Novel insights into the interplay between peripheral reactions encoded by *xyl* genes and the chlorocatechol pathway encoded by *tfd* genes for the degradation of chlorobenzoates by *Ralstonia eutropha* JMP134

Thomas Ledger,¹ Dietmar H. Pieper,² Danilo Pérez-Pantoja¹ and Bernardo González¹

Author for correspondence: Bernardo González. Tel: +56 2 6862845. Fax: +56 2 2225515.
e-mail: bgonzalez@genes.bio.puc.cl

¹ Laboratorio de Microbiología, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D Santiago, Chile

² Division of Microbiology, National Research Centre for Biotechnology – GBF, Braunschweig, Germany

Many bacteria can grow on chloroaromatic pollutants because they can transform them into chlorocatechols, which are further degraded by enzymes of a specialized *ortho*-cleavage pathway. *Ralstonia eutropha* JMP134 is able to grow on 3-chlorobenzoate by using two pJP4-encoded, *ortho*-cleavage chlorocatechol degradation gene clusters (*tfdC_ID_IE_IF_I* and *tfdD_{II}C_{II}E_{II}F_{II}*). Very little is known about the acquisition of new catabolic genes encoding enzymes that lead to the formation of chlorocatechols in *R. eutropha* JMP134. The effect on the catabolic properties of an *R. eutropha* JMP134 derivative that received the *xylS*–*xylXYZL* gene module, encoding the *xylS*-regulated expression of the broad-substrate-range toluate 1,2-dioxygenase (*xylXYZ*) and the 1,2-dihydro-1,2-dihydroxytoluate dehydrogenase (*xylL*) from pWW0, which allows the transformation of 4-chlorobenzoate into 4-chlorocatechol, was studied. Such a derivative could efficiently grow on 4-chlorobenzoate. Unexpectedly, this derivative also grew on 3,5-dichlorobenzoate, a substrate for *XylXYZL* but not an inducer of the *XylS* regulatory protein. The ability to grow on 4-chlorobenzoate or 3,5-dichlorobenzoate was also observed in derivatives of strain JMP134 containing the *xyl* gene module but lacking *xylS*, indicating the presence of an *xylS*-like element in *R. eutropha* with an inducer profile different from that of the pWW0-encoded regulator. Growth on 4-chlorobenzoate was also observed after introduction of the *xyl* gene module into strain JMP222, a JMP134 derivative lacking pJP4, but only if multiple copies of *tfdC_ID_IE_IF_I* or *tfdD_{II}C_{II}E_{II}F_{II}* were present. However, only the derivative containing multiple copies of *tfdD_{II}C_{II}E_{II}F_{II}* was able to grow on 3,5-dichlorobenzoate. These observations indicate that although the acquisition of new catabolic genes actually enhances the catabolic abilities of *R. eutropha* JMP134, these new properties are strongly influenced by the dosage of the *tfd* genes, the presence of a chromosomal *xylS*-like regulatory element and the different contributions of the *tfd* gene clusters.

Keywords: chloroaromatic compounds, maleylacetate reductase, gene acquisition, AraC/XylS regulatory family, gene dosage

INTRODUCTION

Aerobic bacterial metabolism of chloroaromatic pollutants commonly proceeds via peripheral reactions that

produce chlorocatechols, which are channelled into the central metabolism by a modified *ortho*-cleavage pathway (Reineke & Knackmuss, 1988). It is assumed that the coordinated expression of peripheral reactions and

Abbreviations: CB, chlorobenzoate; 3-CB, 3-chlorobenzoate; 4-CB, 4-chlorobenzoate; 3,5-DCB, 3,5-dichlorobenzoate; 3-MB, 3-methylbenzoate; 4-MB, 4-methylbenzoate; 2,4-D, 2,4-dichlorophenoxyacetate.

Ralstonia eutropha JMP134 is well known for its catabolic properties toward chloroaromatic compounds. This bacterium grows on 2,4-dichlorophenoxyacetate (2,4-D) and 3-chlorobenzoate (3-CB), as well as other chloroaromatic pollutants (Clément *et al.*, 1995; Don & Pemberton, 1981; Pieper *et al.*, 1988). The key catabolic abilities of *R. eutropha* JMP134 are

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enzymes whose activity profiles are different (Fig. 1b) (Laemmli *et al.*, 2000; Pérez-Pantoja *et al.*, 2000; Plumeier *et al.*, 2002).

Acquisition of the full set of *tfd*-encoded genes by bacteria receiving pJP4 has been reported (Don & Pemberton, 1981; Friedrich *et al.*, 1983; Haugland *et al.*, 1990; Kleinsteinuber *et al.*, 2001; Sahasrabudhe & Modi, 1991). These studies have shown that the expression of the ability to grow on 3-CB or 2,4-D, or on both, is strongly dependent on the recipient strain. In this context, the effect of the introduction of genes that expand the peripheral reactions already present in *R. eutropha* JMP134 has not been assessed. The interplay between new peripheral reactions with each of the two *tfd* gene modules encoding chlorocatechol degradation in *R. eutropha* JMP134 is also unknown. Here we report the effect of the acquisition of catabolic genes on the ability of *R. eutropha* JMP134 and derivatives containing single or multiple copies of the *tfdC_ID_IE_IF_I* or *tfdD_{II}C_{II}E_{II}F_{II}* modules to grow on chlorobenzoates (CBs). For such purposes, we chose the *xylS*-*xylXYZL* genes from pWW0, a TOL (toluene degradation) plasmid, which encode the *xylS*-regulated expression of the broad-substrate-range toluate 1,2-dioxygenase (*xylXYZ*) and the 1,2-dihydro-1,2-dihydroxytoluate dehydrogenase (*xylL*), which catalyse reactions for the initial metabolism of substituted benzoates (Fig. 1a, striped arrows) (Ramos *et al.*, 1986; Reineke & Knackmuss, 1978).

METHODS

Bacterial strains and growth conditions. The *R. eutropha* strains used in this work are listed in Table 1. *R. eutropha* JMP134 and JMP222 were grown in minimal saline medium (Kröckel & Focht, 1987), containing 2 mM 3-CB or 1 mM benzoate plus 1000 µg streptomycin ml⁻¹, respectively. All other *R. eutropha* JMP134 derivatives (Table 1) were maintained in minimal medium containing 1 mM benzoate plus the appropriate selective antibiotic. *Escherichia coli* CC118λpir, *E. coli* HB101 and *E. coli* DH5α derivatives harbouring the plasmids listed in Table 1 were grown in Luria-Bertani medium containing the appropriate selective antibiotic: 50 µg ampicillin ml⁻¹ or 50 µg kanamycin ml⁻¹; 20 µg gentamicin ml⁻¹ or 20 µg chloramphenicol ml⁻¹; 30 µg streptomycin ml⁻¹ or 40 µg tellurite ml⁻¹.

