Growth of the genetically engineered strain *Cupriavidus necator* RW112 with chlorobenzoates and technical chlorobiphenyls

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Cupriavidus necator (formerly *Ralstonia eutropha*) strain H850 is known to grow on biphenyl, and to co-oxidize congeners of polychlorinated biphenyls (PCBs). Using a Tn5-based minitransposon shuttle system and the TOL plasmid, the rational construction of hybrids of H850 was achieved by subsequent introduction of three distinct elements carrying 11 catabolic loci from three other biodegrading bacteria into the parent strain, finally yielding *C. necator* RW112. The new genetic elements introduced into H850 and its derivatives were *tcbRCDEF*, which encode the catabolic enzymes needed for chlorocatechol biodegradation under the control of a transcriptional regulator, followed by *cbdABC*, encoding a 2-halobenzoate dioxygenase, and *xylXYZ*, encoding a broad-spectrum toluate dioxygenase. The expression of the introduced genes was demonstrated by measuring the corresponding enzymic activities. The engineered strain RW112 gained the ability to grow on all isomeric monochlorobenzoates and 3,5-dichlorobenzoate, all monochlorobiphenyls, and 3,5-dichloro-, 2,3'-dichloro- and 2,4'-dichlorobiphenyl, without accumulation of chlorobenzoates. It also grew and utilized two commercial PCB formulations, Aroclor 1221 and Aroclor 1232, as sole carbon and energy sources for growth. This is the first report on the aerobic growth of a genetically improved bacterial strain at the expense of technical Aroclor mixtures.

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INTRODUCTION

Polychlorinated biphenyls (PCBs) represent highly persistent and toxic halo-organic compounds in the biosphere (Carpenter, 1998; Harris *et al.*, 1993); therefore, they are found on the priority pollutant lists of many countries. PCBs are not easily degraded by microbes, and thus have accumulated in the ecosphere. Highly chlorinated congeners can be subject to anaerobic bacterial dehalogenation (Tiedje *et al.*, 1993). However, anaerobic dehalogenation reactions on haloaliphatic and haloaromatic environmental contaminants proceed slowly due to the relatively low energy yield. They do, however, yield less-chlorinated PCBs, which can be more easily attacked by aerobic bacteria. The aerobic mineralization of oligo- or polyhalogenated aromatics is typically more rapid due to the much higher amount of energy theoretically available (Abramowicz, 1990; Higson, 1992; Potrawfke *et al.*, 1998b; Sander *et al.*, 1991). However, a new problem emerges in that some aerobic strains are only able to co-oxidize structurally related haloaromatics because they are not equipped with dehalogenating enzyme systems.

As a consequence, these strains encounter the problem of misrouting halogenated metabolites into unproductive catabolic pathways, forming toxic compounds, such as protoanemonin, or accumulating toxic halogenated dead-end products, such as halosalicylates, halocatechols and others (Bartels *et al.*, 1984; Blasco *et al.*, 1997; Davison *et al.*, 1999; Camara *et al.*, 2004; Pieper, 2005).

Although microbial communities are seemingly able to reduce the amount of PCBs in the environment (Abraham *et al.*, 2002), all attempts to enrich and isolate bacteria able to grow on commercial PCB mixtures under aerobic conditions have been unsuccessful to date. The aerobic mineralization of PCBs is restricted to monochlorinated and very few dichlorinated congeners. Bacteria capable of degrading monochlorobiphenyls and several dichlorobiphenyls have been obtained by direct isolation (Kim & Picardal, 2001), and by enhancing the natural genetic exchange between biphenyl- and chlorobenzoate-mineralizing species (Mokross *et al.*, 1990; Havel & Reineke, 1991; Adams *et al.*, 1992), and by applying plasmid-based genetic approaches (Havel & Reineke, 1993; Reineke, 1998; Hrywna *et al.*, 1999; Rodrigues *et al.*, 2001). Very recently, bacterial isolates have been reported to be able to aerobically utilize substrates that have hitherto not been seen to be

Abbreviation: PCB, polychlorinated biphenyl.

A supplementary table summarizing the data in Fig. 4 is available with the online version of this paper.

mineralized; these substrates include 2,6-dichloro-, 2,3,6-trichloro- and 2,4,6-trichlorobenzoate (Abraham *et al.*, 2005). These isolates may represent interesting organisms for the further improvement of PCB-degrading strains if they are shown to be able to degrade these halobenzoates in liquid culture also. However, none of these strains is able to grow on technical mixtures of PCBs.

