. strain DJ-12 induced with 4CBA. These RT-PCR experiments thus demonstrate that expression of the transporter genes is dependent on growth on 4CBA and that the

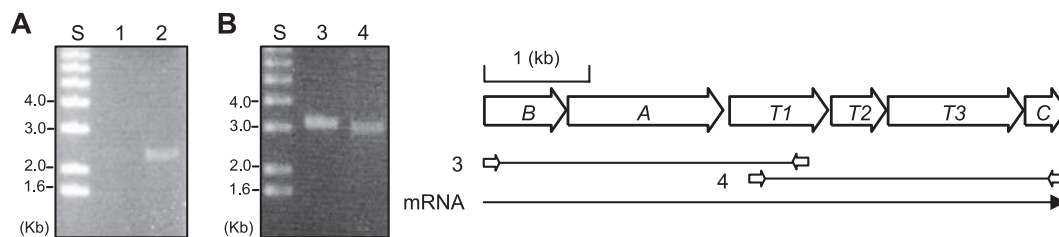


FIG. 1. Transcriptional analysis indicating inducible (A) and operonic (B) expression of the *fcb* gene cluster in *Comamonas* sp. strain DJ-12. Lanes 1 and 2 (A) show RT-PCR results amplified with total RNA extracted from cells grown on 10 mM succinate or 1 mM 4CBA, respectively. Lanes 3 and 4 (B) indicate RT-PCR products obtained using RNA prepared from cells induced with 1 mM 4CBA. *fcbA*, *-B*, and *-C* encode 4CBA-CoA ligase, 4CBA-CoA dehalogenase, and 4HBA-CoA thioesterase, respectively. *fcbT1*, *-T2*, and *-T3* represent putative transporter genes. S, 1-kb DNA ladder.

*fcbT1T2T3* genes are present on the same mRNA as the *fcbBAC* genes. The *fcbBAT1T2T3C* genes are thus coregulated and in the same operon.

In order to verify the inducibility and the effect of different substrates, the upstream region of *fcbB* was fused with the *lacZ* reporter gene of the pKRZ-1 vector using *SalI* and *XbaI* as described in the Materials and Methods. The *SalI*-*XbaI* fragment includes the putative ribosome binding site and promoter. The resulting construct, pFCBP1, was transformed into the wild-type DJ-12 strain by triparental conjugation and used for the  $\beta$ -galactosidase assay (Fig. 2). DJ-12 with pFCBP1 was grown on succinate and exposed to different benzoate derivatives. Cells induced by 4CBA, 4-bromobenzoate, and 4-iodobenzoate showed the highest  $\beta$ -galactosidase activity. Interestingly, cells exposed to 2-chlorobenzoate and 4-fluorobenzoate have approximately double the  $\beta$ -galactosidase activity seen with the control with no substituted benzoate added. This demonstrates that 2-chlorobenzoate and 4-fluorobenzoate act as weak inducible substrates.

**Disruption of the *fcbT1T2T3* genes by homologous recombination.** An approximately 7-kb *Bam*HI-*Not*I DNA fragment containing the *fcb* gene cluster was moved into the pJP5603

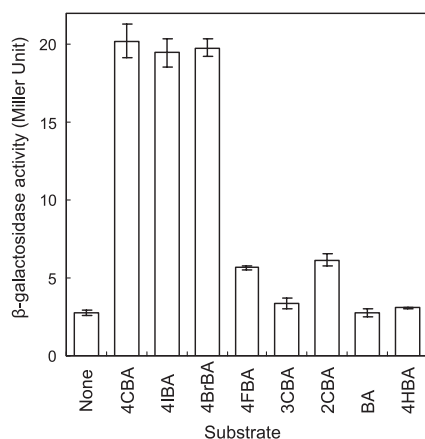


FIG. 2. Expression of the *fcb* gene operon in response to benzoate derivatives. The enzyme activities are the average of triplicate experiments. Cells were grown on succinate and exposed to one of the following inducing compounds: 4CBA, 4-chlorobenzoate; 4IBA, 4-iodobenzoate; 4BrBA, 4-bromobenzoate; 4FBA, 4-fluorobenzoate; 3CBA, 3-chlorobenzoate; 2CBA, 2-chlorobenzoate; BA, benzoate; 4HBA, 4-hydroxybenzoate.

