EJB Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.5 No.2, Issue of August 15, 2002

Received February 25, 2002 / Accepted August 6, 2002
RESEARCH ARTICLE

Engineering bacterial strains through the chromosomal insertion of the chlorocatechol catabolism *tfd*$_I$CDEF gene cluster, to improve degradation of typical bleached Kraft pulp mill effluent pollutants

Roberto Bobadilla *
Departamento de Prevención de Riesgos y Medio Ambiente
Facultad de Ciencias de la Construcción y Ordenamiento Territorial
Universidad Tecnológica Metropolitana
Calle Dieciocho Nº 390, piso 4
Santiago, Chile
Tel: 56 2 7877300
Fax: 56 2 7877321
E-mail: rbobadi@utem.cl

Cristián Varela#
Unidad de Microbiología
Departamento de Genética Molecular y Microbiología
Facultad de Ciencias Biológicas
Pontificia Universidad Católica de Chile
Santiago, Chile
E-mail: cvarela@ing.puc.cl

Ricardo Céspedes§
Unidad de Microbiología
Departamento de Genética Molecular y Microbiología
Facultad de Ciencias Biológicas
Pontificia Universidad Católica de Chile
Santiago, Chile
E-mail: Ricardo.cespedesaraneda@epfl.ch

Bernardo González
Unidad de Microbiología
Departamento de Genética Molecular y Microbiología
Facultad de Ciencias Biológicas
Pontificia Universidad Católica de Chile
Av Libertador Bernardo O'Higgins Nº 340, piso 3
Santiago, Chile
Tel: 56 2 686 2842
Fax: 56 2 222 5515
E-mail: bgonzale@genes.bio.puc.cl

Financial support: This work was supported by grants 1960262 and 8990004 from FONDECYT-Chile.

Keywords: chloroaromatics, chlorocatechols, metabolic complementation, *Ralstonia eutropha*, ortho ring cleavage, *tfd* genes.

Present addresses: # Laboratorio de Microbiología Industrial, Departamento de Ingeniería, Facultad de Ingeniería, Pontificia Universidad Católica de Chile, Av Libertador Bernardo O'Higgins Nº 340, piso 3, Santiago, Chile. Tel: 56 2 686 4053. § Environmental Biotechnology (DGR), Swiss Federal Institute of Technology Laussane (EPFL), CH-1015 Laussane, Switzerland. Tel: 41 21 693 6231. Fax: 41 21 693 4722.

Chloroaromatic pollutants from bleached Kraft pulp mill effluents (BKME) are difficult to degrade, because bacterial strains present in BKME aerobic treatments, only partially degrade these compounds, accumulating the corresponding chlorocatechol intermediates. To improve the catabolic performance of chlorocatechol-accumulating strains, we introduced, by chromosomal insertion, the *tfd*$_I$CDEF gene cluster from *Ralstonia eutropha* JMP134 (pJP4). This gene cluster allows dechlorination and channelling of chlorocatechols into the intermediate metabolism. Two bacterial strains, *R. eutropha* JMP222 and *Pseudomonas putida* KT2442, able to produce chlorocatechols from 3-chlorobenzoate (3-CB) were used. *Acinetobacter lwoffii* RB2 isolated from BKME by its ability to grow on guaiacol as sole carbon source and shown to be able to produce the corresponding chlorocatechols from the BKME pollutants 4-, and 5-chloroguaiacol, was also used. The

* Corresponding author

tfd_ICDEF gene cluster was inserted in the chromosome of these strains using mini*Tn*5-derived vectors that allow expression of the Tfd enzymes driven by the $lacI^q/P_{trc}$ or $tfdR/P_{tfd-I}$ regulatory systems, and therefore, responding to the inducers isopropyl-ß-D-thiogalactopyranoside (IPTG) or 3-CB, respectively. Crude extracts of cells from strains JMP222, KT2442 or RB2 engineered with the *tfd* genes, grown on benzoate and induced with IPTG or 3-CB showed Tfd specific activities of about 15% - 80% of that of the strain JMP134. Dechlorination rates for 3-CB or chloroguaiacols correlated with levels of Tfd enzymes. However, none of the strains containing the chromosomal copy of the tfd_ICDEF cluster grew on monochloroaromatics as sole carbon source. Experiments with BKME aerobic treatment microcosms showed that the catabolic performance of the engineered bacteria was also lower than the wild-type *R. eutropha* strain JMP134.

Bleached Kraft pulp mill effluents (BKME) are complex wastes containing organic matter and chloroorganic compounds generated in the chlorine bleaching process (Leuenberger et al. 1985). Low molecular weight compounds such as chlorophenols, chlorobenzoates, chlorocatechols and chloroguaiacols, among others, are generated in small but significant quantities (Kringstad and Lindström, 1984). BKME are usually treated in aerobic systems where the microbial activity substantially decreases the organic matter content, but is less effective degrading low, and specially, high molecular weight chlorinated material (Graves et al. 1995). The presence and adaptation of chloroorganic degrading microorganisms, especially bacteria, in such polluted systems, makes BKME a suitable place to search for new strains with catabolic activity towards chloroaromatic pollutants (Fulthorpe et al. 1993; González et al. 1993; Acevedo et al. 1995).

