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Functional characterization of genes involved in alkane oxidation by *Pseudomonas aeruginosa*

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Abstract

Most clinical isolates identified as *Pseudomonas aeruginosa* grow on long-chain *n*-alkanes, while environmental *P. aeruginosa* isolates often grow on medium- as well as long-chain *n*-alkanes. Heterologous expression showed that the two alkane hydroxylase homologs of *P. aeruginosa* PAO1 (AlkB1 and AlkB2) oxidize C_{12}-C_{16}*n*-alkanes, while two rubredoxin (RubA1 and RubA2) and a rubredoxin reductase (RubB) homologs can replace their *P. putida* GPo1 counterparts in *n*-octane oxidation. The two long-chain alkane hydroxylase genes are present in all environmental and clinical isolates of *P. aeruginosa* strains tested in this study.

Abbreviations: CF – cystic fibrosis, C_6 – *n*-hexane, C_8 – *n*-octane, C_{10} – *n*-decane, C_{12} – *n*-dodecane, C_{14} – *n*-tetradecane, C_{16} – *n*-hexadecane, C_{18} – *n*-octadecane, DMMZ – Department of Medical Microbiology, Zürich, PAGP – *Pseudomonas aeruginosa* Genome Project

Introduction

Pseudomonas aeruginosa is of clinical importance as the primary opportunistic pathogen among the pseudomonads, but is also a common organism in soil, water and on plants (Botzenhart and Döring 1993). Environmental and clinical isolates are indistinguishable by most chemotaxonomic and molecular techniques (Hilligan 1995; Foght et al. 1996), except that the majority of clinical isolates possess a genomic island named PAG-1 (Liang and Lory 2001). Both in the clinic and in water or soil, alkane oxidation is a relevant property. Enrichments from soil or water with *n*-alkanes as the carbon source often yield *P. aeruginosa* strains. Conversely, the ability of *P. aeruginosa* to use paraffins as sole carbon source is used to identify clinical isolates, in combination with other methods like fluorescence and oxidase assays (Massengale et al. 1999). Most reports on the biochemistry of alkane degradation by *P. aeruginosa* deal with strains able to grow on C_6-C_{10}*n*-alkanes in addition to the longer alkanes (Vandecasteele et al. 1983), and recently it was shown that these particular *P. aeruginosa* strains contain alkane hydroxylases that are virtually identical to the *P. putida* GPo1 alkane hydroxylase (van Beilen et al. 1998). This enzyme system, however, is not involved in growth of these strains on long-chain alkanes, as the *P. putida* GPo1 alkane hydroxylase only oxidizes C_5-C_{12} *n*-alkanes (van Beilen et al. 1994a). *P. aeruginosa* long-chain alkane hydroxylases have not been characterized biochemically or genetically, although compounds which solubilize *n*-alkanes and make them available for uptake, such as rhamnolipids (Koch et al. 1991; Zhang and Miller 1992) and the so-called ''protein activator of alkane oxidation'' PraA (Hisatsuka et al.

1972; Hardegger et al. 1994) have been studied in some detail. In this study, we show that *P. aeruginosa* possesses two homologs of the *P. putida* GPo1 alkane hydroxylase, two rubredoxin homologs, and one rubredoxin reductase homolog. We also show that these proteins are functional, and that the two alkane hydroxylase genes occur in all clinical and environmental isolates of *P. aeruginosa*.

Growth of *Pseudomonas aeruginosa* strains on alkanes

To confirm that the environmental and clinical *P. aeruginosa* isolates listed in Table 1 are able to grow on *n*-alkanes, the strains were tested for growth on medium- and long-chain alkanes. *P. aeruginosa* PAO1 and most of the clinical isolates grew on E2 minimal medium plates (Lageveen et al. 1988) with C_{12}, C_{14}, and C_{16} as the sole carbon and energy source, which is consistent with previous data that clinical strains are able to grow on these compounds (Alonso et al. 1999; Massengale et al. 1999). None of the clinical isolates grew on C_8 or C_{10}. In contrast, environmental isolates described earlier (Azoulay et al. 1963; Van Eyk and Bartels 1968; Macham and Heydeman 1974; Vandecasteele et al. 1983) grew on C_8 and C_{10} as well as on the longer *n*-alkanes.

