A linear megaplasmid, p1CP, carrying the genes for chlorocatechol catabolism of *Rhodococcus opacus* 1CP

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The Gram-positive actinobacterium *Rhodococcus opacus* 1CP is able to utilize several (chloro)aromatic compounds as sole carbon sources, and gene clusters for various catabolic enzymes and pathways have previously been identified. Pulsed-field gel electrophoresis indicates the occurrence of a 740 kb megaplasmid, designated p1CP. Linear topology and the presence of covalently bound proteins were shown by the unchanged electrophoretic mobility after S1 nuclease treatment and by the immobility of the native plasmid during non-denaturing agarose gel electrophoresis, respectively. Sequence comparisons of both termini revealed a perfect 13 bp terminal inverted repeat (TIR) as part of an imperfect 583/587 bp TIR, as well as two copies of the highly conserved centre (GCTXCGC) of a palindromic motif. An initial restriction analysis of p1CP was performed. By means of PCR and hybridization techniques, p1CP was screened for several genes encoding enzymes of (chloro)aromatic degradation. A single maleylacetate reductase gene *macA*, the *clc* gene cluster for 4-chloro-/3,5-dichlorocatechol degradation, and the *clc2* gene cluster for 3-chlorocatechol degradation were found on p1CP whereas the *cat* and *pca* gene clusters for the catechol and the protocatechuate pathways, respectively, were not. Prolonged cultivation of the wild-type strain 1CP under non-selective conditions led to the isolation of the *clc*- and *clc2*-deficient mutants 1CP.01 and 1CP.02 harbouring the shortened plasmid variants p1CP.01 (500 kb) and p1CP.02 (400 kb).

INTRODUCTION

Rhodococci are metabolically versatile organisms which play an increasing role in biotransformations and the degradation of toxic compounds. *Rhodococcus opacus* 1CP, a Gram-positive nocardioform soil bacterium, is able to utilize, among other substrates, benzoate, 4-hydroxybenzoate, 4-chlorophenol and 2,4-dichlorophenol as sole sources of carbon and energy (Gorlatov et al., 1989). Even 2-chlorophenol and 3-chlorobenzoate are mineralized by a mutant of strain 1CP, which was selected after prolonged adaptation (Moiseeva et al., 1999). Whereas the non-chlorinated aromatic compounds are degraded via the catechol or the protocatechuate branch of the 3-oxoadipate pathway, encoded by *cat* and *pca* genes, respectively (Eulberg et al., 1997, 1998b), chlorophenols and 3-chlorobenzoate are catabolized via the 4-chloro-/3,5-dichlorocatechol branch or the 3-chlorocatechol branch, which are encoded by the *clc* and the *clc2* gene clusters, respectively (Eulberg et al., 1998a; Moiseeva et al., 2002). While the *clcBRAD* cluster includes genes for a chlorocatechol 1,2-dioxygenase, a chloromuconate cycloisomerase, a dienelactone hydrolase, and a presumed regulator, the *clc2* cluster is unusual in comprising a 5-chloromuconolactone dehalogenase gene in addition to those for dioxygenase, cycloisomerase and dienelactone hydrolase. A separate maleylacetate reductase gene (*macA*) may also be involved (Seibert et al., 1998).

The enzymes of chlorocatechol pathways of proteobacteria are often encoded by circular catabolic plasmids such as pJP4 of *Ralstonia eutropha* JMP134 (Don & Pemberton, 1985), pP51 of *Pseudomonas* sp. P51 (van der Meer et al., 1991a, b), pAC27 of *Pseudomonas putida* AC866 (Ghosal...
et al., 1985), and pEST4011 of P. putida EST4021 (Mae et al., 1993), all of which have been characterized in detail.

Plasmids of rhodococci, which, like their Streptomyces counterparts often possess linear topology, frequently encode catabolic genes, enabling their hosts either to grow with unusual carbon and energy sources such as CO₂/ hydrogen (Kalkus et al., 1990), (chloro)biphenyl (Kosono et al., 1997; Masai et al., 1997), and isopropylbenzene (Dabrock et al., 1994; Stecker et al., 2003) or to just convert such compounds as was shown for trichloroethene (Dabrock et al., 1994; Saeki et al., 1999). In addition, linear plasmids of rhodococci were shown to harbour genes for resistance to thallium (Kalkus et al., 1990) and arsenic (Dabrock et al., 1994; Stecker et al., 2003), as well as genes for phytopathogenicity (Maes et al., 2001).

The high similarities between some genes and gene clusters, located on linear plasmids of different Rhodococcus species, suggest that they may have been distributed in this genus via horizontal gene transfer. Thus, similar bphC genes, expressing 2,3-dihydroxybiphenyl dioxygenase activity, were found to be plasmid encoded in Rhodococcus erythropolis TA421 and in Rhodococcus globularus P6 (Kosono et al., 1997). Extremely high ratios of identical positions (96–100%) were found between the deduced catabolic TA421 and in polis Rhodococcus were found to be plasmid encoded in

At present the number of identified linear plasmids in rhodococci is still limited and characterization has usually been restricted to the determination of their termini and of special phenotypic properties. The only and very recently available complete sequence is that of pBD2 (210 kb) of the isopropylbenzene degrader R. erythropolis strain BD2 (Stecker et al., 2003). On plasmids of rhodococci or on other linear plasmids of actinobacteria, chlorocatechol catabolic genes have so far not been identified.

The investigation reported in the present paper aimed at elucidating whether the chlorophenol-degrading R. opacus 1CP carries a linear plasmid and if so, to investigate the localization of chlorocatechol catabolic and other degradative genes on it and to determine the basic properties of the plasmid. Some of the results have been reported previously in a preliminary communication (Eulberg et al., 1998c).

