A genetic system for the rapid isolation of aromatic-ring-hydroxylating dioxygenase activities

Silke Kahl and Bernd Hofer

Aromatic-ring-hydroxylating dioxygenases (ARHDOs) are key enzymes in the aerobic bacterial metabolism of aromatic compounds. They are of biotechnological importance as they function as biocatalysts in the stereospecific synthesis of chiral synthons and the degradation of aromatic pollutants. This report describes the development and validation of a system for the rapid isolation and characterization of specific ARHDO activities. The system is based on the identification of ARHDO gene segments that encode the enzymes' major functional determinants, on consensus primers for the direct amplification of such partial genes and on a 'recipient' ARHDO gene cluster for the insertion of the amplified segments. Previously, it has been shown that neither the N- nor the C-terminal portions but only the core region of the large or \(\alpha\)-subunit of a class II ARHDO significantly influence substrate and product spectra. On the basis of these observations, consensus primers were designed for the amplification of the gene segment encoding the catalytic core of the large subunit. These primers were tested on 11 bacterial isolates known to metabolize aromatic compounds. In 10 cases, a gene fragment of expected length was amplified. DNA sequencing confirmed similarity to ARHDO \(\alpha\)-subunit gene cores. The heterologously well-expressible \(bphA\) gene cluster of \(Burkholderia\) sp. strain LB400 was modified to facilitate the in-frame insertion of amplified segments. It was used successfully to express the resulting hybrid gene clusters and to form catalytically active chimaeric ARHDOs. The metabolic properties of these enzymes differed significantly from each other and from the parental ARHDO of strain LB400. These results indicate that the system described here can be used to rapidly isolate and functionally characterize ARHDO activities, starting from isolated strains, mixtures of organisms or samples of nucleic acids. Applications of the system range from the recruitment of novel ARHDO activities to an improved characterization of natural ARHDO diversity.

INTRODUCTION

Aromatic-ring-hydroxylating dioxygenases (ARHDOs) are key enzymes of the aerobic bacterial metabolism of aromatic compounds. The prototype reaction they catalyse is the addition of two hydroxy groups to vicinal carbons, thereby destroying the aromatic system and yielding dihydrodiol compounds of \(cis, cis\) stereochemistry (Butler & Mason, 1997; Boyd & Sheldrake, 1998). Bacterial ARHDOs are quite versatile, both in terms of the substrates they accept and the oxidations they catalyse. Thus, even small aliphatic compounds such as trichloroethylene may serve as substrates (Wackett & Gibson, 1988; Furukawa et al., 1994) and different types of oxidations including dehydrogenation of vicinal carbons and mono-oxygenation of carbons and hetero atoms have been observed (Resnick et al., 1996; Boyd & Sheldrake, 1998). The subunit characteristics and compositions of such dioxygenase systems vary considerably. However, all of these systems consist of a hydroxylase, which binds and oxidizes the organic substrate, and of a short transport chain for the electron supply of the hydroxylase (Butler & Mason, 1997). Based on the number and some characteristic features of the subunits, ARHDOs have been grouped into five classes, namely IA, IB, IIA, IIB and III (Butler & Mason, 1997).

Previous studies with class II ARHDO systems have demonstrated that the major determinants of the fundamental catalytic properties such as substrate and product spectra reside within the C-terminal part of the large or \(\beta\)-subunit (Erickson & Mondello, 1993; Kimura et al., 1997; Mondello et al., 1997; Beil et al., 1998; Zielinski et al., 2002), although the small or \(\beta\)-subunit has occasionally been reported to exert some influence on these properties (Furukawa et al., 1994; Hurtubise et al., 1998). Recently, it has been shown that the C-terminal 60 aa of the \(\alpha\)-subunit are of minor importance for these properties (Zielinski et al.,...
2002). Thus, if consensus oligonucleotide primers could be derived from conserved sequences flanking the part of the gene that encodes the catalytic centre, such segments could be rapidly amplified from, for example, bacterial isolates, microbial consortia or just samples of nucleic acids. These segments could be reconstituted into complete ARHDO gene clusters by fusion with the missing sequences from a ‘helper’ operon. If the resulting hybrid genes would express catalytically active enzymes, such a system should be a powerful tool for the rapid isolation and characterization of naturally occurring ARHDO activities.