Different metabolic pathways have been described for bacterial chloroaromatic degradation (Reineke and Knackmuss, 1988). In most cases, a specific intermediate is the bottleneck in the metabolic pathway due to impaired enzyme activity and/or toxic effects. This is the case for chlorocatechols, which are key intermediates in the biodegradation of several chloroaromatics. These compounds can be accumulated or, otherwise, further metabolised through the *ortho*, modified ring-cleavage pathway (Reineke and Knackmuss, 1988). One extensively studied example of an efficient chloroaromatic degrading bacteria that use such a chlorocatechol degradation pathway is *Ralstonia eutropha* JMP134 (pJP4), a strain able to grow on 2,4-dichlorophenoxyacetate (2,4-D), and 3-chlorobenzoate (3-CB), as well as other chloroaromatics (Don and Pemberton, 1981; Pieper et al. 1988; Clément et al. 1995). Most of its catabolic abilities are encoded on the *tfd* genes from the plasmid pJP4 (Figure 1a). The enzymes of the catabolism of chloroaromatics in pJP4 have been studied (Pieper et al. 1988; Fukumori and Hausinger, 1993; Kasberg et al. 1995; Vollmer et al. 1999). Catabolism of 2,4-D is started by the 2,4-D/α-ketoglutarate dioxygenase (*tfd*A) and the 2,4-dichlorophenol hydroxylase (*tfd*B) encoded in pJP4, to form 3,5-dichlorocatechol. Metabolism of 3-CB is initiated by a chromosomally encoded-, low specificity- enzyme benzoate dioxygenase and 1,2-dihydro-1,2-dihydroxybenzoate dioxygenase to form 3-, and 4-chlorocatechol (Pieper et al. 1993). Chlorocatechol metabolism is performed by the enzymes encoded on the pJP4 gene clusters: tfd_ICDEF and tfd_{II}DCEF (Figure 1a). *tfd*C, *tfd*D, *tfd*E, and *tfd*F genes encode for chlorocatechol-1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase, respectively (Figure 1b). The first three enzymes encoded in tfd_ICDEF are more active than in tfd_{II}DCEF, whereas the TfdF activity of the latter is more active that in tfd_ICDEF (Pérez-Pantoja et al. 2000). The regulation of the *tfd* genes is not well understood. Recent works indicate that *tfd*R, a LysR- type transcriptional activator, may play the main role in expression of both chlorocatechol-catabolism *tfd* clusters (Matrubutham and Harker, 1994; Leveau and Van der Meer, 1996; Leveau et al. 1999).

In this work, we explored the improvement of the ability to degrade, and eventually, grow on chloroaromatics in bacterial strains that accumulate chlorocatechols, by engineering them with the chlorocatechol degradation tfd_ICDEF gene cluster from *R. eutropha* JMP134. We chose *R. eutropha* JMP222, a pJP4 plasmid-free derivative of strain JMP134, and *Pseudomonas putida* KT2442, both accumulating monochlorocatechols from 3-CB. We also included in this study a not previously reported bacterial strain, *Acinetobacter lwoffii* RB2, isolated from a BKME aerobic treatment system by its ability to grow on guaiacol. This strain accumulates monochlorocatechols from chloroguaiacols. In derivatives of these strains containing a chromosomal insertion of the tfd_ICDEF gene cluster, we assessed the expression of Tfd enzymes, the dechlorination of monochloroaromatic compounds, and the ability to grow on them. With some of these derivatives we studied the ability to remove 3-CB in microcosms of BKME aerobic treatment.

Materials and Methods

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha* JMP134 (pJP4), *R. eutropha* JMP222 (cured of pJP4) and *P. putida* KT2442 were grown at 30ºC in a chlorine-free minimal media (Kröckel and Focht, 1987) with 2 mM 2,4-D, 3 mM benzoate plus 1,000 mg/ml streptomycin, or 3 mM benzoate plus 50 µg/ml

rifampycin, respectively. *A. lwoffii* RB2 was isolated by enrichment culture with 1 mM guaiacol, using BKME as inoculant. The source of BKME has been described elsewhere (Céspedes et al. 1996; Valenzuela et al. 1997). 16S RNA sequencing analysis of strain RB2 was performed in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), resulting in a 97.0% overlap with the species *Acinetobacter lwoffii*. *Escherichia coli* strains were maintained in Luria Bertani (LB) medium plus the appropriate antibiotic: ampicillin: 50 µg/ml, kanamycin: 50 µg/ml, rifampycin: 50 µg/ml, chloramphenicol: 20 µg/ml, or streptomycin: 1,000 µg/ml.

DNA manipulation

Restriction, ligation, and dephosphorylation reactions, purification of DNA, and Southern analysis were performed by standard procedures (Ausubel et al. 1992). Detection by PCR was performed using primers 5'-TCTAGAATTCATGACGGAGGCAAAGTGAAC-3' and 5'-GCCGTGGAATTCGCCAGTGGGAACCTGCAG-3' for a 1.8 kb fragment of the *tfd*$_I$CD sequence and 5'-ACTCGGGATCCGGGATTACGAACTGCAGGT-3', and 5'-CCCGGGATCCATTATTTGAAATCCGGTCTT-3' for a 1.9 kb fragment of the *tfd*$_I$EF sequence (Pérez-Pantoja et al. 2000). The program was: initiation 95ºC x 120 s, 35 cycles of melting 95ºC x 45 s, annealing 55ºC x 30 s, and extension 72ºC x 180 s, final extension 72ºC x 10 min. Competent cells for the introduction of DNA by thermal shock (42ºC x 1 min) were prepared following a described procedure (Nishimura et al. 1990). Triparental mating was performed on LB agar plates inoculated with cells grown overnight on LB medium plus antibiotic (Table 1), centrifuged and washed twice with 50 mM $MgCl_2$. *E. coli* CC118 λpir containing the derivatives of pCNB5 (pITFD), or pUT (pTFD, pDTFD) was used as donor, *E. coli* HB101 (pRK600) as helper, and *A. lwoffii* RB2, *R. eutropha* JMP222, or *P. putida* KT2442 as recipient. A donor to helper to recipient ratio of 1:1:2 was used. After overnight incubation, cells were streaked on a nitrocellulose filter, resuspended, and plated on 2 mM guaiacol or 3 mM benzoate, plus kanamycin.

Construction of *tfd*$_I$CDEF gene clusters

Construction of the *lacI*^{q}/P$_{trc}$*tfd*$_I$CDEF (pITFD) gene cluster: The *tfd*$_I$CDEF genes were cloned into pJRC42, as previously described (Pérez-Pantoja et al. 2000). The 4.2 kb insert of pJRC42 was digested with *Not*I, and introduced into the same restriction site in mini*Tn*5 vector pCNB5 (de Lorenzo et al. 1993), to form pITFD. The proper orientation of the insertion was checked by digestion with *Eco*RI, which produced a fragment of 1.0 kb. Construction of the *tfd*R/P$_{tfd\text{-}I}$*tfd*$_I$CDEF (pDTFD) and the P$_{tfd\text{-}I}$*tfd*$_I$CDEF (pTFD) gene clusters: The *tfd*R/P$_{tfd\text{-}I}$*tfd*$_I$CDEF gene cluster was cloned in pUCLG2, as described elsewhere (Pérez-Pantoja et al. 2000); P$_{tfd\text{-}I}$*tfd*$_I$CDEF was cloned in pUCLG1 during the construction of pUCLG2. Plasmids pUCLG1 and pUCLG2 were digested with *Not*I and the insertions introduced into pUT (de Lorenzo et al. 1990), to give pTFD and pDTFD, respectively.