suicide vector as described in Materials and Methods. The construct was designated pMUT01. The two *Cla*I sites in the middle of *fcbT1* and *fcbT3* were used for disruption of *fcbT1T2T3*. The deletion of *fcbT1T2T3* by *Cla*I digestion and self-ligation generates a hybrid open reading frame containing the 5' end of *fcbT1* and the 3' end of *fcbT3*, so that expression of the downstream *fcbC* gene is not affected by the removal of the *Cla*I fragment. The resultant clone pMUT02 was transferred into *Comamonas* sp. strain DJ-12 by triparental filter mating. A mutated strain with a single recombinational crossover was selected on an LB plate with antibiotic pressure since the insertion of pMUT02 into the chromosome endowed kanamycin resistance on DJ-12. The recombinant strain was cultivated on liquid LB medium without selective pressure overnight. After being subcultured three times on the same medium, the culture was plated on solid LB medium. Double-crossover mutants were selected by inability to grow on LB plus kanamycin due to the removal of vector fragment by a second recombinational event. The selected potential mutants were screened by PCR and Southern hybridization for deletion of the transporter gene fragment.

**4CBA uptake assay.** The ability to transport 4CBA was assayed in the wild-type strain DJ-12 and the knockout mutant strain MUT1. When grown in MSB medium containing 1 mM 4CBA, the knockout mutant MUT1 showed a much slower growth rate (generation time of 120.4 min) with the complete depletion of 4CBA within 12 h compared to the wild type (55.6 min), as shown in Fig. 3. On the other hand, the growth rates of both DJ-12 and MUT1 are identical (20.3-min doubling time) on MSB medium containing 1 mM 4-hydroxybenzoate. These data, combined with our previous bioinformatic analysis (3), indicate that the three *fcbT*-encoded proteins are involved in 4CBA transport into the cell. A 4CBA transport assay with DJ-12 and MUT1 positively proved this conclusion (Fig. 4). Saturation kinetics was established between uptake rate and different 4CBA concentrations with 4CBA-induced strain DJ-12 (Fig. 4A). Nonlinear fit of data to the Michaelis-Menten equation calculated a  $V_{max}$  of  $1.068 \pm 0.0182$  nmol  $\text{min}^{-1}$  mg of protein $^{-1}$  and transport affinity ( $K_m$ ) of  $1.648 \pm 0.1723$   $\mu\text{M}$ . This indicates that 4CBA transport into the cell does not rely on passive diffusion by a concentration gradient but occurs by an enzymatic reaction.

DJ-12 grown in the presence of 4CBA transports 4CBA into the cell at the rate of  $1.08 \pm 0.06$  nmol  $\text{min}^{-1}$  mg of protein $^{-1}$ , as shown in Fig. 4B. In contrast, MUT1 grown under the same

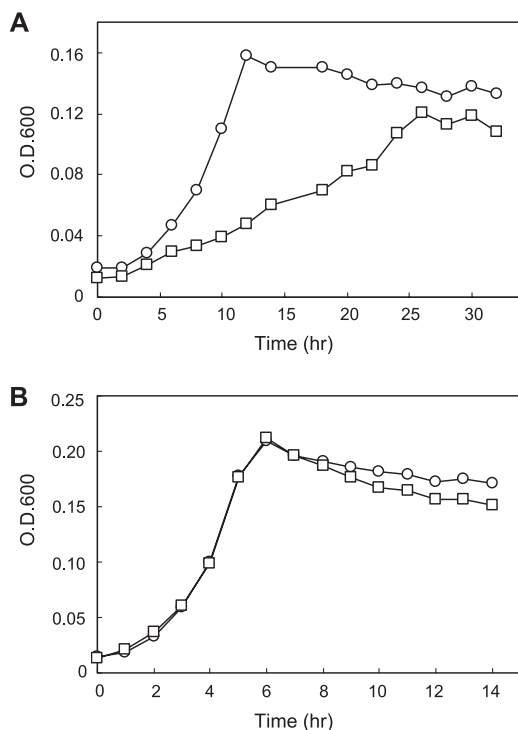


FIG. 3. Growth of *Comamonas* sp. strain DJ-12 (open circle) and MUT1 (open square) in minimal medium with 4-chlorobenzoate (A) and 4-hydroxybenzoate (B) as the sole carbon source. Optical density was measured at 600 nm.

conditions (succinate plus 4CBA) transports 4CBA at the rate of  $0.22 \pm 0.06 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}$ . This is only slightly faster than DJ-12 cells grown only on succinate.