Productive biodegradation of haloaromatic pollutants, such as many pesticides and their correspondingly halogenated precursors, including chlorobenzenes and chlorophenols, requires genetically well-organized and regulated catabolic pathways. The genetic and biochemical bases for the microbial degradation of PCBs have been summarized, as well as the strategies for the improvement of their bioremediation (Brenner *et al.*, 1994; de Lorenzo, 1994; Furukawa, 2000, 2003; Ohtsubo *et al.*, 2004). Here, we report a rational strategy for the construction of a recombinant bacterium capable of more efficient PCB degradation. The non-pathogenic soil bacterium *Cupriavidus necator* (formerly *Wautersia eutropha* > *Ralstonia eutropha* > *Alcaligenes eutrophus*) strain H850 was chosen as the host organism. This bacterial strain shows good survival in soil, and uses benzoate and biphenyl for growth. H850 cannot grow with chlorinated catechols, benzoates or biphenyls, but it co-oxidizes numerous PCBs (Bedard *et al.*, 1987; Bedard & Haberl, 1990; Van Dyke *et al.*, 1996). Therefore, the primary bottlenecks for growth on PCBs are defined by a benzoate dioxygenase of a narrow substrate spectrum, and the lack of an efficient 2-halobenzoate dioxygenase, a chlorocatechol pathway, and other catabolic sequences capable of dehalogenating intermediary haloaliphatic acids, before the carbon from PCBs can be channelled into Krebs cycle intermediates. In this report, we demonstrate that, when equipped with a novel cascade of dehalogenating elements, the biocatalyst grows with mono- and dichlorobenzoates, dichlorobiphenyls (monohalogenated on both aromatic rings), and also with technical Aroclor mixtures, the latter being a feature that has not been described so far.

METHODS

Strains, plasmids and growth conditions. Strain *C. necator* H850 was obtained from the patent culture collection of the US Department of Agriculture, Peoria, IL, USA. The strain was grown in mineral salts solution, under standard conditions (Bedard *et al.*, 1987; Sander *et al.*, 1991), with benzoate or biphenyl, at pH 7.2. The initial pH was raised to 7.5 when growing derivatives on chlorobenzoates or chlorobiphenyls (5 mM), and to 8.0 for growth on Aroclor 1221 and 1232, to compensate for acidification of the culture medium due to the release of protons, and to avoid phosphate-buffer concentrations above 50 mM. Biphenyl as a co-substrate was not added to cultures to be grown on specific congeners or Aroclors. Cultures (1 and 2 ml) of recombinant derivatives and individual chlorobiphenyls or PCB mixtures were grown in 15 ml PTFE-sealed glass tubes on an overhead spinner rotating at a speed of 10–50 r.p.m. at 28 °C. At the time points depicted in the figures, tubes were removed, and 5 µl was taken for the determination of c.f.u. by spreading aliquots of appropriate serial dilutions on solid

Luria–Bertani (LB) medium. Colonies were counted after 4 days incubation at 28 °C. At the same time points, 10 µl aliquots were taken for analytical purposes (HPLC).

Escherichia coli donor strain CC118λpir, harbouring the delivery vector on a suicide plasmid (Tn5-based delivery plasmid, Ap^r Km^r pUTkm; de Lorenzo & Timmis, 1994), with the catabolic inserts on pBJ4::4 and pBpc, and *E. coli* helper strain HB101, with pRK600 (Cm^r ColE1 RK2-Mob⁺ RK2-Tra⁺ derivative of pRK2013; Figurski & Helinski, 1979) for the three-parental mating, were grown in LB medium containing the appropriate antibiotics at 50 µg ml⁻¹, at 37 °C. The plasmids pBJ4::4 and pBpc were obtained from B. Jakobs and A. Lehning, respectively (German Research Centre for Biotechnology, Braunschweig, Germany). Plasmid pBJ4::4 (Jakobs, 1998), which carries genes *tcbRCDEF* (positions 637–6340, GenBank accession no. M57629) encoding the entire halocatechol pathway, and was obtained from plasmid pP51, which carries catabolic operons of the halobenzene-degrading *Pseudomonas* sp. strain P51 (van der Meer *et al.*, 1991). Plasmid pBpc (Lehning, 1998) harbours genes *cbdABC* (positions 288–3221, GenBank accession no. X79076), which encode a 2-halobenzoate dioxygenase from the 2-chlorobenzoate-degrading strain *Pseudomonas cepacia* 2CBS (Haak *et al.*, 1995). *Pseudomonas putida* mt-2, as a donor of genes *xylXYZ* (positions 463–3342, GenBank accession no. M64747), located within the catabolic toluene upper and lower pathway genes of the 40.317 kb TOL region of transposon Tn4653 of the TOL plasmid pWW0 (Williams & Murray, 1974; Greated *et al.*, 2002), was grown on 5 mM 3-methylbenzoate. Bacterial growth of donor and recipient strains was determined by measuring the OD₆₀₀ of 1 ml samples, or appropriate dilutions of the culture medium, in a Shimadzu UV-2100 spectrophotometer, or by enumeration of c.f.u. of generated hybrid strains in the PCB-degradation experiments.