METHODS

Bacterial strains, plasmids and culture conditions. Wild-type strain Rhodococcus opacus 1CP (Gorlatov et al., 1989) as well as its derivatives 1CP.01 and 1CP.02, which carry the shortened plasmid variants p1CP.01 and p1CP.02, were usually grown at 30°C on mineral media (Dorn et al., 1974) with 50 mM phosphate buffer (pH 7.3), containing, unless mentioned otherwise, 5 mM benzoate as the sole energy and carbon source, and, for solid media, 1.5% agar. For some experiments, R. opacus strains were grown on complex media (see below).

Escherichia coli DH5α was obtained from Gibco-BRL and was grown aerobically with constant shaking (120 r.p.m.) at 37°C in baffled Erlemeyer flasks with dYT medium (1·6% tryptone, 1% yeast extract, 0·5% NaCl). When appropriate for selection, ampicillin was added to a final concentration of 100 µg ml⁻¹.

The plasmids used in this study are listed in Table 1.

Detection of plasmid p1CP and its derivatives in R. opacus 1CP. For detection of extrachromosomal DNA, a modified version

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1CP</td>
<td>740 kb linear plasmid of wild-type R. opacus 1CP</td>
<td>This study</td>
</tr>
<tr>
<td>p1CP.01</td>
<td>500 kb linear plasmid of mutant R. opacus 1CP.01 lacking both clc and clc2 gene cluster</td>
<td>This study</td>
</tr>
<tr>
<td>p1CP.02</td>
<td>400 kb linear plasmid of mutant R. opacus 1CP.02 lacking both clc and clc2 gene cluster</td>
<td>This study</td>
</tr>
<tr>
<td>p1CP-J</td>
<td>4-08 kb SstI/EcoRV fragment of p1CP right end in pBluescript II SK(+)</td>
<td>This study</td>
</tr>
<tr>
<td>p1CP-S</td>
<td>1-56 kb SstI/EcoRV fragment of p1CP left end in pBluescript II SK(+)</td>
<td>This study</td>
</tr>
<tr>
<td>pRER1</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 4 kb BamHI fragment of R. opacus 1CP DNA carrying catA, catB and catC in pBluescript II KSI(+)</td>
<td>Eulberg et al. (1997)</td>
</tr>
<tr>
<td>pRER3</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 3-6 kb EcoRI fragment of R. opacus 1CP DNA carrying pcaI&lt;sup&gt;I&lt;/sup&gt;, pcaG, pcaB and pcaL&lt;sup&gt;I&lt;/sup&gt; in pBluescript II KSI(+)</td>
<td>Eulberg et al. (1998b)</td>
</tr>
<tr>
<td>pRER7</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; ~3-7 kb EcoRI fragment of R. opacus 1CP DNA carrying clcB in pBluescript II KSI(+)</td>
<td>Eulberg et al. (1998a)</td>
</tr>
<tr>
<td>pMARRE0</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;; 0·982 kb PCR product with a fragment of macA in the EcoRV site of pBluescript II SK(+)</td>
<td>Seibert et al. (1998)</td>
</tr>
<tr>
<td>pHG201-RSal</td>
<td>2·3 kb SalI fragment of the right end of pHG201 in pBluescript SKM13+</td>
<td>Kalkus et al. (1998)</td>
</tr>
</tbody>
</table>
of the protocol of Kalkus et al. (1990) was used. R. opacus 1CP was cultivated at 30 °C in Luria–Bertani (LB)-sucrose-glycine medium [1·8% (w/v) sucrose, 1·5% (w/v) glycine in LB]. When the cultures had reached an OD₆₀₀ of about 0·7, samples of between 1·5 and 3 ml were harvested by centrifugation and resuspended in 100 μl EET buffer [10 mM Tris/HCl (pH 8), 0·1 M EDTA (pH 8), 10 mM EGTA (pH 8)]. To avoid shearing of the high-molecular-mass DNA, the cells were mixed with equal volumes of low-melting-point agarose [2% (w/v) in H₂O] at 45 °C. The agaro-cell mixture was poured into moulds of approximately 1 x 2 x 4 mm in size, and allowed to solidify. The agaro plugs were incubated for 1·5–12 h at 37 °C with 0·5 mg lysoyzyme ml⁻¹, 0·6 mg sodium N-lauroyl-sarcosine ml⁻¹ in EET, followed by an overnight incubation at 55 °C with 0·1 mg proteinase K ml⁻¹, 10 mg SDS ml⁻¹ in EET. The plugs were rinsed three times with TE10.1, pH 8 (10 mM Tris/HCl, 1 mM EDTA) and stored in ES buffer (pH 8·0), sodium N-lauroylsarcosine at 4 °C until use.

**Determination of linearity.** In order to determine whether the presumed extrachromosomal element was in the closed-circular supercoiled form, DNA-containing agaro plugs were additionally treated with S¹ nuclease (Barton et al., 1995). The gel plugs were washed twice (15 min; room temperature) with freshly prepared 1 mM PMSF in TE10.1 (pH 7·5) to inactivate the proteinase K, and then soaked twice (15 min; 37 °C) in 1 ml 10 mM Tris/HCl (pH 7·6). Single agaro plugs were incubated (30 min; 37 °C) with S¹ nuclease in 200 μl of a solution containing 1 U Aspergillus oryzae S¹ nuclease (Gibco), 50 mM NaCl, 30 mM sodium acetate (pH 4·6), 5 mM ZnSO₄ and 5% (v/v) glycerol. The reaction was stopped by transferring the slices to 100 μl ES buffer on ice.