Growth measurements. Growth on 3-CB, 4-chlorobenzoate (4-CB), 3,5-dichlorobenzoate (3,5-DCB), 3-methylbenzoate (3-MB) or 4-methylbenzoate (4-MB) was determined by the increase in the OD₆₆₀ value when measured at the stationary phase of growth, usually after 48 h of culture. At least three replicates for each growth measurement were carried out.

Degradation of CB by resting cells and enzyme-activity assays. For CB degradation assays, *R. eutropha* derivatives were grown in minimal medium containing 5 mM fructose. At the late-exponential phase of growth, cells were induced with 3 mM 4-CB or 2 mM 3,5-DCB for at least 4 h or were not induced. About 100 ml of each culture were harvested, and the resulting cell pellet was washed three times with minimal medium. The cells were then resuspended in the same medium to an OD₆₆₀ value of 2.0, and incubated with 0.3 mM 4-CB or

3,5-DCB. The degradation of CB was monitored in a HP 8452-A UV spectrophotometer (Hewlett Packard). UV spectral profiles (210–300 nm) were determined in supernatants from each sample. Under the experimental conditions used here, no spectral changes corresponding to potential metabolic products, i.e. chlorocatechols, chloromuconates or dienelactones, were observed. Abiotic controls were routinely performed by monitoring CB degradation in uninoculated minimal-medium-containing flasks. Degradation rates of 3,5-DCB were calculated from the absorbance decrease at 238 nm ($\epsilon_{238} = 3300 \text{ M}^{-1} \text{ cm}^{-1}$) after 20 min incubation, and are expressed in µmol 3,5-DCB degraded min⁻¹ (mg protein)⁻¹. Proteins were determined by the Bradford method (Bradford, 1976), after treatment of cells with 5 M NaOH at 85 °C for 10 min. For activity assays of *R. eutropha* derivatives, cells were grown in 2 mM benzoate and induced at the late-exponential phase of growth with 1 mM 3-CB. Growth on benzoate and induction with 3-CB was used in these assays because not all of the *R. eutropha* derivatives grew on 3-CB. The concentration of 3-CB used was chosen to prevent the accumulation of chlorocatechols in strains that had low levels of chlorocatechol 1,2-dioxygenase. Cell extracts were obtained as follows. About 100 ml of each culture were harvested at the end of the exponential phase of growth and centrifuged. The resulting cell pellets were washed twice and resuspended in 5 ml of 50 mM Tris/acetate (pH 7.5) containing 1 mM MnSO₄. The cells were then disrupted by sonication (Vibracell; Sonics & Materials). The soluble protein fraction was obtained after 1 h centrifugation at 130 000 g, in a Beckman L-80 ultracentrifuge. Cell extracts were used without further purification. Enzyme assays for chlorocatechol 1,2-dioxygenase, maleylacetate reductase and chloromuconate cycloisomerase were performed as described previously (Pérez-Pantoja *et al.*, 2000).

Cloning of the *tfd* gene modules. Restrictions, ligations, dephosphorylation reactions, DNA purification, electroporation conditions and Southern analysis were carried out following standard protocols (Ausubel *et al.*, 1992). Different chlorocatechol catabolism *tfd* gene modules were prepared for chromosomal insertions (using vector pUT or pBSL202) or for cloning into a medium-copy-number vector (pBBR1MCS-2). The plasmids constructed and the vectors used in this study are listed in Table 1. The cloning of the *tfdR*-*tfdC_ID_IE_IF_I* gene module into the pUC18*NotI* derivative pUCLG2 has been described previously (Pérez-Pantoja *et al.*, 2000). Plasmid pUCLG2 was digested with *NotI* and the resulting insert was introduced into pUT, to give pR1TFD. Cloning of *tfdR*-*tfdC_ID_IE_IF_I* into the medium-copy-number vector pBBR1MCS-2 to give pBBR1M-I has also been described previously (Pérez-Pantoja *et al.*, 2000). To clone *tfdR*-*tfdD_{II}C_{II}E_{II}F_{II}*, pBSDP4 was digested with *SacI/KpnI* to generate a 5.9 kb fragment containing the *tfdR*-*tfdD_{II}C_{II}E_{II}F_{II}* gene module; this fragment was then introduced into pHRP316 to give pHRPM-II. pHRP316 was only used for intermediate cloning steps. The 6 kb *HindIII* fragment of pHRPM-II was cloned into pBBR1MCS-2 to yield pBBRHM-II. The *tfd* gene module was extracted from pBBRHM-II with a *SpeI* digestion, and then introduced into pBSL202 to give pR2TFDG. Additionally, the *SpeI* fragment from pBBRHM-II was introduced into pR1TFDG, a pBSL202 derivative in which a 5.1 kb *NotI* fragment from pUCLG2 containing the *tfdR*-*tfdC_ID_IE_IF_I* gene module had been cloned previously, to produce pR12TFDG. The latter contains tandem, direct copies of each *tfd* module. Cloning of *tfdR*-*tfdD_{II}C_{II}E_{II}F_{II}* into pBBR1MCS-2, to give pBBR1M-II, has been described previously (Pérez-Pantoja *et al.*, 2000). The maleylacetate reductase gene encoded on pBBR1M-I was inactivated by a 0.6 kb deletion corresponding