Chemicals. Biphenyl was from Aldrich. Individual chlorobiphenyl congeners and Aroclor mixtures were from Promochem (Aroclor 1232, Lot no. N32SI). All other chemicals were of the highest purity commercially available.

Construction of hybrid strains. Using a previously described protocol (Klemba *et al.*, 2000), with minor modifications, the recipient strain *C. necator* H850 was mixed with the donor strain *E. coli* CC118λpir and *E. coli* HB101 helper strain, in a 1:1:1 ratio, and incubated on solid LB medium, in the absence of antibiotics, for 1 week. Aliquots were scraped from the surface of the agar medium, diluted, and plated directly onto solid mineral salts medium containing the target carbon source, which was 2-chlorobenzoate in the case of transfer of *cbdABC*, and 3-chlorobenzoate in case of *tcbRCDEF*. Colonies that appeared after incubation for about 3–4 weeks were subcultured on the same medium, then purified by passage on solid LB medium, and transferred to solid selective medium.

For the transfer of the TOL plasmid with the catabolic genes *xylXYZ*, the recipient strain was incubated overnight with *P. putida* mt-2 TOL on LB plates, and processed as described above, selecting for additional growth on either 4-chloro- or 3,5-dichlorobenzoate. Additional details for the construction of the catabolic inserts of the suicide vectors, according to a published procedure (de Lorenzo & Timmis, 1994), can be found elsewhere (Jakobs, 1998; Lehning, 1998; Klemba *et al.*, 2000). The resulting final hybrid, termed RW112, was used for further studies, and deposited in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), under accession number DSM 13439.

Enzyme assays. The harvest of bacterial cells, preparation of crude cell extracts, and determination of enzyme activities and protein content, were performed as reported previously (Potrawfke *et al.*, 1998b; Sander *et al.*, 1991). Enzyme activities were determined in

crude cell extracts at 25 °C by using a Shimadzu UV-2100 dual-beam spectrophotometer. Specific enzyme activities are expressed in $\mu\text{mol per min per mg protein}$. Specific rates of oxygen uptake were determined polarographically with a DW-1 liquid-phase oxygen electrode from Hansatech Instruments.

Analytical techniques. From 10 ml culture medium, residual chlorobiphenyls or PCB mixtures were extracted with hexane, and analysed by HPLC and/or GC. HPLC with UV detection on a Merck-Hitachi liquid chromatograph system was used to determine the relative concentration of PCB mixtures as a sum parameter, and to screen for polar metabolites. Separation of the PCB fraction from polar compounds, such as chlorobenzoates, was achieved on a $4 \times 125 \text{ mm}$ column, with $5 \mu\text{m}$ Lichrosphere 100 RP8 as the solid phase, and detection at 220 nm; the liquid phase was 80 % methanol in water (v/v) at pH 1.5 (phosphoric acid, 0.1 %). GC was performed with flame ionization detection, in order to include non-halogenated biphenyl, and it was used to determine the concentrations of individual congeners. PCB congeners were analysed on a Hewlett Packard 5890 series II gas chromatograph equipped with a $50 \text{ m} \times 0.2 \text{ mm}$ Hewlett Packard Ultra2 capillary column (thickness of film, 0.11 mm), and hydrogen as the carrier. The injector temperature was 250 °C, and that of the detector, 300 °C. The temperature programme started with 80 °C for 3 min, followed by a ramp to 290 °C at $6 \text{ }^\circ\text{C min}^{-1}$. The final temperature was kept constant for an additional 20 min. Peaks generated by either an electron capture detector or a flame ionization detector were identified by comparison with commercial standards and data reported in the literature (Frame *et al.*, 1996), which also served for the correction of incompletely dissolved signals. Chloride ion concentrations were determined by use of a chloride electrode within a flow-injection system developed by the Fraunhofer-Institut für Grenzflächen- und Bioverfahrenstechnik, Stuttgart, Germany.

RESULTS AND DISCUSSION

Rational construction and genetic stability of the PCB degrader

The construction of efficient PCB degraders was realized by stable chromosomal integration of the necessary additional catabolic genes. Using primers specific for the three vectors, the miniTn5-derived shuttle vectors and pWW0 were not detected in strain RW112 (data not shown). The transfer of plasmids carrying the required catabolic elements requires growth of recipient strains in the presence of antibiotics in order to maintain the desired genotype and phenotype. Such constructs, however, are not useful as field-application vectors in bioremediation. The generally applied selection for antibiotic resistance markers, even that of the transposon gene shuttle system, was also avoided because of the very high number of resistant transformants formed, from which only very few are able to productively express the new genes, and grow on the target substrates (see Klemmba *et al.*, 2000). Therefore, selection of new clones (genotypes) took place under the regimen of pressure for growth with the new substrates, ensuring expression of the desired phenotype. In this selection system, however, incubation times of about 1 month are necessary for positive colonies to appear.