For the isolation of native megaplasmid and to detect covalently linked proteins, which are a general property of actinobacterial linear plasmids (Sakuguchi, 1990), a plasmid preparation was made as described above but omitting the proteinase K treatment. Genomic DNA in which covalently linked proteins remained attached to plasmid DNA was obtained as described above but omitting the proteinase K treatment. Genomic DNA or the inserts of plasmids pRER1, pRER3, pRER7 and pMARRE0 (Table 1) as template. All probes were hybridized under high-stringency conditions as described in the Boehringer manual together with dot blots of genomic DNA or p1CP DNA, or with Southern blots of p1CP or its deletion mutants p1CP.01 and p1CP.02 from PFGE agarose gels. To enhance the transfer efficiency of the Southern blots, the PFGE gels were exposed to UV radiation for a few minutes to nick the DNA, followed by a downward alkaline transfer (Sambrook et al., 2001).

**Electrophoresis.** Conventional electrophoresis was performed by standard methods (Sambrook et al., 2001). PFGE was performed by contour-clamped homogeneous field electrophoresis (Chu et al., 1986), initially using a CHEF-DR II system from Bio-Rad and at a later point a 2015 Pulsaphor system from Pharmacia LKB. Slab gels with 0·8% or 1% (w/v) agarose in TBE buffer [44·5 mM Tris-base, 44·5 mM boric acid, 1 mM EDTA (pH 8·5)] were used at 14 °C for the separations. In general, a constant pulse time duration of 80 s was maintained during the total run time (24 h; 6 V cm⁻¹). For restriction analysis, a linear increasing pulse time from 1 to 25 s during the total run time (24 h; 6 V cm⁻¹) was used. At a later point an additional step of increasing pulse time from 25 to 30 s over 6 h was performed. Lambda Ladder PFGE Marker, MidRange I PFGE Marker obtained from New England Biolabs and chromosomes of Saccharomyces cerevisiae obtained from Pharmacia were used as high-molecular-mass DNA standards. Gels were stained by ethidium bromide or SYBR Gold. To visualize the mobility of the native plasmid, and of restriction fragments not treated with proteinase K, 0·2% SDS was added to slab gels and electrophoresis buffer.

**Localization of genes by PCR.** For experiments on the localization of catabolic genes, genomic DNA of R. opacus 1CP was obtained as described previously (Eulberg et al., 1997). Plasmid DNA was prepared in sufficient amounts by excision of the plasmid band, which was visible after PFGE of lysed, agarose-embedded cells of R. opacus 1CP, and elution from the gel with an Easy Pure kit (Biozym). Specific and degenerate primers, which were used for PCR experiments are listed in Table 2.

Reaction mixtures for regular or touchdown PCR contained 0·6–0·7 units Taq polymerase (MBI Fermentas), 0·4 μM of each primer, 200–400 μM dNTPs, 1·5 mM MgCl₂, 5% DMSO and 150–300 ng DNA in different PCR buffers. For primer pair BDHHf/BDDHHr, 1 unit Taq, 1 mM of each primer and 300 μM dNTPs, and for primer pair ClicA2-Rop-fw84/ClicA2-Rop-rev552, 3 mM MgCl₂ was necessary to get a fragment of correct size. The regular PCR was performed with the following standard thermocycle programme: initial denaturation (95 °C, 5 min); 30 cycles of denaturation (95 °C, 30 s), annealing (temperature mentioned in Table 2, 30 s), and polymerization (72 °C, 1 min; last cycle additional 5 min). The touchdown PCR was done by the following thermocycle programme: initial denaturation (95 °C, 5 min) before addition of the polymerase, 14 cycles with decreasing annealing temperature (65 to 52 °C, 1 min), polymerization (72 °C, 2 min), and denaturation (95 °C, 40 s), 20 more cycles with the same parameters but annealing at 50 °C for 1 min (last cycle with 5 min of polymerization).

**Localization of genes by hybridization.** In hybridization experiments, all labelling, dot blot, hybridization and detection procedures were done using a DIG DNA Labelling and Detection Kit Nonradioactive (Roche) and positively charged nylon membranes from Qiagen or Roth. DIG-labelled probes were either prepared by PCR or generated by randomly primed labelling with Klenow polymerase using genomic DNA or the inserts of plasmids pRER1, pRER3, pRER7 and pMARRE0 (Table 1) as template. All probes were hybridized under high-stringency conditions as described in the Boehringer manual together with dot blots of genomic DNA or p1CP DNA, or with Southern blots of p1CP or its deletion mutants p1CP.01 and p1CP.02 from PFGE agarose gels. To enhance the transfer efficiency of the Southern blots, the PFGE gels were exposed to UV radiation for a few minutes to nick the DNA, followed by a downward alkaline transfer (Sambrook et al., 2001).

**Initial restriction analysis and exonuclease digestion.** After equilibration of agaro plugs in TE buffer (pH 8) and then in restriction buffer, incubation in the presence of 30 units of the restriction enzyme took place for at least 16 h. The reaction was terminated by incubation for 1 h in 1 ml 20 mM Tris/HCl (pH 8)/50 mM EDTA (pH 8). Plugs were then equilibrated with 0·5 × TBE and analysed for restriction fragments formed by PFGE as well as (for fragments <100 kb) by agarose gel electrophoresis. The size estimation was performed with the software TotalLab version 1.1 (Nonlinear Dynamics). To determine the localization of genes or plasmid ends on restriction fragments, Southern blots were performed as described above, followed by hybridization with probes specific for macA, clcA, clcA2, clcE, or the ends of p1CP, respectively.