Dechlorination and enzyme activity assays

Dechlorination studies were performed with cell suspensions (O.D.$_{660\ nm}$=1.0) prepared from cells grown on 2 mM guaiacol or LB, in the absence or presence of 1 mM of isopropyl-ß-D-thiogalactopyranoside (IPTG) or 1 mM 3-CB. Cells were incubated for up to 6 h with 5 ml of free-chlorine minimal medium, containing 1 mM of a monochloroaromatic compound. Chloride released was determined by the mercury thiocyanate method (Florence and Farrar, 1971). For enzyme activity assays, cultures grown on minimal medium containing 3 mM benzoate, with or without inducer, were obtained. About 100 ml of each culture was centrifuged, and cells were washed and resuspended in 5 ml of 50 mM Tris-acetate (pH 7.5), supplemented with 1 mM $MnCl_2$. Cells were disrupted by sonication with a Vibracell apparatus (Sonics & Materials Inc.). The soluble protein fraction was obtained after ultracentrifugation at 130,000 x g, for 60 min, in a Beckman ultracentrifuge L-80. Crude extracts (0.1 - 5.3 mg of proteins per ml) were used without further purification. Enzyme activities were determined in spectrophotometrical assays as described previously (Pérez-Pantoja et al. 2000).

BKME microcosms experiments

BKME microcosms consisted on BKME or sterile BKME 10-ml batch cultures (Valenzuela et al. 1997). The microcosms were aerated at a constant rate (1 ml air/min/ml), and separately inoculated (50% v/v) with late log cultures of strains JMP134, JMP222, RB2 or their *tfd* genes containing derivatives, and incubated at room temperature (20-25ºC), with or without addition of 1 mM 3-CB. Survival of each introduced strain was determined by viable counts plating samples taken after 6 days of incubation in the corresponding selective medium. Removal of 3-CB was followed by UV spectrophotometry, after 0, 3 and 6 days of incubation.

Results

Isolation and characterisation of a chloroguaiacol-degrading bacterial strain

A. lwoffii RB2 was isolated from BKME by its ability to use guaiacol as sole carbon source. The ability to grow and/or dechlorinate typical BKME chloroaromatic pollutants was studied. Growth tests were carried out in minimal medium liquid cultures with 0.2-2 mM carbon source, and incubated for up to 10 days. The strain was

only able to grow in simple, non-chlorinated aromatic compounds like benzoate and phenol (Table 2). However, the strain was able to dechlorinate some chloroguaiacols and chlorocatechols (Table 2). These observations suggest that strain RB2 has the ability to produce and partially metabolise chlorocatechols from some representative BKME chloroaromatic compounds. In fact, a gas chromatography- mass spectra analysis, performed as previously described (Céspedes et al. 1996), of supernatant of resting cells pre-grown on guaiacol and exposed to 4- or 5-chloroguaiacol showed the formation of the respective chlorocatechol (data not shown). The ability to produce chlorocatechols and dechlorinate chloroguaiacols was dependent on the growth substrate, because cells grown on LB rich medium were less efficient than cells of strain RB2 grown on guaiacol (Figure 2).

Catabolic properties of bacterial strains engineered with a chlorocatechol-degrading *tfd* gene cluster

Three mini*Tn*5 derived plasmids containing a differentially regulated *tfd*$_I$CDEF gene cluster were constructed: *lac*I^q/P$_{trc}$*tfd*$_I$CDEF (pITFD), *tfd*R/P$_{tfd-I}$*tfd*$_I$CDEF (pDTFD) and P$_{tfd-I}$*tfd*$_I$CDEF (pTFD). pITFD harbors a *tfd* gene cluster that is responsive to IPTG through the heterologous *lac*I regulator. The other two contains the upstream controlling region of the *tfd*$_I$CDEF, P$_{tfd-I}$, but only one of them (pDTFD) has also the putative master regulatory gene (*tfd*R) for *tfd* gene expression. The appropriate expression of *tfd* genes would allow chlorocatechol produced by initial reactions encoded in the recipient strains to be further metabolised (Figure 1c), and eventually, allow growth of bacterial strains on monochloroaromatic compounds.