The slow rate of growth of the knockout mutant MUT1 on 4CBA and the extremely slow transport of 4CBA into MUT1 may be due either to the presence of a second inefficient transporter or simple diffusion of 4CBA into the cell driven by metabolic enzymes since 4CBA was completely depleted by MUT1. For instance, Chang and Zylstra (5) reported that a phthalate permease knockout mutant strain could take up phthalate at the same rate as the wild type, suggesting the existence of a second phthalate transport system. PcaK in *Pseudomonas putida* is characterized as a multifunctional transporter for 4-hydroxybenzoate as well as protocatechuate (22). Metabolic enzymes can accelerate the simple diffusion of the undissociated form of benzoate and 4-hydroxybenzoate across biological membranes (6, 34).

**Transporter specificity.** The substrate specificity of the transporter was examined with *Comamonas* sp. strain DJ-12 through a substrate inhibition study which investigated the competitive uptake of radioactive 4CBA in the presence of related compounds. *para*-Substituted halogens inhibited transport the most in the order bromo > chloro > iodo > fluoro (Table 1). This indicates that the 4CBA transporter has strong substrate specificity for benzoates substituted in the 4 position. This is correlated with inducibility of the *pcb* operon by various substituted aromatic acids (Fig. 2). Interestingly, other substrates tested in this study also show an inhibitory effect on 4CBA transport. A 50 to 60% reduction in transport was ex-

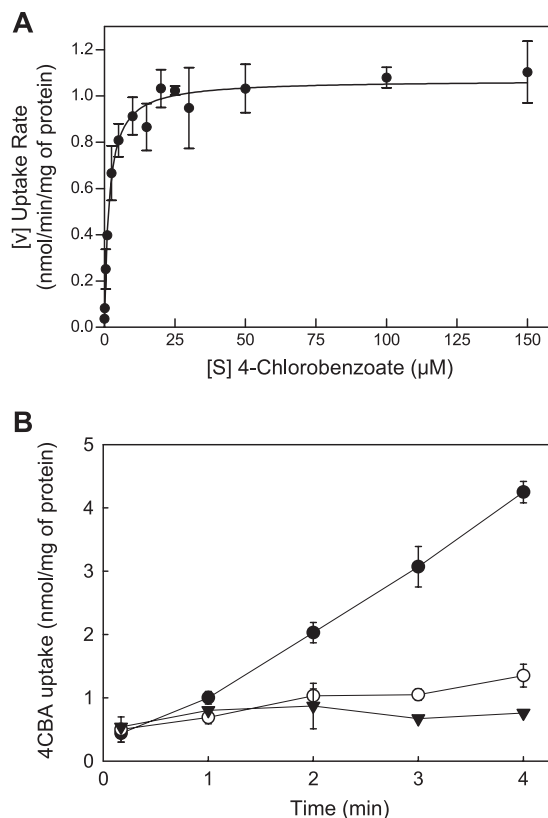


FIG. 4. Uptake of 4-chlorobenzoate by induced cells of *Comamonas* sp. strain DJ-12. (A) The curve shows a nonlinear regression fit of data to the Michaelis-Menten equation. (B) The uptake assay was performed with 4CBA-induced cells of *Comamonas* sp. strain DJ-12 (closed circle) and MUT1 (open circle) and uninduced DJ-12 (closed triangle). Each point in panels A and B is the average value from triplicate experiments. Error bars indicate standard deviation.

hibited with 3-chlorobenzoate, 2-chlorobenzoate, 4-hydroxybenzoate, 3-hydroxybenzoate, and benzoate. It is thus possible that this TRAP-type transporter has broad substrate affinity for many types of aromatic compounds.