Cloning of *P. aeruginosa* PAO1 genes involved in alkane oxidation

Highly degenerate primers based on conserved regions of alkane hydroxylase genes yielded almost identical PCR fragments with *P. aeruginosa* PAO1 and PG201 (Smits et al. 1999). The PG201 PCR fragment was used as a probe in Southern blots, which showed that the *P. aeruginosa* PAO1 genome contains two related *alkB* gene homologs (Figure 2). Two independent cosmids were isolated by screening a PAO1 genebank (Visca et al. 1994) with the same probe. Cosmid pTS200 contained an *alkB* homolog, corresponding to the PG201 degenerate PCR fragment, which was designated *alkB1*. Cosmid pTS100 contained a second *alkB* homolog, designated *alkB2* (Belhaj et al. 2002), with only 65% overall DNA sequence identity to *alkB1*, but significantly higher homology in the internal gene segment that was used as a probe, explaining the cross-reactivity in the Southern blot. Neither *alkB1* nor *alkB2* showed significant DNA sequence identity with the *P. putida* GPo1 *alkB* gene in a pairwise alignment by the Wilbur-Lipman method (Wilbur and Lipman 1983). The encoded proteins, AlkB1 and AlkB2, showed 37.4% amino acid sequence identity to the GPo1 alkane hydroxylase and 67.7% to each other. The two genes are also present in the *Pseudomonas aeruginosa*

Table 1. Growth behavior of *P. aeruginosa* strains on E2 minimal medium with 0.2% citrate or *n*-alkane vapor as carbon source

Isolate (Labname)	CuHure[a] collection number	Isolated from	Growth on E2 medium with[b]						Reference
			Citr.	C8	C10	C12	C14	C16	
PAO1	ATCC 15692	Infected wound	++[c]	-	+	++	+++	+++	(Holloway 1969)
PG201	DSM 2659	Soil	++	+	+	++	+++	+++	(Guerra-Santos et al. 1986)
KSLA 473	KSLA 473	Y-harbor, Amsterdam	++	+++	++	++	+++	+++	(Van Eyk and Bartels 1968)
Sol 20	NCIMB 8704	Soil	++	+++	++	++	+++	+++	(Azoulay et al. 1963)
196Aa	NCIMB 9571	Soil	++	+++	++	++	+++	+++	(Vandecasteele et al. 1983)
ATCC 17423	ATCC 17423	Soil	++	+++	++	++	+++	+++	(Macham and Heydeman 1974)
CPA1[d]	DMMZ V10 18600	Urine	++	-	-	+	++	++	This study
CPA2	DMMZ V07 19924-1	Ethmoid tissue, CF	++	-	-	-	-	-	This study
CPA3	DMMZ V07 19924-4	Ethmoid tissue, CF	-	-	-	-	-	-	This study
CPA4	DMMZ V07 19925-2	Ethmoid tissue, CF	++	-	-	-	-	-	This study
CPA5	DMMZ V07 19939	Urine	++	-	+	+	++	++	This study
CPA6	DMMZ V07 19941	Urine	++	-	-	+	++	++	This study
CPA7	DMMZ V07 19965	Pleural fluid	++	-	-	+	+	+	This study
CPA8	DMMZ V09 20207	Urine	++	-	-	+	++	+	This study
CPA9	DMMZ V09 20227-1	Bronchial secretion	++	-	-	+	+	+	This study
CPA10	DMMZ V05 20348-2	Urine	++	-	-	+	+	+	This study
CPA11	DMMZ V05 20391-2	Tracheal aspirate	++	-	-	+	++	++	This study
CPA12	DMMZ V07 21517	Tracheal aspirate	++	-	-	+	+	++	This study

[a] DMMZ: Department of Medical Microbiology Zürich; CF: cystic fibrosis[b] Citr.: 0.2% citrate; C8: octane; C10: decane; C12: dodecane; C14: tetradecane; C16: hexadecane[c] Growth was analyzed after 90 h. incubation. +++ good growth, ++ average growth, + weak growth, - no growth[d] Clinical *P. aeruginosa* strains were identified according to (Hilligan 1995) at DMMZ, Zürich