For exonuclease digestion, agaro plugs containing a visible amount p1CP DNA after equilibration for 1 h with the appropriate exonuclease buffer were incubated for 4 h at 37 °C in 250 μl buffer with 30 units of exonuclease III (MBI Fermentas) or λ exonuclease (New England Biolabs). After stopping the reaction by addition of 5 μl 0·5 M EDTA, plugs equilibrated in 0·5 × TBE were analysed on a PFGE gel. For digestion experiments with native p1CP, plugs of an SDS-PFGE gel were used after having been washed in 10 mM Tris/HCl, 1 mM EDTA (pH 8) to eliminate the SDS.

**DNA sequence analysis.** Sequencing was done on an ALFExpress Sequencer (Pharmacia) or a LI-COR 4200 IR Sequencer using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia; both sequencers) or the Cycle Reader Auto DNA Sequencing kit (MBI Fermentas; LI-COR only) with labelled T3 or T7 primer. Extensive secondary structures hampered sequencing of the outer 100 bp of p1CP-I. Therefore, the respective subclone was commercially sequenced by SEQLAB. Sequence analysis was done using the Lasergene 99 program package v4.05 (DNASTAR) and BLAST provided by the National Center of Biotechnology Information (NCBI) (Altschul et al., 1990, 1997).
RESULTS

Detection of a linear megaplasmid p1CP

By PFGE under the conditions described above, an extrachromosomal DNA element with a size of 740 kb, assuming linear topology, could be detected in strain 1CP. That the plasmid, designated p1CP, is in fact linear, was originally concluded from the observation that its electrophoretic mobility was not altered by treatment with S1 nuclease (Fig. 1). This nuclease specifically digests single-stranded DNA and thus linearizes supercoiled DNA which always contains small single-stranded regions due to torsional stress. Large supercoiled plasmids during PFGE migrate much more slowly than their linear form (Barton et al., 1995).

Covalently bound proteins at the termini of p1CP

The presence of covalently linked proteins at the termini of linear replicons of actinomycetes is a feature which can be determined by various approaches. We tested the electrophoretic immobility after omission of proteolytic and denaturating treatments in plasmid preparation or separation (Picardeau & Vincent, 1998) as well as the protection of protein-bearing DNA against exonuclease digestion (Kalkus et al., 1993).

In contrast to p1CP obtained from cell lysis with proteinase K treatment, the native plasmid (obtained from proteinase K-free cell lysis) did not migrate into the agarose gel (Fig. 2a), indicating the presence of a covalently linked protein. During PFGE under denaturing conditions (0-2 % SDS), mobility of native (i.e. not protease treated) p1CP was restored (Fig. 2b).

A PFGE preparation of proteinase K-treated p1CP was completely digested by \( \lambda \) exonuclease, whereas in the absence of proteinase K, the activity of \( \lambda \) exonuclease was completely inhibited, indicating the presence of a protein on the 5’ terminus of p1CP (data not shown).

Identification of genes for (chloro)aromatic catabolism on p1CP

PCR and hybridization experiments with PFGE-separated p1CP DNA were performed for a number of genes involved

### Table 2. Oligonucleotides used for PCR experiments

| Oligonucleotide | Specificity for | Approx. expected product size (kb) | Nucleotide sequence 5’→3’ | Reference for primer or sequence | Annealing temp. \( ^\circ \mathrm{C} \)
<table>
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<tbody>
<tr>
<td>CatB-St</td>
<td>catB</td>
<td>1-1</td>
<td>CGT CAT ATG ACC GAC CGT TCG ATC GTC TCC CGC GGA TCC TTA GGT CCT GGT CCA GGT</td>
<td>Eulberg (1997)</td>
<td>–</td>
</tr>
<tr>
<td>CatB-Sp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fwcA-R-op</td>
<td>catA</td>
<td>0-5</td>
<td>CTC CGC CGC CAC CCA AAA GGT CA</td>
<td>Eulberg et al. (1997)</td>
<td>60</td>
</tr>
<tr>
<td>rev-catA-R-op</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fwc-diox-rhod</td>
<td>clcA</td>
<td>0-4</td>
<td>TGC CGA TGC GCG GA (AC) (AT) GAG AG CAG AGT AGT GCT GGG TGA TCA GT</td>
<td>Thiel et al. (unpublished)</td>
<td>60</td>
</tr>
<tr>
<td>clcA-rev-Rhod-op</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ClcB-St</td>
<td>clcB</td>
<td>1-1</td>
<td>CGT CAT ATG CGC ACC TCC ACC GTC A</td>
<td>Eulberg (1997)</td>
<td>–</td>
</tr>
<tr>
<td>ClcB-Sp</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ClcA2-R-op-fw8</td>
<td>clcA2</td>
<td>0-5</td>
<td>TGC CGA ATA TAA AGC ATC AAC C</td>
<td>Moiseeva et al. (2002)</td>
<td>50§</td>
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<tr>
<td>ClcA2-R-op-r552</td>
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<tr>
<td>ClcF-fw</td>
<td>clcF</td>
<td>0-25</td>
<td>GCT TCG ACA TAT GTC GTA CCT AGT TC ATG GAT CCT CAG TCT TT GGC GAC</td>
<td>Krauß (2002)</td>
<td>40</td>
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<tr>
<td>ClcF-rev</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PND</td>
<td>macA</td>
<td>1-0</td>
<td>GAT CAT ATG GTC GAC CGC TCC TCC GAG ATG GAT CCC TAT GTC GCG GGC GGA GGA TGG</td>
<td>Seibert et al. (1998)</td>
<td></td>
</tr>
<tr>
<td>PBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3_1106_1245</td>
<td>pcatGBl</td>
<td>2-3</td>
<td>CGA CCA CAC CGC CGA CAA CTG</td>
<td>Eulberg et al. (1998b)</td>
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<td>3_3293_3005</td>
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<td>BDHH f</td>
<td>benL</td>
<td>0-8</td>
<td>TGA TGG TAC TCC TCA CCA GGA TCC GCA GCT AGC GGA G</td>
<td>Gröning et al. (unpublished)</td>
<td>60§</td>
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<tr>
<td>BDHH r</td>
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<tr>
<td>p1CP-end end1-fw</td>
<td>Left end</td>
<td>1-6</td>
<td>CCG CGG CGG CAT ACT TCC CCG CGG CGG TGC TCC</td>
<td>This work</td>
<td>60</td>
</tr>
<tr>
<td>p1CP-end end1-rev</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p1CP-end end2-fw</td>
<td>Right end</td>
<td>0-7</td>
<td>CCG CGG CGG TGC TGC TCC T TCC C</td>
<td>This work</td>
<td>60</td>
</tr>
<tr>
<td>p1CP-end end2-rev</td>
<td></td>
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</table>