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant phenotype/genotype*	Source/reference†
<i>R. eutropha</i>		
JMP134	2,4-D, 3-CB	DSMZ
JMP134::X	2,4-D, 3-CB, 4-CB, 3,5-DCB, 3-MB, 4-MB; <i>xylS-xylXYZL</i>	This work
JMP134::XΔS	2,4-D, 3-CB, 4-CB, 3,5-DCB, 3-MB, 4-MB; <i>xylXYZL</i>	This work
JMP134::X(pJP4ΔF _{II})	3-CB, 4-CB, 3-MB, 4-MB; <i>xylS-xylXYZL</i>	This work
JMP222	Sm ^r ; a pJP4-cured JMP134 derivative	Don & Pemberton (1981)
JMP222::X	Sm ^r ; <i>xylS-xylXYZL</i>	This work
JMP222::R1TFD	Sm ^r ; <i>tfdR-tfdC_ID_IE_IF_I</i>	This work
JMP222::R2TFD	Sm ^r ; <i>tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i>	This work
JMP222::R12TFD	Sm ^r ; <i>tfdR-tfdC_ID_IE_IF_I, tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i>	This work
JMP222::X::R1TFD	Sm ^r , Te ^r ; <i>xylS-xylXYZL, tfdR-tfdC_ID_IE_IF_I</i>	This work
JMP222::X::R2TFD	Sm ^r , Te ^r ; <i>xylS-xylXYZL, tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i>	This work
JMP222::X::R12TFD	Sm ^r , Te ^r ; <i>xylS-xylXYZL, tfdR-tfdC_ID_IE_IF_I, tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i>	This work
JMP222::X(pBBR1M-I)	3-CB, 4-CB; Sm ^r , Te ^r , Km ^r ; <i>xylS-xylXYZL, tfdR-tfdC_ID_IE_IF_I</i>	This work
JMP222::X(pBBR1M-II)	3-CB, 4-CB, 3,5-DCB; Sm ^r , Te ^r , Km ^r ; <i>xylS-xylXYZL, tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i>	This work
JMP222::X(pBBRΔF _I)	3-CB, 4-CB; Sm ^r , Te ^r , Km ^r ; <i>xylS-xylXYZL, tfdR-tfdC_ID_IE_I</i>	This work
JMP222::X(pBBRΔF _{II})	3-CB, 4-CB, Sm ^r , Te ^r , Km ^r ; <i>xylS-xylXYZL, tfdR-tfdD_{II}C_{II}E_{II}</i>	This work
Plasmid		
pRK600	Cm ^r ; <i>tra</i> ⁺	de Lorenzo <i>et al.</i> (1990)
pBSL202	Gm ^r , Ap ^r	Alexeyev <i>et al.</i> (1995)
pHRP316	Sm/Sp ^r , Ap ^r	Parales & Harwood (1993)
pBBR1MCS-2	Km ^r ; broad-host-range	Kovach <i>et al.</i> (1995)
pUT	Km ^r , Ap ^r	de Lorenzo <i>et al.</i> (1990)
pSPM100	Ap ^r , Te ^r ; <i>xylS-xylXYZL</i> , pUT derivative	V. de Lorenzo
pUCLG2	Ap ^r ; <i>tfdR-tfdC_ID_IE_IF_I</i> , pUC18 <i>NotI</i> derivative	Pérez-Pantoja <i>et al.</i> (2000)
pBSDP4	Ap ^r ; <i>tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i> , pBSKSII derivative	Pérez-Pantoja <i>et al.</i> (2000)
pSPMAS	Ap ^r , Te ^r ; <i>xylXYZL</i> , pUT derivative	This work
pR1TFD	Km ^r , Ap ^r ; <i>tfdR-tfdC_ID_IE_IF_I</i> , pUT derivative	This work
pR1TFDGD	Gm ^r , Ap ^r ; <i>tfdR-tfdC_ID_IE_IF_I</i> , pBSL202 derivative	This work
pR2TFDGD	Gm ^r , Ap ^r ; <i>tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i> , pBSL202 derivative	This work
pR12TFDGD	Gm ^r , Ap ^r ; <i>tfdR-tfdD_{II}C_{II}E_{II}F_{II}, tfdR-tfdC_ID_IE_IF_I</i> , pBSL202 derivative	This work
pBBRHM-II	Km ^r ; <i>tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i> , pBBR1MCS-2 derivative	This work
pHRPM-II	Sm/Sp ^r , Ap ^r ; <i>tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i> , pHRP316 derivative	This work
pBBR1M-I	Km ^r ; <i>tfdR-tfdC_ID_IE_IF_I</i> , pBBR1MCS-2 derivative	Pérez-Pantoja <i>et al.</i> (2000)
pBBR1M-II	Km ^r ; <i>tfdR-tfdD_{II}C_{II}E_{II}F_{II}</i> , pBBR1MCS-2 derivative	Pérez-Pantoja <i>et al.</i> (2000)
pBBR1ΔF _I	Km ^r ; <i>tfdR-tfdC_ID_IE_I</i> , pBBR1MCS-2 derivative	This work
pBBR1ΔF _{II}	Km ^r ; <i>tfdR-tfdD_{II}C_{II}E_{II}</i> , pBBR1MCS-2 derivative	This work

* Able to grow on the chloro and methyl aromatic compounds shown. Ap^r, ampicillin-resistant; Km^r, kanamycin-resistant; Cm^r, chloramphenicol-resistant; Sm^r, streptomycin-resistant; Sp^r, spectinomycin-resistant; Gm^r, gentamicin-resistant; Te^r, tellurite-resistant; *tra*, transfer functions.

† DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

to the internal *AatII* sites in the *tfdF_I* gene, to produce pBBRΔF_I. To construct a pBBR1M-II derivative lacking the maleylacetate reductase gene, the 8.3 kb *EcoRI* fragment of pJP4 was cloned in pUC18*NotI*, and its 4.5 kb *SacI* fragment containing the *tfdR-tfdD_{II}C_{II}E_{II}F_{II}* genes was directly cloned into pBBR1MCS-2, to give pBBRΔF_{II}. A 10-fold decrease in maleylacetate reductase activity was determined for strain JMP222(pBBRΔF_{II}) with respect to strain JMP222(pBBR1M-II). The determination of this activity in strain JMP222(pBBRΔF_I) was not performed because JMP222(pBBR1M-I) has a maleylacetate reductase activity level similar to that found in the chromosome of strain JMP222 (Pérez-Pantoja *et al.*, 2000).

Construction of a *xylXYZL* gene module. A pUT derivative, pSPM100, containing the *xylS-xylXYZL* gene module of the TOL plasmid pWW0 and resistance to tellurite as a selective marker was obtained from V. de Lorenzo (S. Panke & V. de Lorenzo, unpublished data). To construct an unregulated *xyl* gene module, pSPM100 was digested with *BstEII*, which produces three cuts in this plasmid, to remove a 725 bp internal *xylS* fragment that included the translation initiation site of the gene. The remaining two fragments of pSPM100 were re-ligated in the proper orientation and designated as pSPMAS. The *BstEII* and *NotI* restriction profiles of pSPMAS were determined and were in agreement with the deletion of the internal *xylS* fragment. The absence or presence of an

active XylS regulatory protein was verified in *E. coli* cells containing pSPMΔS or pSPM100, respectively, by visual detection of the production of an intense brown colour due to chlorocatechol accumulation when these cells were exposed to 3-CB.