Table 2. Other strains and plasmids used in this study

Name	Characteristics	Reference
Strains		
E. coli DH10B	cloning strain	Gibco BRL
E. coli GEc137	*thi*, *fadR*	(Eggink et al. 1987a)
P. fluorescens KOB2Δ1	*alkB1*$^-$ (C_{12}-C_{16}^-, C_{18}-C_{24}^+)	(Smits et al. 2002)
Plasmids		
pGEM7-Zf(+)	Cloning vector, Ap^r	Promega
pKKPalk	Expression vector with *alk*-promoter, Ap^r	(Smits et al. 2001)
pCom8	*E. coli-Pseudomonas* expression vector with *alk*-promoter, Gm^r, *oriT*, *alkS*	(Smits et al. 2001)
pGEc47	*alkBFGHJKL/alkST* in pLAFR1	(Eggink et al. 1987a)
pGEc47ΔG	pGEc47, deletion in *alkG*	(van Beilen et al. 2002)
pGEc47ΔT	pGEc47, deletion in *alkT*	this study
pGEc48	*alkBFGH'* in pBR322	(Eggink et al. 1987b)
pTS2	PG201 PCR fragment in pGEM7-Zf(+)	(Smits et al. 1999)
pTS100	Cosmid harboring *alkB2* gene	this study
pTS200	Cosmid harboring *alkB1* gene	this study
pKKRubA1(PAO1)	*rubA1* gene in pKKPalk	this study
pKKRubA2(PAO1)	*rubA2* gene in pKKPalk	this study
pKKRubB(PAO1)	*rubB* gene in pKKPalk	this study
pCom8B1(PAO1)	*alkB1* gene in pCom8	(Smits et al. 2002)
pCom8B2(PAO1)	*alkB2* gene in pCom8	(Smits et al. 2002)

Genome Project (PAGP) sequence (Stover 2000). No additional *alkB* homologs were found (Figure 1A, 1B).

The *P. putida* GPo1 alkane hydroxylase requires two electron transfer components for activity: a rubredoxin and a rubredoxin reductase (van Beilen et al. 1994b). We inspected the PAGP database for the presence of corresponding homologs, and found a possible operon consisting of two rubredoxin gene homologs (*rubA1* and *rubA2*) and one rubredoxin reductase gene homolog (*rubB*) (Figure 1C). The two rubredoxins were most closely related to RubA of *Acinetobacter* sp. ADP1 (70–72% protein sequence identity) (Geißdörfer et al. 1995) and showed between 50–65% sequence identity with other rubredoxins involved in alkane oxidation (van Beilen et al. 2002). All extant rubredoxin sequences were more distantly related. The putative rubredoxin reductase was most closely related to the rubredoxin reductase (RubB) of *Acinetobacter* sp. ADP1 (40.1% protein sequence identity) (Geißdörfer et al. 1995) and the rubredoxin reductase (AlkT) of *P. putida* GPo1 (37.1%) (Eggink et al. 1990).

Functional analysis

Based on the observation that the electron transfer components of alkane hydroxylase systems can be exchanged (van Beilen et al. 2002), we have developed recombinant hosts that express two of the three alkane hydroxylase components. To test whether the two PAO1 *alkB* genes encode functional alkane hydroxylases, we used a derivative of *P. fluorescens* CHA0, named KOB2Δ1, which lacks an alkane hydroxylase that allows the wild-type strain to grow on C12-C16 alkanes (Smits et al. 2002). Rubredoxin genes were tested in *E. coli* GEc137[pGEc47ΔG] (van Beilen et al. 2002), while the rubredoxin reductase gene was tested in *E. coli* GEc137[pGEc47ΔT]. To construct pGEc47ΔT, a *Sac*I fragment containing all of the *alkT* gene and part of *alkS* was cloned in pGEM-7Zf(+). The resulting plasmid, pBG211, was digested with *Acc*I and *Hpa*I (blunt), and the *Acc*I site was filled in with Klenow DNA polymerase. Religation resulted in a 355 basepair deletion covering the startcodon and the putative FAD-binding fold. The deletion was then transferred to pGEc47 by homologous recombination as described previously for pGEc47ΔB (van Beilen et al. 1992).