*Parts of the primers which are not homologous to the template DNA are shown in italics.
†For primer pairs with undetermined optimal annealing temperature, a touchdown PCR was performed.
§PCR contained 3 mM MgCl₂.
§PCR contained 1 U Taq polymerase, 1 μM of each primer and 300 μM dNTPs.
in catabolism of catechol, 3-chlorocatechol, 4-chloro-/3,5-dichlorocatechol, protocatechuate, and benzoate, in order to find out whether they are localized on p1CP or on the chromosome.

For clcA (encoding 4-chloro-/3,5-dichlorocatechol 1,2-dioxygenase), clcA2 (encoding 3-chlorocatechol 1,2-dioxygenase), clcB (encoding 3-chloro-/2,4-dichloro-cis,cis-muconate cycloisomerase), clcF (encoding 5-chloromuconolactone dehalogenase), and macA (encoding maleylacetate reductase), p1CP DNA gave PCR products of expected size with the respective specific primers (Table 2). Because of the possible existence of more than one maleylacetate reductase gene in strain 1CP (Seibert et al., 1998), the PCR product obtained was ligated into pBluescript II SK(+) and after transformation eight clones were sequenced. All gave sequences that were identical to that of macA (Seibert et al., 1998), indicating that the detected gene is in fact the one already described.

The positive hybridizations of PFGE-separated p1CP DNA with DIG-labelled probes for clcA, clcB and macA (Fig. 3) clearly support the PCR results. In addition, positive hybridizations were obtained for clcA2 and clcF, indicating that not only the 4-chloro-/3,5-dichlorocatechol pathway and at least one maleylacetate reductase gene but also the 3-chlorocatechol pathway is located on the megaplasmid.

In contrast, PCR as well as hybridization experiments with primers or probes specific for catA (encoding catechol 1,2-dioxygenase), catB (encoding muconate cycloisomerase) or pcaGBL9 (encoding enzymes of the protocatechuate pathway) were negative with p1CP DNA (Fig. 3) but positive with genomic DNA. Therefore, the catabolic pathways for catechol and protocatechuate are not encoded on p1CP. According to PCR results this also seems to apply to benD (encoding a benzoate dihydrodiol dehydrogenase).

**Cloning and sequence analysis of terminal fragments of p1CP**

Terminal sequences of linear replicons in actinomycetes are characterized by the occurrence of differently sized inverted repeats and palindromic motifs important for replication and propagation of linearity (Chen, 1996; Sakaguchi, 1990). In order to characterize p1CP in this respect, both ends of p1CP were cloned and sequenced.

One terminal fragment was identified by hybridization of SstI-digested purified p1CP DNA using a DIG-labelled 2·3 kb Sall fragment of the right end of the linear Aut plasmid pHG201 from R. opacus MR11 (Kalkus et al., 1998). Ligation into SstI/EcoRV-digested pBluescript II KS(+) and transformation into E. coli DH5α, followed by colony hybridization, led to the identification of one positive clone,
**Fig. 3.** Southern blots of PFGE-separated p1CP with DIG-labelled gene probes specific to different gene loci involved in catabolism of (chloro)aromatic compounds. M, λ ladder; lane 1, PFGE of genomic DNA preparation of wild-type strain 1CP.

**Fig. 4.** Hybridization pattern of the DIG-labelled 798 bp *ClaI*/EcoRV fragment of p1CP-S with digested genomic DNA of *R. opacus* 1CP. The 4.08 kb *SstI* fragment, marked by the arrow, was cloned into EcoRV/SstI-digested pBluescript II SK(+) to yield p1CP-J.

**Fig. 5.** (a) Rough restriction map of the total linear megaplasmid p1CP. Restriction fragments found to harbour genes for chlorocatechol catabolism are shaded grey. Dashed lines indicate unknown order of the respective fragments. (b) Restriction maps of the 1.56 kb insert of p1CP-S and the 4.08 kb insert of p1CP-J designated left and right end of p1CP, respectively. The black bar represents the 798 bp *ClaI*/EcoRV fragment which after DIG-labelling was used as a probe to obtain p1CP-J. (c) Patterns of DraI and VspI digestion as well as DraI/VspI double digestion of p1CP. M, MidRange I PFG Marker.
p1CP-S, containing a 1.56 kb SstII/blunt-end insert. Its sequence was determined after restriction analysis (see Fig. 5b) and subcloning.

To identify the second end of p1CP, the blunt-ended 798 bp ClaI/EcoRV fragment of the 1.56 kb insert of p1CP-S (see Fig. 5b) was DIG-labelled and used as a probe in hybridization against genomic DNA of strain 1CP, digested with different enzymes of the multicloning site of pBluescript II SK(+) and pUC19. As shown in Fig. 4, nearly every restriction digest produced a typical hybridization pattern consisting of a strong and an additional weak signal. Assuming that the strong signals belong to the plasmid consisting of a strong and an additional weak signal. Cloning of an approximately 4·1 kb SstI fragment into SstI/EcoRV-digested pBluescript II SK(+) and transformation into E. coli DH5α, followed by colony hybridization, led to the identification of 15 positive clones, of which one (p1CP-J) was further characterized. Sequence analyses of the 4·08 kb insert of p1CP-J was performed after restriction analysis (Fig. 5b) and subcloning.