METHODS

Chemicals and oligonucleotides. Chemicals were of analytical grade, if available. Oligonucleotides were synthesized by Gibco/Life Technologies. Their sequences (5′−→3′) were as follows: BPH2454M, ATGACGATCTTTCCGAGATCCTGACACCTCTGGACCCTTCCCAAATC; BPH-2651M, TGGCCCTGGTACCCCTTAAAGCCGCCTTCGGTATCCTCC; BPH2632M, CTTAAGAGGGTACAAGGCCAAGAGCCAGC; BPH-2711, AATCCAGGGTACCCGCTTGCAG; BHD202, GAGCCAGGGTACCCGCTTGCAG; BPH2632M and BPH-2711.

Bacterial strains and plasmids. The following strains were used in this study: Escherichia coli strains DH5α (Grant et al., 1990), DH10B (Grant et al., 1990) and BL21(DE3) (pLysS) (Studier, 1991); Burkholderia sp. strain LB400 (Bopp; 1986; Fain & Haddock, 2001); Pseudomonas sp. strains B2A, B3B, B4, B6K and B7A (Bartels et al., 1999); Ralstonia eutropha H850 (Bedard et al., 1987; Williams et al., 1997); Ralstonia sp. strains B11 and B15 (Bartels et al., 1999); Rhodococcus globularus P6 (Furukawa et al., 1978; Asturias et al., 1994); Rhodococcus opacus BIE-20 (Wagner-Dobler et al., 1998); Sphingomonas yanoikuyae Q1 (Furukawa et al., 1983; Wang & Lau, 1996). pAIA6000 is a pT7-6-based expression plasmid harboring genes bphA1m2A3A4BC of strain LB400; bphA1m contains silent mutations that generate NdeI and XhoI sites (Zielinski et al., 2002).

Bacterial cultures and preparation of resting cells. Soil bacteria were grown at 30°C inuria broth (Bopp et al., 1983) or in minimal medium with biphenyl as carbon source as described previously (Bartels et al., 1999). E. coli DH strains harbouring a pAIA plasmid were grown at 37°C in LB medium (Sambrook et al., 1989) containing 100 μg ampicillin ml−1. E. coli BL21(DE3)(pLysS) strains harbouring a pAIA plasmid were grown at 30°C in LB medium containing 50 μg chloramphenicol ml−1 and 100 μg ampicillin ml−1. For the preparation of resting cells, the latter strains were grown to an OD600 value of about 11 and cells were harvested, washed with 50 mM sodium phosphate buffer (pH 7.5) and then resuspended in the same buffer to an OD600 value of 5-10. IPTG was added to 0-4 mM (final concentration), and the incubation was continued for another 40-45 min. Cells were harvested, washed with 50 mM sodium phosphate buffer (pH 7-5) and then resuspended in the same buffer to an OD600 value of 5-10.

DNA techniques. In vitro DNA modifications, agarose gel electrophoresis (AGE) and transformations were carried out according to standard protocols (Sambrook et al., 1989), unless described in detail.

The introduction of an AflII site into bphA1m−LB400 was done as follows. Plasmid pAIA6000 was used as template to amplify two overlapping parts of its bphA1m gene by PCR (Mullis & Faloona, 1987), using primers BPH2454M and BPH-2651M or BPH2632M and BPH-2711, respectively. Both PCR products (198 and 80 bp, respectively) were purified via AGE and then used as template for overlap extension PCR (Higuchi et al., 1988) with primers BPH2454M and BPH-2711. The gel-purified product (258 bp) was cut with XhoI and AgeI and then cloned into identically cleaved, dephosphorylated pAIA6100. This yielded plasmid pAIA6100. The integrity of its insert was verified by DNA sequencing.

For PCRs with consensus primers, a small sample of cells was suspended in 20 μl of water and heated to 95°C for 4 min. After spinning down cell debris with a table top centrifuge, 2 μl of supernatant were used as template in the PCR. Thirty cycles with an annealing temperature of 60°C, an elongation time of 90 s (with an increment of 3 s per cycle) and otherwise standard conditions were used.

To generate ARHDO x-subunit fusion genes, PCR products of the consensus primers were purified by AGE, cut with MluI and AflII and then ligated with identically cleaved, dephosphorylated pAIA6100. The resulting clones were screened for ARHDO activity (see below) and further analysed by restriction and insert sequencing.

For DNA sequence determinations, about 1 μg of plasmid or 0-05–0-2 μg of PCR product was subjected to Taq DNA polymerase-catalysed cycle sequencing as described previously (Bartels et al., 1999). The sequences of the novel bphA1 core segments have been deposited in the EMBL/GenBank/DBJ data libraries under accession numbers AJ544517–AJ544523.