The PAO1 alkane hydroxylase genes *alkB1* and *alkB2* were amplified by PCR from chromosomal DNA using primer combinations AlkBpaFwd (aactggaattcacgatgtttga) and AlkBpaRv2 (ctgcccgaagcttgagctat) and AlkBpaBfw (ggagaattctcagacaatct) and AlkBpaBrv (gaggcgaatctagaaaaaactg) respectively. The *alkB1* PCR fragment was digested with *Eco*RI

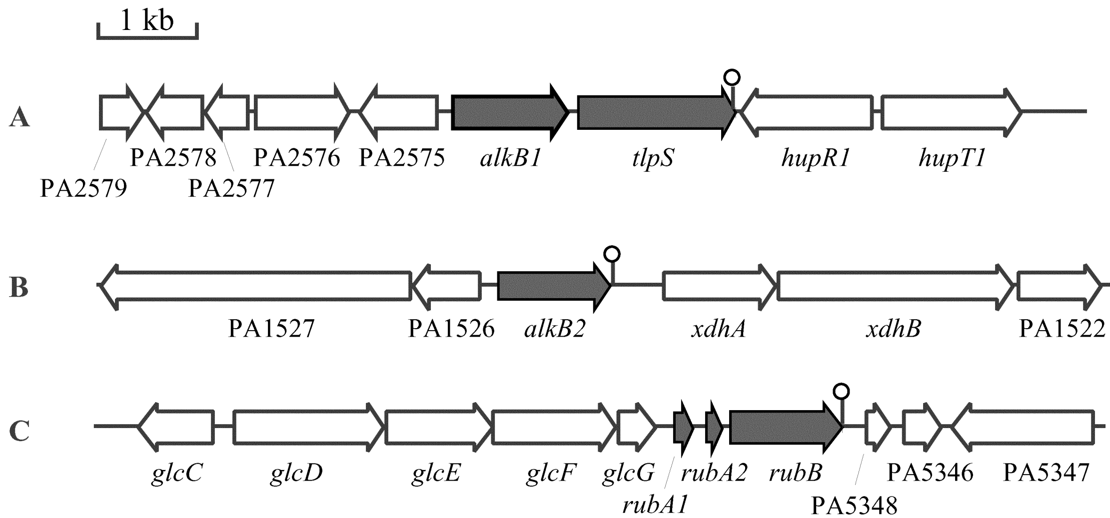

Figure 1. Analysis of open reading frames flanking the *P. aeruginosa* PAO1 genes involved in alkane degradation. The data were obtained from the *P. aeruginosa* genome sequence (www.pseudomonas.com) (Stover 2000).1a: Alkane hydroxylase 1 (*alkB1*; PA2574; from 2911876 to 2910728) and flanking region. Other genes: *tlpS*: methyl-accepting chemotaxis protein; *hupR1*: two-component regulatory system involved in the regulation of the [NiFe] hydrogenase activity; *hupT1*: sensor protein involved in repression of hydrogenase synthesis. PA2577: putative transcriptional regulator of the AsnC family; PA2579: homologous to human tryptophan-2,3-dioxygenase; PA2578, PA2576, PA2575: hypothetical proteins.1b: Alkane hydroxylase 2 (*alkB2*; PA1525, from 1660546 to 1659413) and flanking region. Other genes: PA1527: homologous to yeast chromosome separation protein SMC; PA1526: putative transcriptional regulator of the GntR family; *xdhA*: homologous to the N-terminal domain of eukaryotic xanthine dehydrogenases (XDH); *xdhB*: homologous to internal fragments of eukaryotic XDHs and total XDH of *Rhodobacter capsulatus*; PA1522: homologous to the N-terminal domain of *R. capsulatus* XDH.1c: The *rubA1A2B* gene cluster (*rubA1*: PA5351; from 6019347 to 6019180; *rubA2*: PA5350; from 6018996 to 6018829; *rubB*: PA5349; from 6018777 to 6017623) and flanking region. Other genes: *glcCDEFG*: genes homologous to the *glcRDEFG* genes of *E. coli* involved in glycolate oxidation; PA5348: homologous to histone-like protein HU from *P. aeruginosa*; PA5347, PA5346: hypothetical proteins.