Introduction of the *tfd* and/or *xyl* gene modules into *R. eutropha*. The introduction of the *tfd* and/or *xyl* gene modules was performed by using mini-Tn5-derived vectors (de Lorenzo *et al.*, 1990) or pBBR1MCS-2, which were transferred by triparental mating with *E. coli* CC118λpir containing pBBR1MCS-2 (pBBR1M-I, pBBR1M-II), pUT (pR1TFD), pSPM100, pSPMΔS or pBSL202 (pR2TFDG, pR12TFDG) derivatives as donors (Table 1), *E. coli* HB101(pRK600) as the helper and *R. eutropha* JMP134 or JMP222, or derivatives thereof, as recipients. A donor-to-helper-to-recipient ratio of 1:1:2 was used. After incubation, cells were resuspended and the transconjugants were selected on agar plates containing minimal medium supplemented with 3 mM benzoate plus 50 µg kanamycin ml⁻¹ (pUT and pBBR1MCS-2 derivatives), 3 mM benzoate plus 20 µg gentamicin ml⁻¹ (pBSL202 derivatives) or 2 mM 2,4-D plus 40 µg tellurite ml⁻¹ (pSPM100 derivatives). Six to ten derivatives from each mating were analysed for the absence of plasmid DNA, ampicillin sensitivity and stable maintenance (20 generations) of kanamycin, gentamicin or tellurite resistance after growth in Luria–Bertani medium without a selective marker. To detect the *tnp* Tn5 IS50R transposase gene, primer pair TNP-1 (5'-CGGCGGC-GCTGGGTGATCCT-3') and TNP-2 (5'-GCCCCAGCTGG-CAATTCCGG-3') (nucleotides 148–167 and 1414–1433, respectively, of GenBank accession no. U15573) was used (Ahmed & Podemski, 1995). Failure to detect this gene was an indication that the vector had been lost and that transposition had occurred (de Lorenzo *et al.*, 1990). The absence of *tnp*, sensitivity to ampicillin, stable inheritance of kanamycin, gentamicin or tellurite resistance and the absence of the corresponding plasmid indicated that insertion of cloned genes had occurred. Southern analysis with a biotinylated pR1TFD, pR2TFDG or pSPM100 DNA probe was carried out to confirm that the selected derivatives carried single insertions of the corresponding *tfd* or *xyl* gene module. All transconjugants showed single insertions of the *tfd* or *xyl* genes, as determined by Southern hybridization, indicating successful establishment of the genes in the transconjugants. Southern analysis performed with a pSPM100 probe showed the presence of single insertions of the *xyl* genes, into either the chromosome or pJP4. To confirm the presence of the chlorocatechol genes in the transconjugants, PCR was performed using the primer pair BDO-1 and VAL-2 (Pérez-Pantoja *et al.*, 2000) to amplify a 1.8 kb *tfdC₁D₁* fragment. The primer pair RBA-1 (5'-CAACGAAATAGCGAAGCTGT-CGA-3') and RDB-1 (5'-ATGAGCACGCTGCTCTGATG-CTTG-3') (nucleotides 863–885 and 1141–1164, respectively, of GenBank accession no. M98445) was used to amplify a 302 bp fragment of the *tfdR*–*tfdD_{II}* intergenic region. Conditions for PCR were: denaturation at 95 °C for 2 min, followed by 35 cycles at 95 °C for 45 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 10 min.

Detection of a *xylS* gene homologue in *R. eutropha*. Southern hybridization of *R. eutropha* JMP134 genomic DNA that had been digested with *Eco*RI was performed using the 725 bp *Bst*EII-generated fragment from *xylS* as a biotinylated probe. A 684 bp region from an ORF taken from the genomic sequence of *Ralstonia metallidurans* CH34, encoding a *xylS* gene homologue, was amplified by PCR using primers FOR130 (5'-TCCTTCAACCGGCTCAGTTA-3') and REV814 (5'-ATGCGTCATTGAGCAGATCC-3'). *R. metallidurans*

CH34 is a strain closely related to *R. eutropha* JMP134, whose genome sequence is available at the Department of Energy (DOE) Joint Genome Institute (http://www.jgi.doe.gov/JGI_microbial/html/). Conditions for PCR were: denaturation at 94 °C for 5 min, followed by 28 cycles at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 90 s, with a final extension at 72 °C for 10 min. The resulting PCR product was also used as a probe for the *R. eutropha* JMP134 genomic DNA that had been digested with *Eco*RI.

Construction of an *R. eutropha* JMP134 derivative harbouring a *tfdF_{II}*-inactivated pJP4. To inactivate the *tfdF_{II}* gene in pJP4, the 1.0 kb pBSL202 fragment encoding gentamicin resistance was introduced into the *Mlu*I sites of the 1.0 kb *Eco*RI–*Nde*I fragment from the *tfdF_{II}* gene, and the resulting fragment was cloned into pLITMUS. The resulting plasmid was introduced into *R. eutropha* JMP134 by electroporation; homologous recombination derivatives were selected on the basis of a gentamicin-resistant, ampicillin-sensitive phenotype. The insertion in the *tfdF_{II}* gene was verified by Southern analysis of plasmid DNA that had been digested with *Eco*RI, which showed positive hybridization with the gentamicin-resistance probe only in the pJP4 derivative (pJP4Δ*F_{II}*). DNA fragment that had altered mobility with respect to the wild-type pJP4 DNA. A sevenfold decrease in maleylacetate reductase activity was found in strain JMP134(pJP4Δ*F_{II}*), when compared to the wild-type strain.

RESULTS AND DISCUSSION

Growth of *R. eutropha* JMP134 on CBs after acquisition of the pWW0-derived *xylS*–*xyl*XYZL gene module

R. eutropha JMP134 does not grow on 4-CB. This may be due to the narrow substrate range of its benzoate 1,2-dioxygenase, which would only transform benzoate and 3-CB at significant rates, as has been reported for the closely related strain B9, a mutant of strain 335, the type strain of *R. eutropha* (formerly *Alcaligenes eutrophus*) (Reineke & Knackmuss, 1978). We determined whether the acquisition of the broad-substrate-range enzyme toluate (methylbenzoate) 1,2-dioxygenase (*xyl*XYZ) and the 1,2-dihydro-1,2-dihydroxytoluate dehydrogenase (*xyl*L), regulated by their transcriptional activator *xylS* and, therefore, providing a better peripheral function for CB degradation, can actually expand the growth substrate range of strain JMP134. The pWW0-encoded enzymes can efficiently transform alkyl-substituted benzoates such as 3-MB and 4-MB, as well as chlorinated compounds such as 3-CB, 4-CB and 3,5-DCB, to their corresponding catechols (Reineke & Knackmuss, 1978), although 3,5-DCB is not an inducer of XylS (Ramos *et al.*, 1986). The *xylS*–*xyl*XYZL genes were introduced into *R. eutropha* JMP134 and JMP222 using mini-Tn5-derived vectors. All of the derivatives of *R. eutropha* JMP134 containing the *xylS*–*xyl*XYZL genes inserted into the chromosome, or into pJP4 (*R. eutropha* JMP134::X), could grow on 0.5–10 mM 4-CB (Fig. 2a) and on 2 mM 3-MB or 4-MB (not shown). Unexpectedly, the presence of *xylS*–*xyl*XYZL also allowed growth of all of these derivatives on 0.5–10 mM 3,5-DCB (Fig. 2b), a substrate of the toluate dioxygenase system but not an inducer of the XylS regulator. Control growth tests for the wild-type strain JMP134, performed