The presence of covalently linked protein on the 1.56 kb SstII/blunt-end fragment and the 4·08 kb SstI/blunt-end fragment was proven by comparison of the electrophoretic mobility of the restriction fragments obtained from proteinase K-treated and non-proteinase K-treated DNA in denaturing and non-denaturing gels (Fig. 6). While proteinase K-treated DNA of both plasmid ends migrated into and through denaturing as well as non-denaturing gels, DNA not treated with proteinase K migrated only into SDS gels (Fig. 6). This clearly indicated the presence of protein on p1CP-S as well as on p1CP-J. Moreover, a significant size difference for the 1.56 kb SstII/blunt-end fragment representing p1CP-S was observed during electrophoresis in the presence of SDS. This band shift obviously resulted from the protein on the terminal DNA fragment in the sample not treated with proteinase K. For the 4·08 kb SstI/blunt-end fragment the band shift was not obvious probably because of insufficient resolution of the gel at this size range.

The sequences of the 1.56 kb insert of p1CP-S and the 4·08 kb insert of p1CP-J as given in Fig. 7 do, in fact, represent the plasmid ends. Sequencing showed that the presumed blunt ends of the linear plasmids had been ligated to the EcoRV site while the inner SstII or SstI sites had been ligated to the respective vector sites. The SstII recognition sequences at the blunt ends of p1CP (Fig. 5b) were obviously not cut during the cloning.

Later it was shown that DIG-labelled probes, which were prepared from the templates p1CP-S and p1CP-J by means of PCR with the primer pairs p1CP-end1-fw/rev and p1CP-end2-fw/rev (Table 2), respectively, both hybridized with PFGE-separated restriction fragments of purified p1CP (see below), providing evidence that the cloned fragments in fact originated from plasmid p1CP.

Sequence alignment of the 1.56 kb insert of p1CP-S and the 4·08 kb insert of p1CP-J, designated the left and right end of p1CP, respectively, showed identity of the terminal 13 nucleotides (Fig. 7). In addition to this short perfect inverted repeat, a similarity of 89 % identical positions between the terminal 587 bp of the left end and the terminal 583 bp of the right end indicated the presence of a larger imperfect TIR. The homologous regions are sharply separated from the residual sequences which do not show significant similarities to each other.

BLASTN screening of NCBI and EBI databases with the inserts of p1CP-S and p1CP-J showed high similarities to the ends of other linear plasmids of rhodococci (Table 3). Highest scores were obtained for the right end of pHG204 (AF001834), which showed 92 % identical positions over 710 and 331 bp to p1CP-S, and the 3′ end of pHG207 (L14442), which shared 95 %, 91 % and 87 % identical positions over 239, 992 and 339 bp, respectively, with p1CP-J. As indicated by these data, regions of high similarity
Fig. 7. Manual sequence alignment between the terminal nucleotides of the left and right end of p1CP and all other available terminal sequences of linear plasmids of rhodococci. Positions identical in all sequences are highlighted by black background. Palindromic sequences are indicated both by arrows on top of the alignment and by bold letters. Letters at the bottom indicate conserved motifs of presumed function. The perfect 13 bp inverted repeat of p1CP is indicated by a black box. The accession numbers are as follows: AY569453, p1CP-J (right end); AY569454, p1CP-S (left end); AF007825, pHG201, 5' end; AF001835, pHG201, 3' end; AF001834, pHG204, right end; L14443, pHG207, 5' end; L14442, pHG207, 3' end; AB048370, pRHL2, right end; AB048369, pRHL2, left end; AY223810, pBD2 complete sequence. A full sequence alignment of p1CP-J and p1CP-S is available as supplementary data with the online version of this paper (at http://mic.sgmjournals.org).

Table 3. Comparison of the sequences of both ends of p1CP with database entries

The comparison was made on February 28, 2004 with BLASTN using the standard parameters. In cases where BLASTN searches revealed more than one region of similarity, these are all given.

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*Blastn results indicated very short or even no regions of similarity; however, manual alignment led to identification of strongly conserved (palindromic) motifs within the first ~115 nucleotides.
are often interrupted, obviously by deletions or insertions. The fact that only one of the two ends of plasmids pHG201, pRHL2 and pBD2 is detected by BLASTN searches reflects the fact that linear plasmids of rhodococci, like their counterparts from streptomycetes (Chen et al., 1993, 1996; Lin et al., 1993; Pandza et al., 1998), may exchange their ends (Kalkus et al., 1993). Even plasmids with very similar ends may be the product of a recombination process, as was shown for pHG207. The two ends of pHG207 share 95 and 91% identical base pairs over two regions of 237 and 329 positions, respectively; however, restriction analysis confirmed that the left and right end in fact originate from pHG204 and pHG205, respectively (Kalkus et al., 1993).

Palindromic sequences, whose hairpin structures are thought to be potential binding sites for proteins required for replication, are common structural features of linear replicons of actinomycetes. p1CP-S shows two perfect palindromic motifs (sequence positions 12–34 and 65–81) which are almost identical to the corresponding sequence positions in p1CP-I (Fig. 7). However, in p1CP-I the palindrome of position 12–34 is imperfect because of a mismatch at position 14. Both palindromic structures contain the central motif GCTXCGC, already obtained for other plasmid termini of rhodococci (Kalkus et al., 1998; Shimizu et al., 2001) and streptomycetes (Bey et al., 2000; Huang et al., 1998). Several other regions of striking similarity were identified (alignment positions 1–2, 5–7 and 49–62). However, nothing is known yet of their functional implication.