Each *tfd* gene cluster was introduced into the chromosome of the recipient cells by triparental mating which allows mini*Tn*5 mediated integration of each cluster due to the use of a suicide plasmid in recipients lacking the λpir protein (Herrero et al. 1990; de Lorenzo et al. 1990). Transconjugants of strains JMP222 or KT2442 were selected in agar plates with benzoate plus kanamycin, whereas transconjugants of strain RB2 were selected in guaiacol plus kanamycin. All clones tested gave positive PCR amplification for *tfd*$_I$CD and *tfd*$_I$EF sequences. Confirmation of chromosomal *tfd*$_I$CDEF insertions in selected clones was obtained by Southern analysis of a genomic *Eco*RI digestion probed with biotinylated pITFD DNA (data not shown). Furthermore, the absence of plasmid DNA in cell lysates prepared by a described procedure (Kado and Liu, 1981), the Ap^s phenotype, and the stability of the kanamycin marker after growth on LB medium without antibiotic indicated chromosomal insertion of the internal sequences of each mini*Tn*5 derived vector. Six to ten clones from each strain/gene cluster were checked as indicated above, and no significant differences were found among them. Therefore, two clones of each type were selected for enzyme activities and dechlorination tests. For simplicity, only the results of one clone of each strain/gene cluster are given in Table 3. To make a proper comparison we used cells grown on benzoate in the presence of 3-CB or IPTG as inducers. Induced cells of *R. eutropha* JMP222 containing the regulated clusters, exhibited 63% to 78% of the chlorocatechol 1,2-dioxygenase specific activity detected in strain JPM134 (0.51 U/mg, Table 3). Non-induced cells gave 0% to 6% of the TfdC activity of the induced strain JMP134, and were as low as in the non-induced strain JMP134 (0.04 U/mg). The chloromuconate cycloisomerase assay indicated that induced cells expressed as high as 38% of the level of the wild type strain (0.08 U/mg). Rates of dechlorination of 3-CB were in agreement with the level of Tfd enzymes (last column, Table 3). Cells of *P. putida* KT2442 containing the *tfd*$_I$CDEF cluster regulated by IPTG or 3-CB, were also analysed (Table 3). TfdC specific activities were also lower than in wild type strain JMP134. Again, rates of dechlorination of 3-CB agreed with Tfd activities. On the other hand, the activity of Tfd enzymes in strains containing P$_{tfd-I}$*tfd*$_I$CDEF insertions, (therefore lacking *tfd*R), was five to ten-fold lower than those induced clones containing a regulated *tfd*$_I$ cluster (Table 3). In the case of *A. lwoffii* RB2 derivatives containing the *tfd*$_I$CDEF cluster, the activity of the Tfd enzymes were almost negligible (Table 3, only results with the *lac*I^q/P$_{trc}$*tfd*$_I$CDEF gene cluster are shown). Despite of that, 3-CB dechlorination tests gave significant results for the ITFD clone (Table 3, last row). In addition, cells of this derivative grown on guaiacol, but not on LB medium, showed higher dechlorination of 4- and 5-chloroguaiacol in the presence of the inducer IPTG (Figure 2).

Two hundred clones of *R. eutropha* JMP222, *P. putida* KT2442 or *A. lwoffii* RB2 containing one of each regulated *tfd*$_I$CDEF cluster, were streaked in agar plates containing 3 mM 3-CB or monochloroguaiacols, respectively, with or without the corresponding inducer. In spite of up to 7 days of incubation only very small colonies were observed. No growth was attained after transfer of these colonies to fresh medium. Liquid cultures were also unsuccessful to allow these clones to proliferate. In contrast, under these conditions, liquid cultures or agar plates containing 1-5 mM 3-CB allowed growth of strain JMP134 after 1-2 days.

Catabolic performance of *tfd* genes-engineered derivatives in BKME microcosms

The ability to degrade 3-CB in complex systems by the *tfd* genes-engineered strains *R. eutropha* JMP222::DTFD4, and *A. lwoffii* RB2::ITFD3 was assessed in BKME microcosms. The presence of *R. eutropha* JMP222::DTFD4 provoked significant removal of 3-CB (Table 4). Microcosms non inoculated, or inoculated with the parental strain JMP222,

were completely unable to remove this compound. The wild type strain JMP134, containing the full set of *tfd* genes showed a significantly higher catabolic performance in these microcosms (Table 4). On the other hand, the presence of *tfd* genes in the *A. lwoffii* derivative did not have much effect in removal of 3-CB in BKME microcosms (Table 4). No significant reduction of the introduced population was detected with any of the bacterial strains used as inoculant, with the sole exception of *R. eutropha* JMP222::DTFD4 (Table 4). In sterile BKME microcosms all strains survive, after six days of incubation.

Discussion

In this work we assessed if the introduction of a specialized gene cluster for chlorocatechol degradation in strains accumulating these intermediates, would improve their catabolic properties. Although expression of Tfd enzymes was obtained, the level of activity with the three catabolic strains used, was always lower than with *R. eutropha* JMP134, the source of catabolic genes. This lower activity was also evident in dechlorination, which is produced by the presence of Tfd enzymes and, in the catabolic performance of derivatives introduced into BKME microcosms. The presence of one copy of the *tfd*$_I$CDEF cluster was also not enough to allow *R. eutropha* JMP222, *P. putida* KT2442 or *A. lwoffii* RB2 to grow on 3-CB. There are several possible explanations for this catabolic behaviour. One possibility is that the regulatory systems used here do not allow adequate gene expression. However, as similar Tfd expression levels were obtained with two different regulatory systems used, this explanation may be disregarded. It is also possible that a particular chromosomal position of the inserted cluster prevented adequate gene expression. However, integration driven by *Tn*5 does not have strong sequence preference (Ahmed and Podemski, 1995; de Lorenzo et al. 1990), and we analysed two hundred clones of each type, without any success to find growth on 3-CB or monochloroguaiacols. On the other hand, an interesting possibility is that the *tfd*$_I$CDEF cluster itself is not enough to support adequate rates of monochlorocatechol transformation *in vivo*. During growth on 3-CB, part of it is transformed into 3-chlorocatechol, and then to 2-chloromuconate (Pieper et al. 1993). The latter is non-efficiently degraded due to poor activity of chloromuconate cycloisomerases (Pieper et al. 1993; Vollmer et al. 1999; Pérez-Pantoja et al. 2000). This limitation may be avoided in strain JMP134 by the presence of two *tfd* clusters. Interestingly, a pJP4 rearrangement leading to an enlarged plasmid that includes a duplication of the *tfd*$_I$CDEF and *tfd*$_{II}$CDEF sequences has been recently reported to improve growth of *R. eutropha* JMP134 on 3-CB (Clément et al. 2001). Furthermore, another recent study from our lab showed that the introduction of each *tfd* gene cluster into *R. eutropha* JMP222 or *P. putida* KT2442, cloned in the medium-copy-number plasmid vector pBBR1MCS-2 under the control of the *tfd*R regulatory gene, allows these strains to use 3-CB as sole carbon source (Pérez-Pantoja et al. 2000), suggesting a gene dosage effect.

From a biotechnological and an evolutionary point of view, this work suggests that the sole acquisition of the *tfd*$_I$CDEF cluster, which has a similar organization to those clusters from plasmids p51 (Van der Meer et al. 1991) and pAC27 (Ghosal et al. 1985), is not necessarily enough to allow growth on 3-CB. Introduction into the chromosome of *P. putida* KT2442 of a *tcb*CDEF gene cluster encoding chlorocatechol degradation neither allow selection of derivatives growing on 3-CB, unless direct selection on 3-CB was applied. In such case, only derivatives containing more than one copy of the *tcb*CDEF element were selected (Klemba et al. 2000).