Fig. 2. Growth of *R. eutropha* derivatives containing the *xyl* and *tfd* gene modules on CBs. Effects of the CB concentration on growth with (a) 4-CB or (b) 3,5-DCB of *R. eutropha* JMP134 and its derivative containing the *xylS*-*xylXYZL* genes, namely JMP134::X and derivatives of strain JMP222(pBBR1M-I), JMP222(pBBR1M-II) and JMP222::R1TFD. Samples were taken at the stationary phase of growth, usually after 48 h cultivation. Each point corresponds to the mean $\pm$ SD of three independent experiments. In (a) and (b): ●, JMP134::X(pJP4); ○, JMP134(pJP4); ■, JMP222::X(pBBR1M-I); □, JMP222::X(pBBR1M-II); ▲, JMP222::X::R1TFD.

on plates containing 4-CB or 3,5-DCB, were negative, indicating that these new catabolic abilities are not due to spontaneous mutations, but to the presence of the *xyl* genes plus the *tfd* genes. Accordingly, the introduction of the *xyl* genes into strain JMP222, which lacked the *tfd* genes, did not enable cells to grow on 4-MB, 4-CB or 3,5-DCB, even after more than 15 days incubation, but it did permit growth on 3-MB, because this compound is mainly transformed to 3-methylcatechol. A chromosomal *meta*-ring-cleavage pathway (Pieper *et al.*, 1988) can subsequently metabolize this catechol.

The transfer of pWW0 to *Pseudomonas* sp. B13, another chlorocatechol-degrading bacterium, has been described, and allows transconjugants to grow on 4-CB and 3,5-DCB (Reineke & Knackmuss, 1979). However, these transconjugants were obtained at low frequencies (10^{-8} transconjugants per donor cell, or spontaneous mutation frequency) by direct plating onto each CB, selecting for mutations in addition to the transference of the *xyl* genes. This is not the case for *R. eutropha* containing the *xyl* genes, because the initial selection of transconjugants was performed in 2,4-D plus tellurite and, therefore, did not select for mutations involved in CB-degrading abilities.

Table 2. Relative rates of 3,5-DCB degradation by derivatives of *R. eutropha* JMP134

Cells were grown on fructose, exposed or not to 4-CB or 3,5-DCB, and degradation rates of CB as substrate were measured. Only results using 3,5-DCB as substrate are shown, because no significant differences in degradation were found using 4-CB or 3,5-DCB as substrates. Relative degradation rates were calculated with respect to strain JMP134::X, which was induced with 4-CB. The absolute degradation rate for this strain/condition was 39 μ mol 3,5-DCB degraded min^{-1} (mg protein) $^{-1}$. Results for three separate experiments are shown as the mean $\pm$ SD.

<i>R. eutropha</i> strain	Inducer		
	None	4-CB	3,5-DCB
JMP134::X	3 $\pm$ 1	100 $\pm$ 12	57 $\pm$ 11
JMP134::XΔS	3 $\pm$ 1	90 $\pm$ 3	75 $\pm$ 3
JMP134	3 $\pm$ 1	5 $\pm$ 1	3 $\pm$ 1
JMP222::X	1 $\pm$ 1	89 $\pm$ 3	61 $\pm$ 7

Evidence for the presence of a putative AraC/XylS family regulator capable of activating *xyl* gene expression in *R. eutropha* JMP134

The ability of *R. eutropha* JMP134::X to grow on 3,5-DCB suggests that this compound acts as an inducer with the XylS regulatory protein and/or that another regulatory system present in *R. eutropha* replaces XylS, activating transcription of the *xyl* operon. The first possibility does not seem correct, because it has been reported that 3,5-DCB is unable to activate the regulator in *Pseudomonas putida* (Ramos *et al.*, 1986). Furthermore, 3,5-DCB can also act as an inhibitor of the XylS-mediated induction of the *xylXYZL* genes in the presence of other substituted benzoates (Ramos *et al.*, 1986). To test the second possibility (i.e. that another regulatory system replaces XylS), we constructed a pSPM100 derivative that has a 0.7 kb deletion in *xylS* and, therefore, lacks the possibility of induction mediated by this regulator. When the *xylXYZL* gene module was introduced into *R. eutropha* JMP134, the resulting derivative, *R. eutropha* JMP134::XΔS, was still able to grow on 4-CB and 3,5-DCB to the same extent as the strain harbouring the complete *xyl* gene module. The degradation of CBs in *R. eutropha* JMP134 derivatives containing or lacking *xylS* (strains JMP134::X and JMP134::XΔS, respectively) was studied in cells grown on fructose and induced with 4-CB or 3,5-DCB (Table 2). The results showed that XylXYZ activity in these derivatives is due to the acquisition of the *xyl* genes, because only negligible activity was observed in the wild-type strain. The results also show that XylXYZ activity is inducible by 4-CB or 3,5-DCB and, more significantly, that XylS was not required for such an induction, indicating the presence of another regulatory system in *R. eutropha* JMP134. When the rate of CB degradation was determined for *R. eutropha* JMP222 harbouring the *xylS*-*xylXYZL* module (*R. eutropha*

JMP222::X), essentially the same pattern of degradation was found (Table 2), indicating that the activity of a putative XylS element in *R. eutropha* is located in the chromosome and is not part of the pJP4-encoded genes. Therefore, it is possible that in the chromosome of strain JMP134 a regulatory function is responding to CBs and is cross-activating the expression of the *xylXYZL* module. This kind of cross-regulation has been shown in *P. putida* for benzoate catabolism, between the pWW0-encoded proteins XylS and BenR, the regulator of the chromosomal benzoate-degradation pathway and both proteins belonging to the AraC/XylS family of regulators (Cowles *et al.*, 2000; Jeffrey *et al.*, 1992). To obtain further evidence for the presence of a putative AraC/XylS family regulator similar to XylS from pWW0 in the chromosome of *R. eutropha*, Southern hybridization of *EcoRI*-digested JMP134 and JMP222 genomic DNA was performed using the 725 bp *BstEII*-generated fragment containing part of *xylS*. No hybridization signal was observed for either derivative, even under low-stringency conditions. Nevertheless, the use of the 684 bp PCR product from a *xylS* homologue of *R. metallidurans* CH34 as a probe revealed a single hybridization band of approximately 4 kb in size in both the *R. eutropha* JMP134 and the JMP222 genomic DNA that had been digested with *EcoRI*. It is worth mentioning that the putative protein found in the *R. metallidurans* genome has only 24 % nucleotide identity (30 % amino-acid identity) with *xylS* from pWW0. If it is assumed that the gene from *R. eutropha* JMP134 is closely related to that of *R. metallidurans*, such low identity would explain the failure to find the *xylS* sequence in the *R. eutropha* genome when using *xylS* from pWW0 as the probe. Taken together, these results strongly suggest that the chromosome of strain JMP134 has a BenR/XylS-like element with an inducer profile different to that reported for XylS from pWW0.