For stable chromosomal introduction of genes *tcbRCDEF* and *cbdABC* carrying dehalogenating sequences, we used a minitransposon-based gene shuttle system derived from a

natural transposon system (de Lorenzo & Timmis, 1994). Such miniTn5-based systems can integrate with their IS-element-flanked regions upon transposition at single or multiple sites of the targeted bacterial chromosomes of several taxa of Gram-negative bacteria, and the information is expressed. For efficient production of new enzymes, and growth on new target compounds upon expression of new catabolic genes, the benefits of multiple copies have been discussed previously (Klemmba *et al.*, 2000). However, a more important prerequisite for productive expression of the newly integrated genes seems to be their integration behind an appropriate promoter of the host cell chromosome, preferably downstream of a constitutively expressed gene or operon. Our efforts to generate hybrids based on constructs with fused promoters, either to be constitutive or externally triggered, such as *psaI*, *ptrc* and several others, have not resulted in clones that could productively degrade the new target compounds, and this might explain our poor rate of success in the construction of novel and efficient catabolic capabilities in past years. It is probable that the recipient cell may require a certain period of time (numerous generations), and also some regulatory flexibility, in order to accommodate the desired phenotype into its metabolism, and it is further possible that, by forcing the expression under the above-specified promoters, this flexibility is lost.

The native catechol-degradation pathway of H850, expressed during growth on benzoate, biphenyl, and probably other non-halogenated substrates, cannot mineralize the halogenated derivatives. Since chlorocatechols represent key catabolic intermediates of a huge number of haloaromatic compounds found to be biodegradable by aerobic bacterial strains, expansion of the catechol-degradative capacity to halogenated derivatives was seen as the first objective. To achieve this, we first introduced into the parent strain H850 the key genes of a chlorocatechol pathway that efficiently metabolizes chlorocatechols other than monochlorocatechols and 3,5-dichlorocatechol (van der Meer *et al.*, 1991; Fig. 1). These genes, *tcbRCDEF*, enable degradation of catechols containing up to four halogen substituents, and are predicted to confer the ability to degrade monochlorocatechols, and up to 3,4,6-trichloro-, and even tetrachlorocatechol (van der Meer *et al.*, 1991; Potrawfke *et al.*, 2001). After introduction of the mini-transposon shuttle vector, the first colonies took about 4 weeks to appear on 3-chlorobenzoate plates. It should be noted here that the benzoate dioxygenase of H850, like that of many other strains, is able to transform 3-chlorobenzoate to 4-chlorocatechol, but is not able to transform other isomers of chlorobenzoates. A single transconjugant obtained upon incubation in the presence of 5 mM 3-chlorobenzoate as the selective substrate was selected, and termed strain RW110. RW110 could also grow on 3-chlorobiphenyl and 2,3'-dichlorobiphenyl (Table 1). It is a common feature of initial biphenyl dioxygenases that they eliminate a first chlorine atom gratuitously from position 2 when the other aromatic ring is also chlorinated, but

Fig. 1. General catabolic pathways for the degradation of (halogenated) biphenyls. Left, enzymic steps for the degradation of the non-halogenated biphenyl are depicted as inferred from published data of other biphenyl-degrading bacteria. Note that catechol could also be degraded through a *meta*-fission pathway not shown here. The substrate range for the catabolic enzymes may vary with respect to halogenated derivatives. Right, pathway for degradation of chlorinated derivatives. Co-metabolism of PCBs by strain H850 results in the accumulation of chlorobenzoates. The introduced genes and their additional catabolic (dehalogenating) functions permit the engineered strains *C. necator* RW110, RW111 and RW112 to grow on chlorinated compounds, as shown. Note that dehalogenation of 2-halobenzoate(s) (RW111 and RW112) circumvents the formation of the 1,2-dihydrodiol of benzoate.

generally the non-halogenated ring system is the preferred site of initial attack by biphenyl 2,3-dioxygenase (Fig. 1).

Despite its enhanced chlorocatechol degradative capacity, strain RW110 was not able to grow on all tested monochlorinated benzoates (Table 1). This was predicted

to reflect limited capacity to generate the corresponding catechols from chlorinated benzoates. In order to overcome the catabolic bottleneck for chlorobenzoate halogenated in position 2, genes *cbdABC*, encoding the 2-halobenzoate dioxygenase system of a *Burkholderia cepacia* strain (Haak *et al.*, 1995), were introduced by the technique described

Table 1. Growth of *C. necator* H850 and derivatives on chlorobenzoates and chlorobiphenyls

Growth of 850 and derivative strains (genes introduced are in parentheses) is shown: +, growth; –, no growth. Values in parentheses are the doubling times (h) for growth on the substrate in liquid culture. The sequence of construction of derivative strains from H850 was as follows: H850→RW110→RW111→RW112.