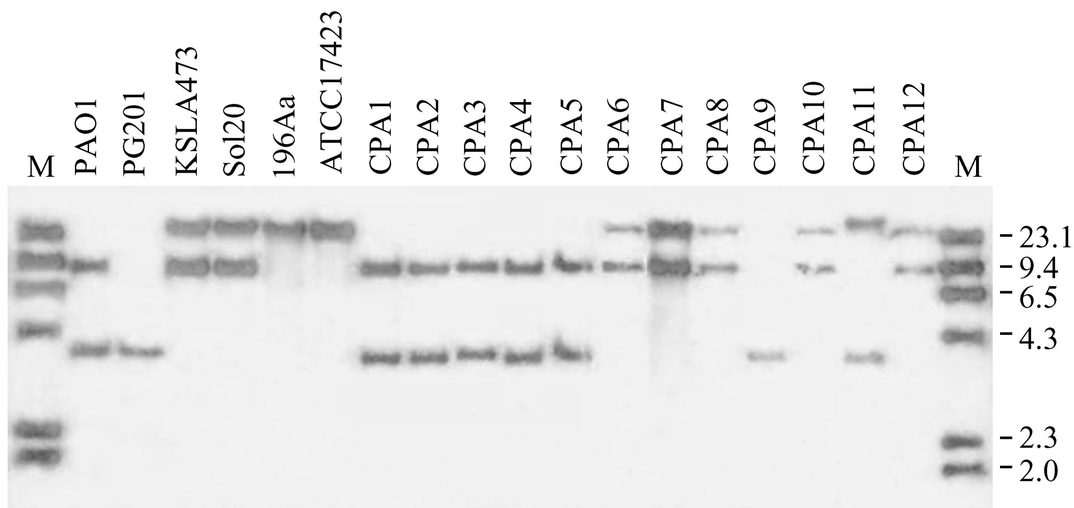

Figure 2. Southern blot of chromosomal DNA of environmental and clinical isolates of *P. aeruginosa* digested with *Bam*HI. As a probe, the 550 bp internal segment of *alkB1* from *P. aeruginosa* PG201 was used. The marker (M) was digoxigenin-labeled lambda DNA digested with *Hind*III; marker sizes are indicated in kb. For strains PG201, 196Aa, ATCC 17423, and CPA.9, bands of around 0.7 kb were also observed but these were too weak to be shown in the figure.

and *Hin*dIII and inserted in pCom8, a *Pseudomonas*-*E. coli* expression vector (Smits et al. 2001). The *alkB2* gene was first cloned between the *Eco*RI and *Xba*I sites of pUC18Sfi (Herrero et al. 1990), and recloned in pCom8 using *Eco*RI and *Hin*dIII. The resulting plasmids pCom8B1(PAO1) and pCom8B2(PAO1) were introduced in *P. fluorescens* KOB2Δ1 as described before (Højberg et al. 1999).

In liquid cultures (E2 minimal medium with 0.05% dicyclopropylketone, a gratuitous inducer of the *alkB* promoter (Grund et al. 1975), and 1% (v/v) alkane), the two recombinant strains KOB2Δ1[pCom8B2(PAO1)] and KOB2Δ1[pCom8B1(PAO1)] grew on C_{12}-C_{16} alkanes, unlike *P. fluorescens* KOB2Δ1 (Table 3). The growth rates of the *alkB2* recombinant were about half of those of the wild-type *P. aeruginosa* PAO1, and slightly higher than those of *P. fluorescens* CHA0, the parent strain of KOB2Δ1. Thus, the two alkane hydroxylases appear to have overlapping substrate specificities, but the two recombinants have different growth rates. The exact substrate range of the two alkane hydroxylases cannot be determined at this point as KOB2Δ1 grows on alkanes longer than C_{18} by virtue of an alkane hydroxylase that has not been characterized yet.