Growth of *R. eutropha* JMP222 derivatives harbouring the *xylS*-*xylXYZL* gene module on CBs requires the presence of multiple copies of the *tfdC_ID_IE_IF_I* or *tfdD_{II}C_{II}E_{II}F_{II}* gene modules

Plasmid pJP4 possesses an uncommon catabolic gene organization (van der Meer *et al.*, 1992), with two different, but almost iso-functional, gene modules encoding chlorocatechol degradation (Leveau *et al.*, 1999). We determined whether each of the *tfd* gene modules was enough to allow growth on CBs when expressed separately in strain JMP222 harbouring the *xyl* genes. First, *tfdR*-*tfdC_ID_IE_IF_I*, *tfdR*-*tfdD_{II}C_{II}E_{II}F_{II}* or both gene modules were introduced into strain JMP222 by using mini-Tn5-derived vectors (these vectors produced single copies of each gene module). When the derivatives *R. eutropha* JMP222::R1TFD, JMP222::R2TFD and JMP222::R12TFD (Table 1), respectively, were used as recipients for the *xylS*-*xylXYZL* genes, the resulting transconjugants [*R. eutropha* JMP222::X::R1TFD, JMP222::X::R2TFD and JMP222::X::R12TFD (Table 1), respectively] were unable to grow on 0.5–10 mM 4-CB or 3,5-DCB (Fig. 2a, b; only

Table 3. Specific activities of chlorocatechol 1,2-dioxygenase (TfdC) in crude extracts of derivatives of *R. eutropha* JMP134 harbouring chromosomal insertions of the *tfd* and *xyl* genes

Cells were grown on benzoate and induced for 4 h with 1 mM 3-CB. Only results using 3,5-dichlorocatechol as substrate are shown. Results for three separate experiments are shown as the mean $\pm$ SD.

<i>R. eutropha</i> strain	TfdC activity [U (mg protein) ⁻¹]
JMP134::X	0.24 $\pm$ 0.01
JMP222::X	< 0.02
JMP222::R1TFD::X	0.05 $\pm$ 0.01
JMP222::R2TFD::X	0.02 $\pm$ 0.01
JMP222::R12TFD::X	0.07 $\pm$ 0.02

JMP222::X::R1TFD is shown). These derivatives were also unable to grow in liquid cultures containing 0.5–10 mM 3-CB. An effect on expression due to the chromosomal position of the introduced genes can be disregarded, because the derivatives analysed (usually more than 50) and the Tn5-promoted transposition events did not show a strong sequence preference (de Lorenzo *et al.*, 1990). The effect observed with monocopies of the *tfd* modules is probably due to poor gene expression, or a low gene copy number, resulting in low enzyme activity, as was determined for chlorocatechol 1,2-dioxygenase (Table 3) and chloromuconate cycloisomerase (not shown). In this context, Pérez-Pantoja *et al.* (2000) have shown that the presence of multiple copies of each *tfd* module allows strain JMP222 to grow on 3-CB as a sole carbon source. When the *xylS*-*xylXYZL* gene module was introduced into strain JMP222 containing multiple copies of *tfdR*-*tfdC_ID_IE_IF_I* or *tfdR*-*tfdD_{II}C_{II}E_{II}F_{II}*, the resulting derivatives, JMP222::X(pBBR1M-I) and JMP222::X(pBBR1M-II), respectively, could grow on 0.5–10 mM 4-CB (Fig. 2a) and on 3-CB (not shown). With regard to the growth of derivatives on 3-CB, derivatives containing the *tfdR*-*tfdD_{II}C_{II}E_{II}F_{II}* gene module were less efficient than those containing the *tfdR*-*tfdC_ID_IE_IF_I* gene module (Pérez-Pantoja *et al.*, 2000; this work). However, the differences between these two *tfd* gene modules were less pronounced when the growth substrate was 4-CB (Fig. 2a). The explanation for this observation is that the main muconate produced by 3-CB is 2-chloromuconate (Pieper *et al.*, 1993). This intermediate, which is poorly metabolized by the enzyme encoded by *tfdD_I*, is an even worse substrate for the enzyme encoded by *tfdF_{II}* (Laemmli *et al.*, 2002; Pérez-Pantoja *et al.*, 2000; Plumeier *et al.*, 2002). In contrast, 3-chloromuconate or 2,4-dichloromuconate, the muconates produced during growth on 4-CB or 3,5-DCB, respectively, are good substrates for the chloromuconate cycloisomerases encoded by *tfdD_I* and *tfdD_{II}* (Kuhm *et al.*, 1990; Pérez-Pantoja *et al.*, 2000; Pieper *et al.*, 1993; Plumeier *et al.*, 2002; Vollmer *et al.*, 1999).

These results indicate that a monocopy insertion of the *tfd* gene modules, alone or in combination, is not enough to support the growth of *R. eutropha* on substrates that produce 3-, 4- or 3,5-dichlorocatechol. This is consistent with two recent observations from our laboratory – (i) that a JMP134 derivative harbouring a chromosomal integration of pJP4 is unable to grow on 3-CB and (ii) that the wild-type strain harbours five copies of pJP4 per genome (Trefault *et al.*, 2002). The copy number of the *clc* genes and the *tcb* genes, which encode chlorocatechol pathways similar to that encoded by the *tfd* genes, has also been shown to be a limiting factor for growth of bacteria on chloroaromatic compounds (Klemba *et al.*, 2000; Ravatn *et al.*, 1998).

The *tfdF_{II}*-encoded maleylacetate reductase plays a major role in the metabolism of 3,5-DCB in *R. eutropha* JMP134

The experiments carried out to study the effect of the presence of multiple copies of the *tfd* genes showed an additional difference between the two *tfd* gene modules. Strain JMP222::X(pBBR1M-I) was unable to grow on 3,5-DCB but strain JMP222::X(pBBR1M-II) was able to grow on this substrate, albeit at lower 3,5-DCB concentrations (Fig. 2b). Such behaviour may be due to the different maleylacetate reductase activities that the two *tfd* gene modules possess (Pérez-Pantoja *et al.*, 2000; Plumeier *et al.*, 2002). When strain JMP222(pBBR1M-II) is grown on 3-CB, it expresses about three times more maleylacetate reductase activity than strain JMP222(pBBR1M-I) (Plumeier *et al.*, 2002). It is worth mentioning that maleylacetate reductase encoded on the chromosome of strain JMP134 is not expressed during growth on 2,4-D or 3-CB (Padilla *et al.*, 2000; Plumeier *et al.*, 2002). Therefore, it is improbable that the chromosomal maleylacetate reductase activity is expressed during growth of strain JMP134 on 4-CB or 3,5-DCB. No significant differences between the activity with maleylacetate and 2-chloromaleylacetate (the intermediates from mono- and dichlorinated substrates, respectively) have been reported for maleylacetate reductases (Padilla *et al.*, 2000; Pérez-Pantoja *et al.*, 2000; Seibert *et al.*, 1993). However, maleylacetate reductase is required twice during the metabolism of dichlorinated compounds, because it converts 2-chloromaleylacetate into maleylacetate, and maleylacetate into 3-oxoadipate (Fig. 1b). Therefore, if TfdF_{II} is more active than TfdF_I, although the activity of these two proteins is not as different as initially reported (Pérez-Pantoja *et al.*, 2000; Plumeier *et al.*, 2002), its contribution to (chloro)maleylacetate turnover would be more important. Evidence for a significant role of TfdF_{II} in the metabolism of 3,5-DCB came from the analysis of additional *R. eutropha* derivatives. When strain JMP222::X harboured pBBRΔF_{II}, a pBBR1M-II-derived plasmid lacking the *tfdF_{II}*-encoded maleylacetate reductase gene, the ability of this strain to grow on 3,5-DCB was completely abolished (data not shown). In contrast, its ability to grow on 4-CB was still present, but the ability to grow on 4-CB decreased to a similar