Compound	Growth			
	H850	RW110 (<i>tcbRCDEF</i>)	RW111 (<i>tcbRCDEF cbdABC</i>)	RW112 (<i>tcbRCDF cbdABC xylXYZ</i>)
Benzoate	+	+	+	+
2-Chlorobenzoate	–	–	+	+
3-Chlorobenzoate	–	+	+	+
4-Chlorobenzoate	–	–	–	+
3,5-Dichlorobenzoate	–	–	–	+
2,4-Dichlorobenzoate	–	–	–	+
Biphenyl	+	+	+	+
2-Chlorobiphenyl	–	–	+	+
3-Chlorobiphenyl	–	+	+	+
4-Chlorobiphenyl	–	–	–	+
3,5-Dichlorobiphenyl	–	–	–	+
2,2'-Dichlorobiphenyl	–	–	+	+
2,3'-Dichlorobiphenyl	–	+	+	+
2,4'-Dichlorobiphenyl	–	–	–	+
Aroclor 1221	–	–	–	+
Aroclor 1232	–	–	–	+

*On 2,4-dichlorobenzoate, RW112 showed growth on solid medium only.

above into RW110, using 2-chlorobenzoate for selection. The time needed for the appearance of the first colonies was similar to that for recovery of RW110, i.e. approximately 4 weeks. We were not able to determine exactly and reproducibly the rates for the conjugal transfer because of the long incubation periods needed for appearance of clones growing on the new substrates used for selection. The rates were roughly between 10^{-9} and 10^{-11} per donor cell, and thus around two orders of magnitude lower when compared with the frequencies reported by Klemba *et al.* (2000) when selecting directly for a new substrate to be degraded (10^{-8} – 10^{-9}), rather than the kanamycin-resistance marker (10^{-6}). A single transconjugant strain was selected, and termed RW111. This strain was found to grow on 2-chlorobenzoate and additional chlorobiphenyls, as shown in Table 1.

Neither of the (at least) two benzoate dioxygenases present in the recombinant strain RW111 transformed 4-chloro- or 3,5-dichlorobenzoate, which are the presumed metabolites of the correspondingly chlorinated biphenyls. Consequently, the relatively narrow substrate specificities of the benzoate dioxygenases represent a bottleneck for the metabolism of many bulky alkyl-, chloro- and bromo-derivatives. Many benzoate dioxygenases transform *meta*-substituted benzoates, such as 3-methyl- or 3-chlorobenzoate, to the corresponding catechols via the intermediary dihydrodiols of the benzoates; the *cis*-dihydrodiol dioxygenases generally exhibit a relaxed substrate specificity. But this type of biotransformation by benzoate dioxygenases does not apply

to *para*- or higher-substituted benzoates, and this deficiency was circumvented by the conjugative integration of the broad-spectrum toluate (methylbenzoate) dioxygenase system from the TOL plasmid of the soil bacterium *P. putida* mt-2. The genes are encoded by *xylXYZ* (Harayama & Rekik, 1990), and have previously been used for the same purpose (Potrawfke *et al.*, 1998a; Reineke, 1998). Transfer of the *xylXYZ* genes located on this TOL plasmid to RW111 by a simple conjugation experiment yielded strain RW112. The conjugal transfer occurred at a relatively high frequency of about 5×10^{-6} to 5.6×10^{-6} per recipient cell. For selection of transconjugants, 4-chlorobenzoate was applied as the selective carbon source in solid medium. RW112 grew also with 3,5-dichlorobenzoate and the chlorobiphenyls listed in Table 1. For growth of the new hybrid in liquid culture, an additional adaptation period of about 2 weeks was necessary. With 2,4-dichlorobenzoate as substrate, only a minute amount of growth was observed on solid medium. Doubling times observed for growth with chlorobenzoates were between four and five times higher than with benzoate. In order to check for the genetic stability of the constructs, cells were grown for at least 100 generations in the absence of selective conditions in liquid LB medium. An aliquot was taken, diluted appropriately, and plated on solid LB medium. After 2 days of incubation at 28 °C, 149 colonies were selected, and transferred to solid mineral salts medium containing 5 mM 3-methyl-, 2-chloro-, 3-chloro- or 4-chlorobenzoate, or biphenyl. All organisms grew well, indicating the overall stability of the integrated genetic elements, including the

capacity to grow stably with biphenyl. Fifty-six per cent of the colonies growing on 4-chlorobenzoate showed some yellowish colouring around the growth zone, indicative of the unwanted and unproductive *meta*-cleavage of 4-chlorocatechol to toxic intermediates by the TOL-plasmid-encoded catechol 2,3-dioxygenase. Finally, we selected a clone that did not express this detrimental *meta*-cleavage activity (XylE, the gene was co-transferred with *xylXYZ* on Tn4653 of the TOL plasmid), which was probably due to a spontaneous mutation (Reineke *et al.*, 1982). This RW112 clone was able to grow with 3-methylbenzoate on solid medium, but cells turned brownish, providing additional evidence that the undesired *meta* pathway from the TOL plasmid was no longer fully functional.