The catabolic complementation for monochloroaromatic degradation in *A. lwoffii* RB2 worked even more poorly than with *R. eutropha* or *P. putida* strains. Unfortunately, the introduction in *A. lwoffii* RB2 of pJP4, or the pBBR1MCS-2 derivatives mentioned above could not be properly assessed because these plasmids are not stably replicated in this strain. Negative effects of heterologous gene expression or the presence of inhibitors may explain such poor performance, and specially the lack of correlation between Tfd enzymes activities and dechlorination rates. The dechlorination substrate profile of *A. lwoffii* RB2 was narrower than a closer strain *A. junii* 5ga (González et al. 1993), or the Gram-positive *Rhodococcus ruber* CA16 (Acevedo et al. 1995). The first strain was only able to dechlorinate monochloroguaiacols whereas the later two also degrade the dichlorinated ones. However, the choice of *A. lwoffii* as recipient for the *tfd* genes was based mostly by the fact that good survival may be also required in a bioremediation procedure. In this context, use of endogenous strains like *A. lwoffii* RB2 may be the first choice with respect to exogenous microorganisms.

Chloroaromatic degrading strains are intended to be used in polluted environments. Therefore, we also tested the catabolic abilities in BKME aerobic treatments microcosms. It has been shown that microbial activity significantly removes several chloroaromatic compounds from BKME, at the low (usually ppb) concentrations found (Céspedes et al. 1996). It has been also shown that BKME microcosms are able to remove higher amounts (usually ppm) of 2,4,6-trichlorophenol, 2,4-dichlorophenol, 3-CB or 2,4-D, if inoculated with *R. eutropha* JMP134 (Valenzuela et al. 1997). These observations along with that reported here indicate that only very efficient catabolic strains should be tested for removal of pollutants from systems like BKME.

Acknowledgments

R. Céspedes was a FUNDACION ANDES Ph.D. fellow.

References

ACEVEDO, C.; BREZNY, R.; JOYCE, T. and GONZÁLEZ, B. Metabolism of mono- and dichlorinated guaiacols by *Rhodococcus ruber* CA16. *Current Microbiology*, 1995, vol. 30, no. 2, p. 63-67.

AHMED, A. and PODEMSKI, L. The revised nucleotide sequence of Tn5. *Gene*, 1995, vol. 154, p. 129-133.

AUSUBEL, Frederick; BRENT, Roger; KINGSTON, Robert; MOORE, David; SEIDMAN, J.G.; SMITH, John and STRUHL, Kevin. *Short Protocols in Molecular Biology*. 2nd ed. New York, Greene Publishing Associate, 1992. 740 p. ISBN 0471577359.

CÉSPEDES, R.; MATURANA, A.; BUMANN, U.; BRONFMAN, M. and GONZÁLEZ, B. Microbial removal of chlorinated phenols during aerobic treatment of effluents from radiata pine kraft pulps bleached with chlorine-based chemicals, with or without hemicellulases. *Applied Microbiology and Biotechnology*, 1996, vol. 46, no. 5-6, p. 631-637.

CLÉMENT, P.; PIEPER, D.H. and GONZÁLEZ, B. Molecular characterization of a deletion/duplication rearrangement in *tfd* genes from *Ralstonia eutropha* JMP134(pJP4) that improves growth on 3-chlorobenzoic acid but abolishes growth on 2,4-dichlorophenoxyacetic acid. *Microbiology*, 2001, vol. 147, no. 8, p. 2141-2148.

CLÉMENT, P.; MATUS, V.; CÁRDENAS, L. and GONZÁLEZ, B. Degradation of trichlorophenols by *Alcaligenes eutrophus* JMP134. *FEMS Microbiology Letters*, 1995, vol. 127, no. 1-2, p. 51-55.

DE LORENZO, V.; ELTIS, L.; KESSLER, B. and TIMMIS, K.N. Analysis of *Pseudomonas* gene products using *lacI*^{q}/Ptrp-lac plasmids and transposons that confers conditional phenotypes. *Gene*, January 1993, vol. 123, no. 1, p. 17-24.

DE LORENZO, V.; HERRERO, M.; JACUBZIK, U. and TIMMIS, K.N. Mini-*Tn5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria. *Journal of Bacteriology*, November 1990, vol. 172, no. 11, p. 6568-6572.

DON, R.H. and PEMBERTON, J.M. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *Journal of Bacteriology*, 1981, vol. 145, p. 681-686.

FLORENCE, T.M. and FARRAR, Y.J. Spectrophotometric determination of chloride at the parts-per-billion level by the mercury(II) thiocyanate method. *Analytica Chimica Acta*, 1971, vol. 54, p. 373-377.

FUKUMORI, Fumiyasu and HAUSINGER, Robert P. *Alcaligenes eutrophus* JMP134 "2,4-dichlorophenoxyacetate monooxygenase" is an alpha-ketoglutarate-dependent dioxygenase. *Journal of Bacteriology*, April 1993, vol. 175, no. 7, p. 2083-2086.

FULTHORPE, Roberta A.; LISS, Steven and ALLEN, Grant. Characterization of bacteria isolated from a bleached Kraft pulp mill wastewater treatment system. *Canadian Journal of Microbiology*, January 1993, vol. 39, no. 1, p. 13-24.

GHOSAL, D.; YOU, I.S.; CHATTERJEE, D.K. and CHAKRABARTY, A.M. Genes specifying degradation of 3-chlorobenzoic acid in plasmids pAC27 and pJP4. *Proceedings of the National Academy of Sciences of the United States of America*, 1985, vol. 82, p. 1638-1642.

GONZÁLEZ, B.; ACEVEDO, C.; BREZNY, R. and JOYCE, T. Metabolism of chlorinated guaiacols by a guaiacol-degrading *Acinetobacter junii* strain. *Applied and Environmental Microbiology*, October 1993, vol. 59, no. 10, p. 3424-3429.