Protein gel electrophoresis. Resting cells were lysed with a cracking buffer (Tabor & Richardson, 1985) and proteins were separated by 0-1% SDS-15% PAGE as described previously (Hofer et al., 1993). Gels were stained with Coomassie brilliant blue R250 (Sambrook et al., 1989).

ARHDO activity measurements. ARHDO activity was assayed with biphenyl as substrate. Colonies were tested by dispensing some crystals of biphenyl into the lid of the Petri dish. As pAIA6100 and its derivatives also expressed bphB and bphC, ARHDO activity led to the conversion of biphenyl into 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, which was observed by eye (λmax =434 nm). ARHDO activity of resting cells was determined by incubation of a cell suspension with an OD600 value of 5-0 with 0-1 mM biphenyl at room temperature. At intervals, the A434 values of the supernatants were measured.

Depletion of aromatic compounds. A mixture of aromatic compounds (see Table 2) and 2,4,6,2′,4′-pentachlorobiphenyl (as internal standard) were added to resting cells with an OD600 value of 5-0 to a final concentration of 12 μM each. After vortexing for 10 s, the teflon-sealed tubes were shaken at 30°C for 24 h. Thereafter, the suspensions were extracted with 1 volume of n-hexane, and 5 μl of the organic phase were analysed in an HP 3890 Series II gas chromatograph (GC) equipped with a flame-ionization detector and an HP Ultra2 column (length, 50 m; inner diameter, 0-2 mm; film thickness, 0-11 μm). The carrier gas was hydrogen. The injector was held at 250°C. The GC temperature programme used was as follows: 5 min at 40°C, linear gradient of 4°C min−1 to 288°C and 10 min at 288°C. The flame-ionization detector was operated at 300°C. GC peak areas were normalized to the internal standard. Background depletion of substrates was determined by using resting cells devoid of ARHDO genes.

RESULTS AND DISCUSSION

Design and construction of a system for the rapid isolation of ARHDO activities

Consensus oligonucleotide primers for the multiplication of gene segments encoding x-subunit cores of enzymes which
Genetic isolation of dioxygenase activities

Genes *bphA1A2A3A4* of the ARHDO (biphenyl dioxygenase) system of *Burkholderia* sp. strain LB400 (encoding hydroxylase α- and β-subunits, a ferredoxin and a ferredoxin reductase) were chosen as the recipient gene cluster for the exchange of α-subunit gene cores. This choice was based on the following findings. It has been shown that the genes for the *bph*-encoded wild-type hydroxylase of strain LB400 can be expressed to high levels in *E. coli* (Seeger et al., 1997). Moreover, it has been demonstrated for the LB400 dioxygenase system that the replacement of various regions of the large subunit by ARHDO segments that share 59–70% sequence identity in most cases resulted in catalytically active enzymes (Zielinski et al., 2002). The *bphA1* gene, which already contains an appropriate recognition sequence for *MluI*, was modified to create an *AflII* site by the introduction of four silent mutations. These alterations, yielding plasmid pAIA6100, did not significantly affect the level of *bphA1* expression (see also Fig. 3).

The basic features of the approach are depicted in Fig. 1. As each of the core segment primers encodes consensus peptides that differ somewhat from the respective BphA1-LB400 sequences, the final hybrid protein consists of five segments which are derived from three different sources, BphA1-LB400, the consensus peptides and the segment...
encoded by the novel gene. If advantageous, the primer-specific sequences of the amplified product may be exchanged, for example, against the respective sequences of the recipient gene cluster. This could, for example, be done by re-amplification of the primary product, using appropriate primers.

**Experimental evaluation of the system**

Bacterial isolates obtained from different locations and known to aerobically metabolize predominantly bicyclic aromatic compounds were chosen to evaluate the approach described here. The ARHDO genes of these organisms were unknown, except for *R. globerulus* P6 (Asturias *et al.*, 1995). In 10 out of 11 cases, PCR fragments of the expected lengths were obtained (Table 1). Only *S. yanoikuyae* Q1 gave no product. The sequence of an extradiol dioxygenase of this strain has been determined (Taira *et al.*, 1988). Interestingly, it is most closely related to sequences found in polycyclic aromatic hydrocarbon (PAH) degraders (Hofer *et al.*, 1993). Thus, the ARHDO gene of this strain may also be of the PAH type. These genes are usually so divergent that they do not permit annealing of the consensus primers. DNA sequencing revealed that the sequences of the amplified segments were typical for the cores of *a*-subunit genes and were devoid of *Mlu*I and *Afl*II sites other than those bestowed by the primer sequences. Some pairs of core segments showed 100 % sequence identity with each other, namely those from strains B6K and B7A, B11 and B15, and H850 and LB400. An alignment of the deduced protein sequences is shown in Fig. 2. Sequence identity of these segments with the replaced polypeptide segment of strain LB400 ranged from 68 to 100 %.