Growth on 2,4'-dichlorobiphenyl and Aroclors

The derivative RW112 almost completely degraded 5 mM 2,4'-dichlorobiphenyl, as a model compound, and released about 10 mM chloride. Only minute amounts of 4-chlorobenzoate, and no 2-chlorobenzoate or other metabolites, were detected by HPLC (Fig. 2). RW112 also grew with the chlorobiphenyls and chlorobenzoates listed in Table 1. This strain degraded commercial Aroclor 1221 almost completely (Aroclor 1221 contains about 34 mol% biphenyl, 60 mol% monochlorobiphenyls, 5 mol% dichlorobiphenyls and 0.3 mol% trichlorobiphenyls; total chlorine content is approximately 22 % by weight). The doubling time was about 2.8 h; >98 % of all chlorobiphenyl congeners showed significant depletion when the spent

medium was analysed, and an estimated 96 % total chlorine was released (specific data from end-point determinations not shown).

Strain RW112 also grew well (Fig. 3) on Aroclor 1232, which is a technical PCB mixture containing around 32 % chlorine by weight: approximately 5 % biphenyl, 30 % monochlorobiphenyls, 22 % dichlorobiphenyls, 22 % trichlorobiphenyls, 15 % tetrachlorobiphenyls and 5 % pentachlorobiphenyls (see also Frame *et al.*, 1996). The new hybrid biocatalyst increased its cell number by about three orders of magnitude within 2 days, with an initial doubling time of about 3.4 h. Growth was associated with consumption of about 42 % of the total Aroclor 1232 PCBs, from an initial amount corresponding to a concentration of about 5.1–5.2 mM in the culture medium. Another 40 % of residual PCB congeners were degraded within an additional 3 days. A relatively high turbidity of the culture broth (OD_{600} 1.1) was reached, indicative of the consumption of the equivalent of about 3 mM biphenyl carbon backbone. After the first week, about 80 % of the degradable PCBs were converted. In the second week, the viable count declined rapidly, while the turbidity of the culture broth remained nearly constant (not shown in the figure; by end-point determination only), probably due to a balanced growth and death of cells. Apparently, only a small amount of lysis occurred in the stationary and death phases. The end-point determination of released chloride ions was 9.4 mM. Although cells from the spent culture were able to reinitiate this process in a new subculture, cells from the first week of culture were considerably more viable, and were able to restart the process without any significant lag phase. In Fig. 4(a)–(d), the depletion of individual PCB congeners of Aroclor 1232 is shown over the incubation period of 2 weeks. It is obvious that the non-chlorinated biphenyl (>4.7 % by weight in the batch of Aroclor 1232), of which a hydrophilic degradation product is known to act as the inductor of the catabolic

Fig. 2. Almost complete degradation of the PCB model compound 2,4'-dichlorobiphenyl (2,4'-DCBPH; ●), by the hybrid biocatalyst *C. necator* RW112, with concomitant formation of cellular biomass, given as c.f.u. (○), and release of chloride ions (■). In the mid-growth phase, up to 0.24 mM 4-chlorobenzoate (▽) was excreted, but then taken up and degraded in the late growth phase; 2-chlorobenzoate or other metabolites were not detected by HPLC.

Fig. 3. Growth of metabolically engineered *C. necator* RW112, shown as c.f.u. (□), at the expense of technical Aroclor 1232 (●). The concentration of substrate initially present corresponded to approximately 5 mM of the biphenyl carbon backbone, estimated on the basis of chlorobiphenyl congener distribution patterns, as given in the legend for Fig. 4.

biphenyl pathway (Ohtsubo *et al.*, 2000), was entirely depleted as the first substrate within about 1.3 days. The rate of further utilization of chlorobiphenyls then strongly depended on the degree and position of chlorine substitution, which was associated with their solubility in water, and their corresponding bioavailability and docking specificity with the corresponding enzyme. In particular, it was evident that the monochlorobiphenyls were completely depleted within about 3 days, the dichlorobiphenyls were depleted to between 20 and 40 % of their original levels, and most tri- and tetrachlorinated biphenyls were depleted to between 40 and 80 % of their original levels. Data from Fig. 4 are also summarized in supplementary Table S1.

The less effective degradation of higher chlorinated congeners is possibly due to the inactivation of catabolic enzymes through accumulation of toxic intermediates (Blasco *et al.*, 1997; Camara *et al.*, 2004; Bartels *et al.*, 1984). The observed drastic decrease in active cells may be associated with a failure of sufficient production of upper-pathway enzymes (the exact half-time is not known), due to a lack of the probable inducer 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate. This compound was shown to trigger the regulating *bphS* element in a *Pseudomonas* sp. (Ohtsubo *et al.*, 2000), although its presence has not yet been confirmed in the parent H850 strain or its derivatives.