GRAVES, John; JAMEEL, Hosan and JOYCE, Thomas. Removal of chlorophenolics from an aerated lagoon. *TAPPI Journal*, 1995, vol. 78, no. 5, p. 99-104.

HERRERO, Marta; DE LORENZO, Victor and TIMMIS, Kenneth N. Transposon vectors containing non-antibiotic selection markers for cloning and stable chromosomal insertion of foreign DNA in Gram-negative bacteria. *Journal of Bacteriology*, November 1990, vol. 172, no. 11, p. 6557-6567.

KADO, C.I. and LIU, S.T. Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology*, 1981, vol. 145, p. 1365-1373.

KASBERG, T.; DAUBARAS, D.L.; CHAKRABARTY, A.M.; KINZELT, D. and REINEKE, W. Evidence that operons *tcb*, *tfd*, and *clc* encode maleylacetate reductase, the fourth enzyme of the modified *ortho* pathway. *Journal of Bacteriology*, 1995, vol. 177, p. 3885-3889.

KLEMBA, Michael; JAKOBS, Barbara; WITTICH, Rolf-Michael and PIEPER, Dietmar H. Chromosomal integration of *tcb* chlorocatechol degradation pathway genes as means of expanding the growth substrate range of bacteria to include haloaromatics. *Applied and Environmental Microbiology*, 2000, vol. 66, no. 8, p. 3255-3261.

KRINGSTAD, K.P. and LINDSTRÖM, K. Spent liquors

from pulp bleaching. *Environmental Science and Technology*, 1984, vol. 18, p. 236A-248A.

KRÖCKEL, L. and FOCHT, D. Construction of chlorobenzene-utilizing recombinants by progenitive manifestation of a rare event. *Applied and Environmental Microbiology*, 1987, vol. 53, p. 2470-2475.

LEUENBERGER, C.; GEGER, W.; CONEY, R.; GRAYDER, J.W. and MOLNAR-KUBICA, E. Persistent chemicals in pulp mill effluents: occurrence and behavior in an activated sludge treatment plant. *Water Research*, 1985, vol. 19, p. 885-894.

LEVEAU, Johan H.; KÖNIG, Franziska; FÜCHSLIN, Hanspeter; WERLEN, Christoph and VAN DER MEER, Jan Roelof. Dynamics of multigene expression during catabolic adaptation of *Ralstonia eutropha* JMP134 (pJP4) to the herbicide 2,4-dichlorophenoxyacetate. *Molecular Microbiology*, 1999, vol. 33, no. 2, p. 396-406.

LEVEAU, Johan H. and VAN DER MEER, Jan Roelof. The *tfd*R gene product can succesfully take over the role of the insertion element-inactivated *tfd*T protein as a transcriptional activator of the *tfd*CDEF gene cluster, which encodes chlorocatechol degradation in *Ralstonia eutropha* JMP134 (pJP4). *Journal of Bacteriology*, 1996, vol. 178, no. 23, p. 6824-6832.

MATRUBUTHAM, Udayakumar and HARKER, Alan R. Analysis of duplicated gene sequences associated with *tfd*R and *tfd*S in *Alcaligenes eutrophus* JMP134. *Journal of Bacteriology*, April 1994, vol.176, no. 8, p. 2348-2353.

NISHIMURA, A.; MORITA, M.; NISHIMURA, Y. and SUGINO, Y. A rapid and highly efficient method of preparation of competent *Escherichia coli* cells. *Nucleic Acids Research*, 1990, vol. 18, p. 6169.

PÉREZ-PANTOJA, D.; GUZMÁN, L.; MANZANO, M.; PIEPER, D.H. and GONZÁLEZ, B. Role of *tfd*$C_ID_IE_IF_I$ and *tfd*$D_{II}C_{II}E_{II}F_{II}$ gene modules in catabolism of 3-chlorobenzoate by *Ralstonia eutropha* JMP134(pJP4). *Applied and Environmental Microbiology*, 2000, vol. 66, no. 4, p. 1602-1608.

PIEPER, D.H.; KNACKMUSS, H.J. and TIMMIS, K.N. Accumulation of 2-chloromuconate during metabolism of 3-chlorobenzoate by *Alcaligenes eutrophus* JMP134. *Applied Microbiology and Biotechnology*, July 1993, vol. 39, no. 4-5, p. 563-567.

PIEPER, D.H.; REINEKE, W.; ENGESSER, K.H. and KNACKMUSS, H.J. Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP134. *Archives of Microbiology*, 1988, vol. 150, p. 95-102.

REINEKE, W. and KNACKMUSS, H.J. Microbial degradation of haloaromatics. *Annual Reviews of Microbiology*, 1988, vol. 42, p. 263-287.

VALENZUELA, Javier; BUMANN, Ursel; CÉSPEDES, Ricardo; PADILLA, Leandro and GONZÁLEZ, Bernardo. Degradation of chlorophenols by *Alcaligenes eutrophus* JMP134 (pJP4) in bleached Kraft mill effluent. *Applied and Environmental Microbiology*, 1997, vol. 63, no. 1, p. 227-232.

VAN DER MEER, Jan Roelof; VAN NEERVEN, Alex R.W.; DE VRIES Ecoo J.; DE VOS Willem M. and ZEHNDER, Alexander J.B. Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro, 1,4-dichloro, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. *Journal of Bacteriology*, January 1991, vol. 173, no. 1, p. 6-15.

VOLLMER, M.D.; SCHELL, U.; SEIBERT, S.; LAKNER, S. and SCHLÖMANN, M. Substrate specificities of the chloromuconate cycloisomerases from *Pseudomonas* sp. B13, *Ralstonia eutropha* JMP134 and *Pseudomonas* sp. P51. *Applied Microbiology Biotechnology*, 1999, vol. 51, no. 5, p. 598-605.

APPENDIX

Tables

Table 1. Strains and plasmids used in this work.