Fig. 3. Growth of *R. eutropha* derivatives containing different maleylacetate-reductase-encoding genes on CBs. Effects of the CB concentration on growth of *R. eutropha* JMP134::X (◆) and its derivative JMP134::X(pJP4ΔF_{II}) (▲), harbouring a *tfdF_{II}*-inactivated pJP4, with (a) 4-CB or (b) 3,5-DCB. Samples were taken at the stationary phase of growth, usually after 48 h cultivation. Each point corresponds to the mean ± SD of three independent experiments.

extent in strains JMP222::X(pBBRΔF_I) and JMP222::X(pBBRΔF_{II}) (data not shown). More significantly, when *tfdF_{II}* was inactivated in pJP4 by the insertion of a gentamicin-resistance gene, the corresponding *R. eutropha* JMP134 derivative, JMP134::X(pJP4ΔF_{II}), was unable to grow on 3,5-DCB, but still grew on 4-CB (Fig. 3). These observations strongly suggest that the presence of TfdF_I and/or the chromosomal maleylacetate reductase is not enough to allow growth of *R. eutropha* JMP134 on 3,5-DCB.

CONCLUSIONS

The results of this work show that even though the acquisition of a new peripheral activity can actually enhance the catabolic abilities of *R. eutropha* JMP134, these new abilities are strongly influenced by the *tfd* gene dosage. The results also show that some of these new properties, such as the unexpected use of 3,5-DCB as a growth substrate, depend on the presence of a putative chromosomal *xylS*-like regulatory element, active in response to chlorinated benzoates, and on the differences in the activities of the maleylacetate reductases encoded by *tfdF_I* and *tfdF_{II}*. The results reported here provide novel insights into understanding the outcome of the acquisition of genes encoding peripheral or central reactions, into the evolution of catabolic abilities toward chlorinated aromatic pollutants and, also, into pre-

dicting the behaviour of strains engineered to contain catabolic genes.

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REFERENCES

- Ahmed, A. & Podemski, L. (1995). The revised nucleotide sequence of Tn5. *Gene* **154**, 129–133.
- Alexeyev, M. F., Shokolenko, I. N. & Croughan, T. P. (1995). New mini-Tn5 derivatives for insertion mutagenesis and genetic engineering in Gram-negative bacteria. *Can J Microbiol* **41**, 1053–1055.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (editors) (1992). *Short Protocols in Molecular Biology*, 2nd edn. New York: Greene Publishing Associates.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- Brinkmann, U. & Reineke, W. (1992). Degradation of chlorotoluenes by *in vivo* constructed hybrid strains: problems of enzyme specificity, induction and prevention of *meta*-pathway. *FEMS Microbiol Lett* **75**, 81–87.
- Clément, P., Matus, V., Cárdenas, L. & González, B. (1995). Degradation of trichlorophenols by *Alcaligenes eutrophus* JMP134. *FEMS Microbiol Lett* **127**, 51–55.
- Cowles, C. E., Nichols, N. N. & Harwood, C. (2000). BenR, a XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida*. *J Bacteriol* **182**, 6339–6346.
- de Lorenzo, V., Herrero, M., Jacobzik, U. & Timmis, K. N. (1990). Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing and chromosomal insertion of cloned DNA in Gram-negative eubacteria. *J Bacteriol* **172**, 6568–6572.
- Don, R. H. & Pemberton, J. M. (1981). Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *J Bacteriol* **145**, 681–686.
- Don, R. H., Weightman, A. J., Knackmuss, H.-J. & Timmis, K. N. (1985). Transposon mutagenesis and cloning analyses of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). *J Bacteriol* **161**, 85–90.
- Friedrich, B., Meyer, M. & Schlegel, H. G. (1983). Transfer and expression of the herbicide-degrading plasmid pJP4 in aerobic autotrophic bacteria. *Arch Microbiol* **134**, 92–97.
- Haugland, R. A., Schlemm, D. J., Lyons, R. P., III, Sferra, P. R. & Chakrabarty, A. M. (1990). Degradation of the chlorinated phenoxyacetate herbicides 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid by pure and mixed bacterial cultures. *Appl Environ Microbiol* **56**, 1357–1362.
- Jeffrey, W. H., Cuskey, S. M., Chapman, P. J., Resnick, S. & Olsen, R. H. (1992). Characterization of *Pseudomonas putida* mutants unable to catabolize benzoate: cloning and characterization of *Pseudomonas* genes involved in benzoate catabolism and isolation of a chromosomal DNA fragment able to substitute for *xylS* in activation of the TOL lower-pathway promoter. *J Bacteriol* **174**, 4986–4996.
- Kasberg, T., Daubaras, D. L., Chakrabarty, A. M., Kinzelt, D. & Reineke, W. (1995). Evidence that operons *tcb* , *tfd* and *clc* encode maleylacetate reductase, the fourth enzyme of the modified *ortho* pathway. *J Bacteriol* **177**, 3885–3889.
- Kleinstaub, S., Müller, R. H. & Babel, W. (2001). Expression of the 2,4-D degradative pathway of pJP4 in an alkaliphilic, moderately halophilic soda lake isolate, *Halomonas* sp. EF43. *Extremophiles* **5**, 375–384.
- Klemba, M., Jakobs, B., Wittich, R.-M. & Pieper, D. H. (2000). Chromosomal integration of *tcb* chlorocatechol degradation pathways genes as a means of expanding the growth substrate range of bacteria to include haloaromatics. *Appl Environ Microbiol* **66**, 3255–3261.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 2nd & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.
- Kröckel, L. & Focht, D. (1987). Construction of chlorobenzene-utilizing recombinants by progenitive manifestation of a rare event. *Appl Environ Microbiol* **53**, 2470–2475.
- Kuhm, A. E., Schlömann, M., Knackmuss, H.-J. & Pieper, D. H. (1990). Purification and characterization of dichloromuconate cycloisomerase from *Alcaligenes eutrophus* JMP134. *Biochem J* **266**, 877–883.
- Laemmli, C. M., Leveau, J. H. J., Zehnder, A. J. B. & van der Meer, J. R. (2000). Characterization of a second *tfd* gene cluster for chlorophenol and chlorocatechol metabolism on plasmid pJP4 in *Ralstonia eutropha* JMP134(pJP4). *J Bacteriol* **182**, 4165–4172.
- Laemmli, C. M., Schönenberger, R., Suter, M., Zehnder, A. J. B. & van der Meer, J. R. (2002). TfdDII, one of the two chloromuconate cycloisomerases of *Ralstonia eutropha* JMP134(pJP4), cannot efficiently convert 2-chloro- *cis, cis* -muconate to *trans* -dienelactone to allow growth on 3-chlorobenzoate. *Arch Microbiol* **178**, 13–25.
- Leveau, J. H. J., König, F., Fuchslin, H., Werlen, C. & van der Meer, J. R. (1999). Dynamics of multigene expression during catabolic adaptation of *Ralstonia eutropha* JMP134(pJP4) to the herbicide 2,4-dichlorophenoxyacetate. *Mol Microbiol* **33**, 396–406.
- Matrubutham, U. & Harker, A. H. (1994). Analysis of duplicated gene sequences associated with *tfdR* and *tfdS* in *Alcaligenes eutrophus* JMP134. *J Bacteriol* **176**, 2348–2353.
- Padilla, L., Matus, V., Zenteno, P. & González, B. (2000). Degradation of 2,4,6-trichlorophenol via chlorohydroxyquinol in *Ralstonia eutropha* JMP134 and JMP222. *J Basic Microbiol* **40**, 243–249.
- Parales, R. E. & Harwood, C. S. (1993). Construction and use of a new broad-host-range *lacZ* transcriptional fusion vector, pHRP309, for gram – bacteria. *Gene* **133**, 23–30.
- Pérez-Pantoja, D., Guzmán, L., Manzano, M., Pieper, D. H. & González, B. (2000). Role of *tfdC_ID_IE_IF_I* and *tfdD_{II}C_{II}E_{II}F_{II}* gene modules in catabolism of 3-chlorobenzoate by *Ralstonia eutropha* JMP134(pJP4). *Appl Environ Microbiol* **66**, 1602–1608.
- Perkins, E. J., Gordon, M. P., Cáceres, O. & Lurquin, P. F. (1990). Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. *J Bacteriol* **172**, 2352–2359.
- Pieper, D. H., Reineke, W., Engesser, K.-H. & Knackmuss, H.-J.