**Energetic inhibition.** To determine the energy dependence of 4CBA uptake, *Comamonas* sp. strain DJ-12 was exposed to vanadate, DNP, CCCP, or DCCD before the transport assay

TABLE 1. Substrate inhibition of 4-chlorobenzoate uptake by *Comamonas* sp. strain DJ-12

Competitor <sup>a</sup>	% Inhibition of 4-chlorobenzoate uptake <sup>b</sup>
4-Bromobenzoate.....	91.5 $\pm$ 3
4-Chlorobenzoate .....	88.1 $\pm$ 3
4-Iodobenzoate .....	86.3 $\pm$ 8
4-Fluorobenzoate.....	76.0 $\pm$ 7
3-Chlorobenzoate .....	61.0 $\pm$ 8
2-Chlorobenzoate .....	55.2 $\pm$ 6
4-Hydroxybenzoate.....	60.0 $\pm$ 7
3-Hydroxybenzoate.....	52.7 $\pm$ 8
Benzoate .....	54.9 $\pm$ 4
2-Aminobenzoate.....	16.5 $\pm$ 2

<sup>a</sup> The concentrations of 4-chlorobenzoate and competing substrate were 50  $\mu\text{M}$  and 1 mM, respectively.

<sup>b</sup> Values are averages from three experiments.

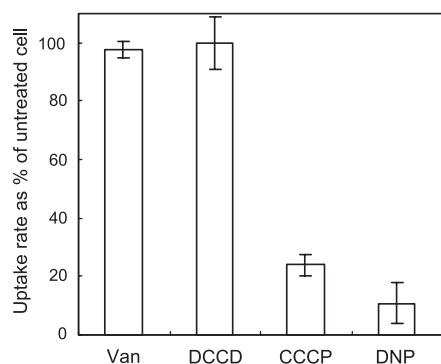


FIG. 5. Effect of energetic inhibitors on 4-chlorobenzoate uptake in *Comamonas* sp. strain DJ-12. Van, sodium orthovanadate.

was performed. DNP and CCCP are ionophores which destroy the formation of a proton motive force, whereas vanadate and DCCD disrupt ATP synthesis by acting on the ATPase complex. Ionophore and ATP synthesis blocking reagents are therefore used to distinguish between a primary and secondary transporter family by dissipating proton motive force or ATP (9, 14, 19, 26). As presented in Fig. 5, vanadate- or DCCD-treated cells retained almost 100% of the ability to transport 4CBA into the cell while DNP or CCCP exposure significantly decreased uptake rate in the cell. Although these compounds were used with rather high concentrations which may have side effects on the transporter or cell, the fact that an obvious difference was seen between the effects of CCCP or DNP and vanadate or DCCD suggests that transport of 4CBA into the cell is dependent on a proton motive force and not ATP. This is in agreement with what is known about other TRAP-type transporters (9, 33).

In addition, this result suggests that uptake of 4CBA in strain DJ-12 is dependent upon the transporter rather than the metabolic drag mechanism (34) caused by metabolic enzymes. Since ATP is required in CoA ligation onto 4CBA catalyzed by FcbA, which is the first step of dehalogenation (30), uptake of 4CBA should be severely inhibited by the vanadate or DCCD in case of the simple diffusion accelerated by the metabolic enzymes.

**Conclusion.** To our knowledge, this is the first demonstration of 4CBA uptake via a TRAP-type transporter. The presence of a transport system for 4CBA has been suggested in the coryneform bacterium NTB-1 (11) and in *Arthrobacter* sp. strain SB8 (31). The uptake of 4CBA in coryneform bacterium NTB-1 was considered to be driven by a proton symport mechanism, and Shimao et al. (31) proposed that the transport of 4CBA requires energy in *Arthrobacter* sp. strain SB8. Interestingly, examination of gene clusters known to be involved in 4CBA dehalogenation in other organisms reveals the presence of a single gene encoding a major facilitator superfamily transporter predicted to transport 4CBA into the cell (accession no. AB041030, AF030397, and AF042490) (10, 29) or two-component transporter highly similar to TRAP family proteins (accession no. AF537222) (17). As documented in the present work, *Comamonas* sp. strain DJ-12 transports 4CBA into the cell through a TRAP-type transporter consisting of three components. It is thus readily apparent that different bacterial

strains have recruited different genes to evolve mechanisms for 4CBA transport into the cell.

#### ACKNOWLEDGMENT

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