Both terminal sequences p1CP-S and p1CP-I were screened with BLASTTX against the February 2004 version of the non-redundant NCBI and EBI databases for open reading frames which would encode polypeptides. However, no ORFs were found to show significant similarity either to proteins of known function or to hypothetical proteins.

Initial restriction analysis of p1CP

In order to get a first view of p1CP, a rough restriction map of p1CP was generated by means of the AT-specific 6 bp cutters VspI and DraI (Fig. 5a, c). DIG-labelled gene probes for macA, clcA, clc2A and clcF as well as for both ends were used as additional markers. While clcA and macA were shown to be both localized on a 160 kb large DraI fragment, clc2A and clcF were found together on the adjoining 190 kb large DraI fragment (Fig. 5a), which also hybridized with a probe for the right end. More detailed analysis led to the detection of the clc2A and clcF genes on an internal 110 kb VspI fragment. However, because of three additional VspI fragments of unknown order, its exact position could not be determined.

Deletion of the clc and clc2 gene cluster in mutants 1CP.01 and 1CP.02

Attempts to cure strain 1CP from p1CP were performed by prolonged cultivation under non-selective conditions in LB medium. After ~130 generations, appropriate dilutions were plated on LB agar plates and incubated for 3 days at 30°C. Five hundred colonies were screened for their ability to convert 4-chlorocatechol, a property which is encoded by the p1CP-located clc gene cluster. For this, material from the colonies was transferred to 200 µl agarized mineral medium in microtitre plates containing 10 mM succinate plus 0.5 mM 4-chlorocatechol. While incubation of such sterile medium for several days at 30°C led to autoxidation of the 4-chlorocatechol, resulting in a brownish colour, wells inoculated with wild-type R. opacus 1CP did not turn dark, due to 4-chlorocatechol degradation prior to autoxidation. Of the 500 picked colonies only two mutants occurred that had lost the ability to degrade 4-chlorocatechol, as indicated by the brown colour of the respective wells after 5–7 days at 30°C. PFGE analysis of the two mutants, designated 1CP.01 and 1CP.02, showed that in both strains a megaplasmid was still present which obviously had undergone a large deletion (Fig. 8).

As for native (i.e. not proteinase K-treated) p1CP, preparations of native p1CP.01 and p1CP.02 did not migrate into the non-denaturing agarose gel during electrophoresis (Fig. 2), while mobility was restored when electrophoresis was performed in the presence of 0.2% SDS (data not shown). Deletions, therefore, must have taken place within the plasmid, leaving both ends intact.

![Fig. 8. PFGE of lysed cells of R. opacus 1CP, 1CP.01 and 1CP.02 embedded in agarose. M, chromosomes of Saccharomyces cerevisiae as molecular mass standard (Pharmacia).](http://mic.sgmjournals.org)
That the deletions within the shortened plasmids p1CP.01 (500 kb) and p1CP.02 (400 kb) in fact include the clc gene region was verified by Southern blot hybridization with a clcB-specific probe as well as by dot blot hybridization and PCR with a clcA-specific probe and primer, respectively, which were all negative. In addition, clcB could not be detected in genomic DNA of the mutants, excluding the possibility that the clc-containing region of p1CP had integrated into the chromosome by some recombination process.

Because of the relative proximity of clc and clc2 genes in p1CP, the deletion mutants 1CP.01 and 1CP.02 were also investigated for the presence of a functional 3-chlorocatechol pathway. The additional absence of the clc2 gene cluster in both mutants was proven by PCR as well as by Southern blot hybridization of digested genomic DNA with clcA2-specific primers and probes (data not shown).

**DISCUSSION**

There are several reasons for the assumption that rhodococci may play a dominant role in bioremediation of xenobiotic compounds under natural conditions. Members of the genus *Rhodococcus* are metabolically extraordinarily versatile (Finnerty, 1992; Warhurst & Fewson, 1994); exhibit slow growth rates but stable cell numbers (Golovlev, 1995), high tolerance to dryness and starvation (Koronelli et al., 1988; Robertson & Batt, 1973), weak catabolic repression by glucose (Warhurst & Fewson, 1994), and long-lasting induction levels in the absence of substrate (Cain, 1981; Dugan & Golovlev, 1983). Another characteristic that enables rhodococci to act as dominant degraders is their extraordinary ability to adapt. Metastable phenotypes of *R. erythropolis* were reported to have been obtained as the result of phase variation at a four- to fivefold higher frequency than spontaneous mutations (Golovlev, 1995).

Despite the obvious metabolic versatility of rhodococci, relatively little is known about the genetics of their degradative pathways for chloroaromatic compounds. In this respect, the strain that has so far received most attention is *R. opacus* 1CP. It was originally isolated due to its ability to utilize 4-chloro- and 2,4-dichlorophenol (Gorlatov et al., 1989), but it also grows with phenol, styrene, benzoate and 4-hydroxybenzoate. Prolonged cultivation in the presence of 2-chlorophenol or 3-chlorobenzoate led to the isolation of 4-hydroxybenzoate. A separate gene for this activity, *macA*, was cloned and sequenced, but turned out not to be involved in 4-chlorophenol degradation (Seibert et al., 1998).