- (1988). Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP134. *Arch Microbiol* **150**, 95–102.
- Pieper, D. H., Engesser, K.-H. & Knackmuss, H.-J. (1989). Regulation of catabolic pathways of phenoxyacetic acids and phenols in *Alcaligenes eutrophus* JMP134. *Arch Microbiol* **151**, 356–371.
- Pieper, D. H., Knackmuss, H.-J. & Timmis, K. N. (1993). Accumulation of 2-chloromuconate during metabolism of 3-chlorobenzoate by *Alcaligenes eutrophus* JMP134. *Appl Microbiol Biotechnol* **39**, 563–567.
- Plumeier, I., Pérez-Pantoja, D., Heim, S., González, B. & Pieper, D. H. (2002). Importance of different *tfd* genes for degradation of chloroaromatics by *Ralstonia eutropha* JMP134. *J Bacteriol* **184**, 4054–4064.
- Ramos, J. L., Stolz, A., Reineke, W. & Timmis, K. N. (1986). Altered effector specificities in regulators of gene expression: TOL plasmid *xylS* mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. *Proc Natl Acad Sci U S A* **83**, 8467–8471.
- Ravatt, R., Studer, S., Springael, D., Zehnder, A. J. B. & van der Meer, J. R. (1998). Chromosomal integration, tandem amplification, and deamplification in *Pseudomonas putida* F1, of a 105-kilobase genetic element containing the chlorocatechol degradative genes from *Pseudomonas* sp. strain B13. *J Bacteriol* **180**, 4360–4369.
- Reineke, W. (1998). Development of hybrid strains for the mineralization of chloroaromatics by patchwork assembly. *Annu Rev Microbiol* **52**, 287–331.
- Reineke, W. & Knackmuss, H.-J. (1978). Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. *Biochim Biophys Acta* **542**, 412–433.
- Reineke, W. & Knackmuss, H.-J. (1979). Construction of haloaromatics utilising bacteria. *Nature* **277**, 385–386.
- Reineke, W. & Knackmuss, H.-J. (1980). Hybrid pathway to chlorobenzoate metabolism in *Pseudomonas* sp. B13 derivatives. *J Bacteriol* **142**, 467–473.
- Reineke, W. & Knackmuss, H.-J. (1988). Microbial degradation of haloaromatics. *Annu Rev Microbiol* **42**, 263–287.
- Rojo, F., Pieper, D. H., Engesser, K.-H., Knackmuss, H.-J. & Timmis, K. N. (1987). Assemblage of *ortho* cleavage route for simultaneous degradation of chloro- and methylaromatics. *Science* **238**, 1395–1398.
- Sahasrabudhe, A. V. & Modi, V. V. (1991). Degradation of isomeric monochlorobenzoates and 2,4-dichlorophenoxyacetic acid by a constructed *Pseudomonas* sp. *Appl Microbiol Biotechnol* **34**, 556–557.
- Seibert, V., Stadler-Fritzsche, K. & Schlömann, M. (1993). Purification and characterization of maleylacetate reductase from *Alcaligenes eutrophus* JMP134(pJP4). *J Bacteriol* **175**, 6745–6754.
- Streber, W., Timmis, K. N. & Zenk, M. H. (1987). Analysis, cloning and high-level expression of 2,4-dichlorophenoxyacetate monooxygenase gene *tfdA* of *Alcaligenes eutrophus* JMP134. *J Bacteriol* **169**, 2950–2955.
- Trefault, N., Clément, P., Manzano, M., Pieper, D. H. & González, B. (2002). The copy number of the catabolic plasmid pJP4 affects growth of *Ralstonia eutropha* JMP134(pJP4) on 3-chlorobenzoate. *FEMS Microbiol Lett* **212**, 95–100.
- van der Meer, J. R., de Vos, W., Harayama, S. & Zehnder, A. J. B. (1992). Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol Rev* **56**, 677–694.
- Vollmer, M. D., Schell, U., Seibert, V., Lakner, S. & Schlömann, M. (1999). Substrate specificities of the chloromuconate cycloisomerases from *Pseudomonas* sp. B13, *Ralstonia eutropha* JMP134 and *Pseudomonas* sp. P51. *Appl Microbiol Biotechnol* **51**, 598–605.

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