Four of the seven different PCR products representing differing degrees of sequence identity with the LB400 gene were selected to replace the *bphA1* core segment in pAIA6100. In each case, ARHDO activity was restored (Table 1). The newly introduced segments of the clones were re-sequenced. All sequences were identical to the

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**Table 1. Amplification, sequencing, cloning and expression of core gene segments of ARHDO a-subunits**

<table>
<thead>
<tr>
<th>Source of template DNA</th>
<th>PCRs</th>
<th>E. coli clone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGE* analysis</td>
<td>Sequencing of product</td>
</tr>
<tr>
<td></td>
<td>Total length (bp)</td>
<td>Length of core (bp)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkholderia sp. LB400</td>
<td>~730</td>
<td>625</td>
</tr>
<tr>
<td>Pseudomonas sp. B2A</td>
<td>~730</td>
<td>625</td>
</tr>
<tr>
<td>Pseudomonas sp. B3B</td>
<td>~730</td>
<td>ND</td>
</tr>
<tr>
<td>Pseudomonas sp. B4</td>
<td>~730</td>
<td>622</td>
</tr>
<tr>
<td>Pseudomonas sp. B6K</td>
<td>~730</td>
<td>625</td>
</tr>
<tr>
<td>Pseudomonas sp. B7A</td>
<td>~730</td>
<td>625</td>
</tr>
<tr>
<td>Ralstonia eutropha HB50</td>
<td>~730</td>
<td>625</td>
</tr>
<tr>
<td>Ralstonia sp. B11</td>
<td>~730</td>
<td>619</td>
</tr>
<tr>
<td>Ralstonia sp. B15</td>
<td>~730</td>
<td>619</td>
</tr>
<tr>
<td>Rhodococcus globerulus P6</td>
<td>~730</td>
<td>622</td>
</tr>
<tr>
<td>Rhodococcus opacus BIE-20</td>
<td>~730</td>
<td>622</td>
</tr>
<tr>
<td>Sphingomonas yanoikuyae Q1</td>
<td>NPO</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, Not applicable; ND, not done; NPO, no product observed.

*AGE, agarose gel electrophoresis.
†Relative to the sequence of the recipient gene cluster from strain LB400.
‡Measured with biphenyl as substrate (see Methods); values are shown ± SD.
§Directs synthesis of wild-type BphA1-LB400 (see Methods).
*Approximately 65 nt encoding 22 aa not sequenced.
uncloned PCR products with one exception; one clone contained a point mutation leading to a substitution of His by Arg in the segment encoded by the reverse primer.

The concentrations of wild-type and hybrid α-subunits and of wild-type β-subunits were analysed by denaturing PAGE of SDS-lysed cells (Fig. 3). After staining with Coomassie blue, bands of both subunits were clearly visible in all cases, indicating high level expression of the respective genes.

Substrate specificities of the hybrid ARHDOs were assessed by incubation of a mixture of aromatic compounds with the respective E. coli recombinants and analysis of their depletion. The data show that the substrate spectra of the hybrid enzymes differed from each other and from that of the strain LB400 ARHDO (Table 2). This result confirms that the substrate range is crucially determined by the replaced core segment. A detailed analysis of substrate and product spectra of selected hybrid ARHDOs will appear elsewhere.

For comparison, the same assays were carried out with the donor strains of the core segments (Table 2). In most cases, similar degrees of substrate depletion were observed with as with the respective E. coli recombinants, indicating that the properties of the hybrid ARHDOs often, but not always, reflect the properties of the donor strains. Minor differences were found occasionally that indicated more depletion by the recombinants. This may result from a higher level of gene expression in the E. coli strains that harbour multiple core segments. A straightforward explanation would be the existence of more than one ARHDO in these strains. This has previously been observed in bacteria (Kim & Zylstra, 1999; Kitagawa et al., 2001).