In proteobacteria the genes of chlorocatechol pathways usually reside on plasmids (Frantz & Chakrabarty, 1987; Köiv et al., 1996; Perkins et al., 1990; van der Meer et al., 1991a) or other transferable genetic elements (van der Meer et al., 2001). In contrast, the genes for the degradation of numerous non-chlorinated aromatic compounds via the catechol and protocatechuate pathways in most strains investigated appear to be located on the chromosome (Doten et al., 1987; Holloway et al., 1994; Sauret-Ignazi et al., 1996; Zylstra et al., 1989). These facts raised the question whether the situation in the Gram-positive *R. opacus* 1CP is similar or different.

In the present paper it is shown that also in *R. opacus* 1CP the chlorocatechol genes are located on a plasmid, p1CP. This is true for both the clc cluster for 4-chlorocatechol and 3,5-dichlorocatechol degradation and the clc2 cluster for 3-chlorocatechol degradation as well as for the *macA* gene. In contrast, the genes for benzoate, catechol and protocatechuate catabolism, as in most proteobacteria, are obviously not located on the plasmid. Plasmid p1CP was shown to be a huge megaplasmid of 740 kb and to have linear topology. Thus, chlorocatechol genes of *R. opacus* 1CP are the first such genes known to be located on a linear plasmid.

Over the last few years, several linear plasmids from rhodococci have been described. The *R. opacus* wild-type strains MR11 and MR22 were each shown to contain three linear plasmids, pHG201 (270 kb), pHG202 (400 kb) and pHG203 (420 kb), as well as pHG204 (190 kb), pHG205 (280 kb) and pHG206 (500 kb), respectively (Kalkus et al., 1990). Two of these plasmids, pHG201 and pHG205, as well as pHG207, obtained from the transconjugant strain MR22533 (Kalkus et al., 1993), enable their hosts to grow chemolithoautotrophically on gaseous hydrogen and carbon dioxide. Furthermore, pHG204 correlates with thallium resistance. Again three linear plasmids, pRHL1 (1100 kb), pRHL2 (450 kb) and pRHL3 (330 kb), were identified in the polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1, and it was shown that gene clusters encoding (chloro)biphenyl conversion are localized on the two largest plasmids (Masai et al., 1997). *Rhodococcus corallinus* B-276 was even found to harbour four linear plasmids, pNC10 (70 kb), pNC20 (85 kb), pNC30 (185 kb) and pNC40 (235 kb). The gene for an alkene monooxygenase, which allows this organism to grow on propene and to co-oxidize trichloroethene, was shown to be located on pHG201 (Saeki et al., 1997). In contrast, only single linear plasmids, pBD2 (210 kb) and pTA421 (500 kb), were detected in the isopropylbenzene-degrading strain *R. erythropolis* BD2 (Kesseler et al., 1996; Stecker et al., 2003).
and the (chloro)biphenyl degrader *R. erythropolis* TA421 (Kosono *et al*., 1997), respectively. However, both plasmids contain at least some of the genes responsible for aromatic catabolism.

Taking into account the 1100 kb pRHL1, p1CP at 740 kb is presently the second largest linear megaplasmid identified from rhodococci.

The propagation of all linear replicons, because of the 5′–3′ polarity of DNA replication, requires a solution to the ‘end replication problem’. Linear plasmids as well as linear chromosomes of actinomycetes have overcome this problem by iterons consisting of perfect or imperfect TIRs and terminal bound proteins (TPs) (Sakaguchi, 1990). Like other linear plasmids of rhodococci, p1CP was shown to have TPs as well as TIRs. The ends of p1CP have a short perfect TIR of 13 bp, which belongs to a larger imperfect TIR of 583/587 bp, in which the two parts share 89% identical positions with each other. Sequence comparison of both p1CP ends with the database revealed significant similarities to the right ends of pHG204, pHG201, pRHL2, the 3′ end of pBD2, and both ends of pHG207, whereas the corresponding left ends of pHG201 and pRHL2, as well as the 5′ end of pBD2 were not revealed by BLASTN searches with the ends of p1CP. However, manual alignment of the terminal 100–117 nucleotides of all hitherto available ends of linear *Rhodococcus* plasmids clearly confirmed the presence of strongly conserved palindromic motifs. Two copies of the motif GCTXCGC were found in each terminus and it was already assumed earlier that these structures are essential for replication (Kalkus *et al*., 1998; Shimizu *et al*., 2001). Essentially the same central motif with the potential to form a stable single C-residue loop close to the sheared T;C pairing was found in the linear *Streptomyces* plasmids pSCL1 (Wu & Roy, 1993), pSLA2 (Hirochika *et al*., 1984) and SLP2 (Chen *et al*., 1993), as well as in the terminal segments of several linear *Streptomyces* chromosomes (Huang *et al*., 1998). In pSLA2 these structures were shown to be essential for replication and propagation of linearity (Qin & Cohen, 1998). The specific binding of TPs (Huang *et al*., 1998) and the patching of the single-stranded gaps at the 3′ termini during replication (Chen, 1996) are two possible functions of palindromes. Additionally, a conserved CXXCGG motif with an unknown, but probably important function, was found at the very end of all termini, including those of p1CP.

Attempts to cure the megaplasmid present in strain 1CP, in order to determine the phenotypic properties encoded on p1CP, have up to the present been unsuccessful. However, prolonged cultivation under non-selective conditions led to the identification of two mutants 1CP.01 and 1CP.02, harbouring shortened megaplasmids p1CP.01 (500 kb) and p1CP.02 (400 kb) which must have undergone large deletions, both covering the *clc* as well as the *clc2* gene cluster, and *macA*. Electrophoretic immobility of the native mutant plasmids indicated the presence of protein-linked termini. The deletions must, therefore, have taken place within the replicons. It will be interesting to elucidate the plasmid regions and mechanisms involved in the occurrence of these deletions.

**ACKNOWLEDGEMENTS**

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M. König and others