Strain/plasmid	Relevant phenotype/genotype	Source
R.eutropha JMP134 (pJP4)	2,4-D, 3-CB	DSMZ
R. eutropha JMP222	Sm^r	H. Knackmuss
P. putida KT2442	Rf^r	(de Lorenzo et al. 1990)
E. coli CC118lpir	Rf^r	(de Lorenzo et al. 1990)
E. coli HB101 (pRK600)	Cm^r, *tra*+	(Herrero et al. 1990)
A. lwoffii RB2	Gu	this work
pUT	Km^r, Ap^r	(de Lorenzo et al. 1990)
pCNB5	Km^r, Ap^r, *lac*I^q/P_{trc}	(de Lorenzo et al. 1993)
pITFD, pCNB5 derivative	Km^r, Ap^r, *lac*I^q /P_{trc} *tfd*$_I$CDEF	this work
pDTFD, pUT derivative	Km^r, Ap^r, *tfd*R/P_{tfd-I} *tfd*$_I$CDEF	this work
pTFD, pUT derivative	Km^r, Ap^r, P_{tfd-I} *tfd*$_I$CDEF	this work

2,4-D, 3-CB, Gu: able to grow on 2,4-dichlorophenoxyacetate, 3-chlorobenzoate, or guaiacol; *tra*+: IncP transference functions; *tfd*: catabolic genes from pJP4; *lac*I^q: *lac* repressor mutant; P_{trc}: *lac*/*trp* hybrid promoter; *tfd*R: regulatory gene of pJP4; P_{tfd-I}: promoter region for *tfd*$_I$CDEF cluster; Ap: ampicillin; Km: kanamycin; Rf: rifampycin; Cm: cloramphenicol; Sm: streptomycin. DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Table 2. Catabolic characterization of *A. lwoffii* RB2.

Substrate	Growth	% Dechlorination*
Phenol	+	n.a.**
3-chlorophenol	-	0
4-chlorophenol	-	6
2,4-dichlorophenol	-	0
Benzoate	+	n.a.
2-chlorobenzoate	-	0
3-chlorobenzoate	-	20
4-chlorobenzoate	-	0
2,4-dichlorobenzoate	-	0
3,5-dichlorobenzoate	-	0
Guaiacol	+	n.a.
4-chloroguaiacol	-	28
5-chloroguaiacol	-	95

6-chloroguaiacol	-	0
3,5-dichloroguaiacol	-	0
4,5-dichloroguaiacol	-	0
4,6-dichloroguaiacol	-	0
Catechol	-	n.a.
3-chlorocatechol	-	0
4-chlorocatechol	-	10
3,5-dichlorocatechol	-	11

*% Dechlorination: calculated from the stoichiometric theoretical value. Growth tests were carried out in minimal medium liquid cultures with 0.2-2 mM carbon source and incubated for up to 10 days. For dechlorination assays cells were pre-grown on the corresponding non-chlorinated substrate. In the case of chlorocatechols cells were pre-grown on 2 mM benzoate. Dechlorination was determined after 1 day with 1 mM substrate.

**n.a.: non applicable.

Table 3. Tfd enzyme activities and rates of 3-CB dechlorination in *R. eutropha*, *P. putida* and *A. lwoffii* strains.

Strain/clone	Inducer	CC-1,2- DO	CMCI	3-CB dechlorination rate
R.eutropha JMP134	3-CB	0.51	0.08	37
R.eutropha JMP134	-	0.04	0.00	19
R.eutropha JMP222	3-CB	0.00	0.00	0
R. e. JMP222::ITFD5	-	0.03	0.00	2
R. e. JMP222::ITFD5	IPTG	0.40	0.03	31
R. e. JMP222::TFD5	-	0.03	0.00	1
R. e. JMP222::TFD5	3-CB	0.02	0.00	1
R. e. JMP222::DTFD4	-	0.03	ND	1
R. e. JMP222::DTFD4	3-CB	0.32	ND	18
P. putida KT2442	3-CB	0.00	0.00	0
P. p. KT2442::ITFD1	-	0.02	0.00	0
P. p. KT2442::ITFD1	IPTG	0.22	0.02	23
P. p. KT2442::TFD7	-	0.13	0.01	20
P. p. KT2442::TFD7	3-CB	0.08	0.01	12
A. lwoffii RB2	-	0.00	0.00	0.4
A. l. RB2::ITFD3	-	0.00	0.00	1
A. l. RB2::ITFD3	IPTG	0.01	0.00	2

Values are averages from two independent measurements. Deviations were less than 10%, and 15%, for determinations of enzyme activities, and dechlorination rates, respectively. Growth substrate concentration was always 3 mM benzoate. CC-1,2-DO: Chlorocatechol-1,2-dioxygenase. CMCI: Chloromuconate cycloisomerase. 3-CB: 3-chlorobenzoate. IPTG: Isopropyl-beta-D-thiogalactopyranoside.
ND: not determined.

Table 4. Expression of catabolic properties and survival of *R. eutropha* and *A. lwofii* in BKME microcosms.

Microcosms conditions	Survival*		3-CB removal (%)
	BKME	BKME sterile	
BKME	n.a.	n.a.	0**
BKME + *R. eutropha* JMP134	+	+	70
BKME + *R. eutropha* JMP222	+	+	0
BKME + *R. e.* JMP222::TFD4	-	+	20
BKME + *A. lwoffii* RB2	+	+	26
BKME + *A. lwoffii* RB2::ITFD3	+	+	11

* : > 10^6 colony forming units per ml after six days.
**% Dechlorination determined after 6 days of incubation and calculated from the stoichiometric theoretical value using 1 mM 3-CB.
n. a.: non applicable.