Fig. 4. Time course of degradation for the individual congeners of technical Aroclor 1232 by the hybrid strain *C. necator* RW112. (a) Depletion of biphenyl, monochloro- and dichlorobiphenyls [○, biphenyl (4.7 %); ●, 2-chlorobiphenyl (17.5 %); □, 3-chlorobiphenyl (1.8 %); ■, 4-chlorobiphenyl (11.1 %); △, 2,2'-dichlorobiphenyl (5.2 %); ▲, 2,5-dichlorobiphenyl (1.8 %); ▽, 2,3'-dichlorobiphenyl (2.3 %); ▼, 2,4'-dichlorobiphenyl (10.7 %); ◇, 3,4'-dichlorobiphenyl (0.8 %); ◆, 4,4'-di-2,2',5-trichlorobiphenyl (3.2/4.8 %)]. (b) Depletion of trichlorobiphenyls [○, 2,2',6-trichlorobiphenyl (0.8 %); ●, 2,2',4-trichlorobiphenyl (3.7 %); □, 2,3',6-trichlorobiphenyl (0.8 %); ■, 2,2',3-trichlorobiphenyl (2.3 %); △, 2,3',5-trichlorobiphenyl (0.8 %); ▲, 2,3',4-trichlorobiphenyl (0.5 %); ▽, 2,4',5/2,4,4'-trichlorobiphenyl (4.1/4.0 %); ▼, 2',3,4-trichlorobiphenyl (2.7 %); ◇, 2,3,4'-trichlorobiphenyl (1.4 %); ◆, 3,4,4'-trichlorobiphenyl (1.2 %)]. (c) Depletion of tetrachlorobiphenyls [○, 2,2',3,6'-tetrachlorobiphenyl (0.8 %); ●, 2,2',5,5'-tetrachlorobiphenyl (1.6 %); □, 2,2',4,5'-tetrachlorobiphenyl (1.4 %); ■, 2,2',4,4'-/2,2',4,5-tetrachlorobiphenyl (0.5/0.6 %); △, 2,2',3,5'-tetrachlorobiphenyl (1.6 %); ▲, 2,2',3,6-tetrachlorobiphenyl (2.8 %); ▽, 2,3,4',6-tetrachlorobiphenyl (1.6 %); ▼, 2,2',3,3'-tetrachlorobiphenyl (0.8 %); ◇, 2,4,4',5-tetrachlorobiphenyl (1.0 %); ◆, 2,3',4',5-tetrachlorobiphenyl (1.7 %)]. (d) Depletion of some residual tetra- and the pentachlorobiphenyls [○, 2,3',4,4'-tetrachlorobiphenyl (1.7 %); ●, 2,3,4,4'-tetrachlorobiphenyl (0.9 %); □, 2,2',3,3',6-pentachlorobiphenyl (0.4 %); ■, 2,2',4,5,5'-pentachlorobiphenyl (0.5 %); △, 2,2',3',4,5-pentachlorobiphenyl (0.5 %); ▲, 2,2',3,4,5'-pentachlorobiphenyl (0.4 %); ▽, 2,3,3',4',6-pentachlorobiphenyl (0.5 %); ▼, 2,3',4,4',5-pentachlorobiphenyl (0.5 %); ◇, 2,3,3',4,4'-pentachlorobiphenyl (0.5 %)].

This inducer can only be generated from non-halogenated biphenyl during its catabolic breakdown, but we have to assume that chlorinated derivatives can also act as the inducing agent, since our constructs were shown to grow on chlorinated biphenyls.

Expression of novel catabolic genes as determined by activities in RW112

Enzyme activities of the benzoate/toluene dioxygenase(s) were determined with an oxygen electrode, whole cells, and (chloro-)benzoates as the substrates, because we were unable to follow the NAD(P)H consuming reaction of these dioxygenases in cell-free extracts. The obtained oxygen-uptake rates for the oxidation of the benzoates, possibly include those for the oxidation of subsequently formed catechols. The parent strain H850 oxidized 3-chlorobenzoate only, with a relative rate of 24 % compared with benzoate, when grown on benzoate or biphenyl. Other isomers, such as 2-chloro- and 4-chlorobenzoate, and isomeric dichlorobenzoates, were not oxidized at all. In contrast to the parent strain H850, RW112 showed significant oxygen-uptake rates with a range of chlorinated substrates. Relative rates for RW112 were: 100 % with benzoate, 4.8 % with 2-chlorobenzoate, 67 % with 3-chlorobenzoate, 39 % with 4-chlorobenzoate, and 39 % with 3,5-dichlorobenzoate, when pre-grown on 2-chloro- or 4-chlorobenzoate. 2,4-Dichloro-, 2,5-dichloro-, and 2,6-dichlorobenzoate were not oxidized at detectable rates. Although the relative rate for the oxidation of 2-chlorobenzoate appeared relatively low with whole-cell suspensions, the doubling time on this substrate (Table 1) was similar to those on the other chlorobenzoates. Further, the doubling time on 2,4'-dichlorobiphenyl of only 2.8 h