The limitations of the approach described here are that it relies upon DNA sequence similarity with the consensus primers and compatibility of protein three-dimensional structures between donor and recipient. An in silico analysis indicated that more than 80 % of the cores of sequenced genes encoding α-subunits of ARHDOs that are known or likely to belong to class II or to accept benzene or benzene derivatives as substrates should be amplified with the described primers. The genes of the entire ARHDO family are too diverse to permit amplification by a single pair of oligonucleotides. However, different sets of primers may be designed for different ARHDO subclasses. A model of the three-dimensional structure of the class II biphenyl dioxygenase of strain LB400 (M. Zielinski, S. Kahl, H.-J. Hecht & B. Hofer, unpublished data), based on the known class III naphthalene dioxygenase structure (Carredano et al., 2000), suggests that the structural similarity between these two classes of ARHDOs is high enough to make it likely that the fusion approach outlined here may also be applied to class III dioxygenases. The formation of a functional ARHDO hybrid will depend upon the structural compatibility of donor and recipient regions. This is not strictly correlated with the amino acid sequence similarity between the fusion components, since rather dissimilar sequences can result in quite similar structures. The class III dioxygenase structure and the class II dioxygenase model show that in the (αβ)₃ hexamer the exchanged core segment has contacts with both terminal parts of its subunit, with the neighbouring β-subunit and with one of the two other α-subunits. It is located at the periphery of the hexamer and forms a fairly compact subdomain in itself. Thus, it is less likely that core replacements will severely disturb the overall structure of the hydroxylase complex.

In summary, our results suggest that the system outlined here will, in many cases, result in catalytically active ARHDOs. These hybrid enzymes possess distinct properties, depending on the donor segment. Therefore, the system could be used to rapidly screen a large number of bacterial isolates for ARHDO activity. It requires no strain selection

### Table 2. Depletion of aromatic compounds by E. coli BL21(DE3)(pLysS) recombinants and parental strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Naphthalene (%)</th>
<th>Biphenyl (%)</th>
<th>Carbazole (%)</th>
<th>Dibenzofuran (%)</th>
<th>Dibenzodioxin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli (pIAA6100)</td>
<td>34 ± 12</td>
<td>99 ± 1</td>
<td>98 ± 2</td>
<td>99 ± 1</td>
<td>63 ± 9</td>
</tr>
<tr>
<td>Burkholderia sp. LB400</td>
<td>39 ± 10</td>
<td>97 ± 3</td>
<td>97 ± 2</td>
<td>95 ± 4</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>E. coli (pIAA6102)</td>
<td>43 ± 9</td>
<td>98 ± 2</td>
<td>58 ± 13</td>
<td>46 ± 15</td>
<td>85 ± 10</td>
</tr>
<tr>
<td>Pseudomonas sp. B2A</td>
<td>26 ± 9</td>
<td>97 ± 2</td>
<td>68 ± 7</td>
<td>26 ± 11</td>
<td>51 ± 13</td>
</tr>
<tr>
<td>E. coli (pIAA6104)</td>
<td>63 ± 18</td>
<td>97 ± 2</td>
<td>70 ± 19</td>
<td>33 ± 12</td>
<td>69 ± 14</td>
</tr>
<tr>
<td>Pseudomonas sp. B4</td>
<td>35 ± 12</td>
<td>88 ± 8</td>
<td>43 ± 11</td>
<td>28 ± 13</td>
<td>83 ± 12</td>
</tr>
<tr>
<td>E. coli (pIAA6115)</td>
<td>7 ± 5</td>
<td>96 ± 4</td>
<td>11 ± 7</td>
<td>5 ± 5</td>
<td>10 ± 7</td>
</tr>
<tr>
<td>Ralstonia sp. B15</td>
<td>11 ± 6</td>
<td>84 ± 8</td>
<td>83 ± 7</td>
<td>12 ± 10</td>
<td>78 ± 7</td>
</tr>
<tr>
<td>E. coli (pIAA6121)</td>
<td>98 ± 2</td>
<td>98 ± 2</td>
<td>93 ± 6</td>
<td>19 ± 12</td>
<td>20 ± 9</td>
</tr>
<tr>
<td>Rhodococcus opacus BIE-20</td>
<td>96 ± 4</td>
<td>96 ± 3</td>
<td>74 ± 13</td>
<td>94 ± 4</td>
<td>93 ± 6</td>
</tr>
</tbody>
</table>
or any knowledge of the induction of ARDHO activity. Moreover, the system may be applied directly to complex mixtures of organisms or their nucleic acids, respectively, such as present in environmental samples. This application may be particularly useful as only a minority of all microbes can currently be cultivated in the laboratory (Amann et al., 1995). Thus, the approach described here may be helpful in a functional characterization of the natural ARDHO diversity and may also allow access to a so-far-unattainable biocatalytic potential.

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REFERENCES