Figures

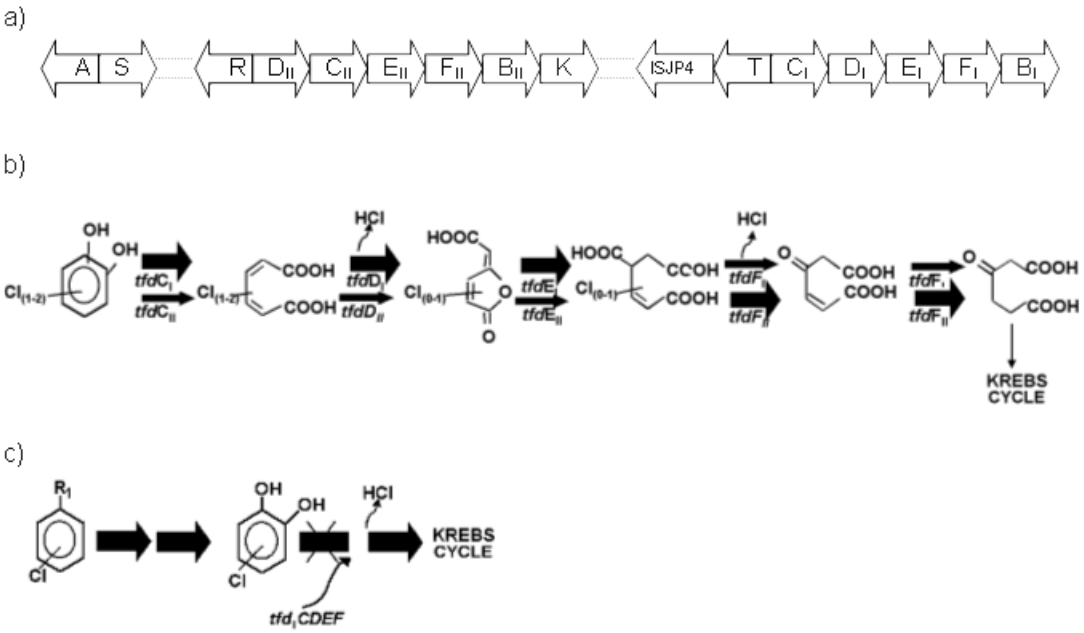

Figure 1. Chloroaromatic catabolism in *R. eutropha* JMP134 (pJP4).

(a) organisation of *tfd* genes in pJP4. Not to scale.
(b) *tfd* genes for the *ortho* ring-cleavage chlorocatechol pathway.
(c) Catabolic complementation for strains producing chlorocatechols from monochloroaromatic compounds.

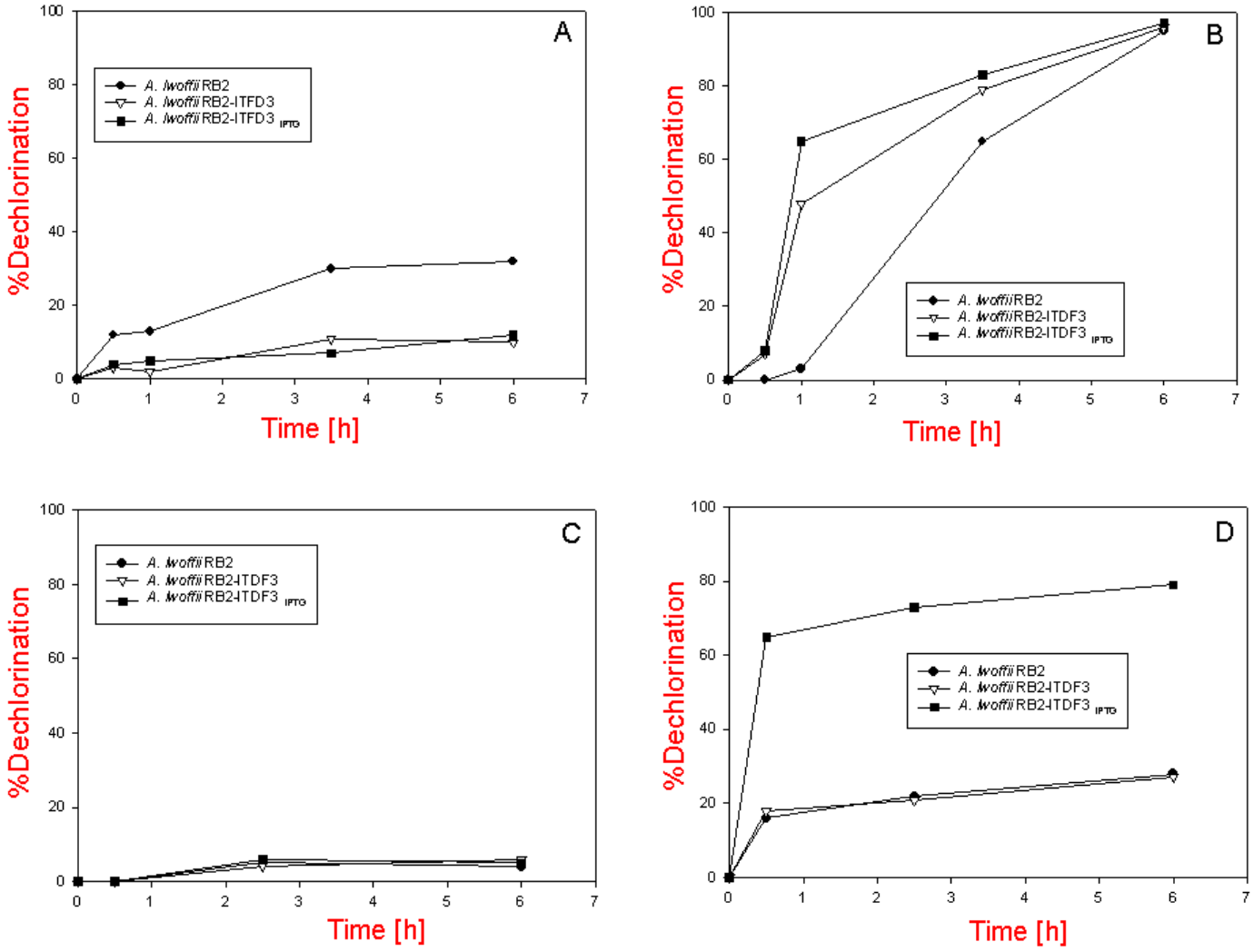

Figure 2. Dechlorination of chloroguaiacols. Dechlorination of 5- (A and B) and 4- (C and D) chloroguaiacol by cells of *A. lwoffii* RB2 and RB2-ITFD3 pre-grown on Luria Bertani (A and C) or guaiacol (B and D), in the presence or absence of inducer (IPTG). % Dechlorination calculated from the stoichiometric theoretical value.