clearly reflects that a transport system for (halo-)benzoates, especially for those substituted in position 2, represents a significant rate-limiting factor for the import and oxidation of the relatively polar benzoates. An ATP-dependent transporter system of a *Pseudomonas huttiensis* strain, specific for 2-halobenzoates, has been characterized recently (Yuroff *et al.*, 2003). Doubling times during growth on the new substrates, and oxygen-uptake rates for the new compounds oxidized, underpin the clear evidence for the expression of the newly introduced genes *cbdABC* and *xylXYZ*.

In Table 2, the catabolic enzyme activities and their potentially coding genes for the parent strain H850 and the final derivative RW112 are listed. When grown on biphenyl or benzoate, minute amounts of *meta*-cleavage activity for catechol were detected, obviously due to the *meta* activity originating from 2,3-dihydroxybiphenyl dioxygenase. Both catechol 1,2-dioxygenase and muconate cycloisomerase activities of a catechol *ortho*-cleavage pathway were present in strain H850 when growing on benzoate and biphenyl, respectively. However, the data clearly show that in RW112 the expansion of the substrate range to include chlorinated derivatives is positively correlated with the introduction of the relevant and inducible *tcb* genes. Catechol 2,3-dioxygenase activities were just above the limit of detection; no characteristic yellow colour was generated when catechol or 4-chlorocatechol was added to cell-free extracts or cell suspensions. Dienelactone hydrolase activities were not present in parent H850, and only minute amounts (background) of activity for maleylacetate reductase were detected. The 3-chlorobenzoate-grown RW112, however, showed high activities of all enzymes encoded by introduced genes *tcbRCDEF*, and continued to show

Table 2. Specific enzyme activities of the parent *C. necator* strain H850, and its derivative RW112

Activity data represent means of two experiments (SD < 12 % in all experiments). For each strain tested, the gene(s) induced and growth substrate are given.

Enzyme and substrate	Specific activity [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]		
	H850 <i>cat</i> benzoate/biphenyl	RW112 <i>cat tcbRCDEF</i> benzoate	RW112 <i>tcbRCDEF</i> 3-chlorobenzoate
Catechol 2,3-dioxygenase			
Catechol	27.6	21.0	19.6
Catechol 1,2-dioxygenase			
Catechol	474.6	243.1	543.6
3-Chlorocatechol	< 1.0	5.5	129.2
4-Chlorocatechol	18.5	46.8	251.0
Muconate cycloisomerase			
Muconate	158.6	71.9	86.1
2-Chloromuconate	< 1.0	3.7	35.5
Dienelactone hydrolase			
<i>cis</i> -Dienelactone	< 1.0	78.4	233.6
Maleylacetate reductase			
Maleylacetate	15.8	61.0	1109.6

significant activity when grown on benzoate, implicating some low-level co-expression of the genes encoding the enzymes of both the cat (catechol) and chlorocatechol (TcbRCDEF) pathways.

Conclusions

Genetic modifications have been suggested in the past as a generic solution to improve existing bacterial biocatalysts, not only for PCB degradation (Brenner *et al.*, 1994; de Lorenzo, 1994; Reineke, 1998). A research article (Haro & de Lorenzo, 2001) and a review article (Pieper & Reineke, 2000) have highlighted that little progress has been made with regard to these goals: introduced genes may be expressed, but growth at the expense of the targeted new substrate(s) has not always been achieved. Here, we have been able to demonstrate that it is indeed possible to design and produce genetically modified organisms with truly novel capabilities, in this case growth on technical PCB mixtures. With regard to our results presented here, further optimization of the initial biphenyl dioxygenase of our hybrid strain towards 4,4'-substituted PCBs and recalcitrant 2,6-dichlorobenzoates, and the correspondingly substituted biphenyls, is needed for the improvement of the performance of the rationally constructed hybrid strain in the laboratory and, later on, in the field. Advances have recently been reported in exchanging more productive variants for the catalytic α subunit of biphenyl dioxygenases (Kumamaru *et al.*, 1998; Barriault *et al.*, 2002). The introduction of more genetic segments encoding additional dehalogenating reactions from the lower, not yet elucidated, chlorocatechol pathway of *Pseudomonas chlororaphis* RW71, which mineralizes the potential pathway intermediate tetrachlorocatechol (Potrawfke *et al.*, 1998b), would be of some interest. The constitutive expression of catabolic genes of the upper biphenyl pathway could also further improve PCB degradation. Additionally, dehalogenating enzyme activities for the mineralization of the halogenated C-5 units obtained upon the action of HOPDA hydrolase on the side chains could efficiently contribute to the construction of what Furukawa (2003) called a 'superbug'.

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