To analyze whether the *P. aeruginosa* PAO1 rubredoxin (*rubA1*, *rubA2*) and rubredoxin reductase (*rubB*) are able to function as electron transfer proteins in alkane oxidation, the three proteins were tested for their ability to replace the corresponding components of the alkane hydroxylase system of *P. putida* GPo1. The *rubA1*, *rubA2* and *rubB* genes were amplified with the primer sets RubA1fwd (cgtggaattccgcccgaggtaa) and RubA1rev (cttcgccggcgcgccgggctcagccg), RubA2fwd (gacgaattcggagggtggcta) and RubA2rev (cgcttctgcagatttcgctcag), and RubBfwd (ccctcacatatgagcgagcgtgccccccctggtaat; introduces a silent mutation in amino acid 5) and RubBrev (ctgcgcaagcttcgtccgacaa). The PCR-fragments were digested with the appropriate restriction enzymes, and cloned separately into the *Eco*RI and *Asc*I, the *Eco*RI and *Pst*I and the *Nde*I and *Hin*dIII sites of pKKPalk, respectively (Smits et al. 2001). The resulting plasmids were transferred to *E. coli* GEc137[pGEc47ΔG] (*rubA1* and *rubA2*) or *E. coli* GEc137[pGEc47ΔT] (*rubB*), and the recombinants were plated on E2 minimal medium containing 0.001% thiamine with *n*-octane as the sole C- and energy source supplied through the gas phase. The positive control was *E. coli* GEc137[pGEc47], while the negative controls were GEc137[pGEc47ΔG] and GEc137[pGEc47ΔT]. Growth on *n*-octane was observed after three days with all constructs and the positive control, while very slight (background) growth was observed with the negative controls. These data show that the two alkane hydroxylases, the two rubredoxins, and the rubredoxin reductase genes encode functional components of alkane hydroxylase systems, and that these enzymes explain the ability of *P. aeruginosa* to grow on alkanes ranging from C12–C16, and most likely also longer alkanes. However, it is still possible that other alkane hydroxylases (not recognized as such in the genome sequence) are present.

Gene organization and flanking genes

Genes that are directly or indirectly involved in the initial alkane oxidation step (*alkB1*, *alkB2*, *rubA1A2B*, *praA*, *rhlABRI*) are distributed over the chromosome of *P. aeruginosa* PAO1 (Stover 2000). Analysis of regions flanking these genes indicates that these have no obvious relation to alkane degradation (Figure 1), with one possible exception, *tlpS*, a gene coding for a methyl-accepting chemotaxis protein (MCP) (Wu et al. 2000) (Figure 1A). The intergenic region between *alkB1* and *tlpS* is only 111 basepairs, and no clear inverted repeats, which could

Table 3. Growth rates of *P. aeruginosa* PAO1, *P. fluorescens* CHA0, and *P. fluorescens* KOB2Δ1 recombinants on alkanes.

Strain	Additional gene	Growth rates (μ) (h^{-1}) C_{12}^{a}	C_{14}	C_{16}
PAO1	-	0.041	0.077	0.10
CHA0	-	0.019	0.019	0.053
KOB2Δ1	*alkB1* (PAO1)	0.004	0.019	0.016
	alkB2 (PAO1)	0.033	0.032	0.046

[a] C_{12}, *n*-dodecane; C_{14},*n*-tetradecane; C_{16},*n*-hexadecane

point towards a *rho*-dependent terminator, could be found between the two ORFs. Directly downstream of the MCP, two clear inverted repeats are located at the end of the *hupR1* gene, which is transcribed in the opposite orientation. TlpS could be involved in chemotaxis towards long-chain *n*-alkanes as *P. aeruginosa* PAO1 and other *P. aeruginosa* strains show strong chemotaxis towards hexadecane (J.B. van Beilen, unpublished data).

Genes involved in the regulation of the *P. aeruginosa* PAO1 *alkB1*, *alkB2* or *rubA1A2B* genes could not be identified, although the *alkB2* gene is preceded by a putative regulatory gene, which is transcribed in the opposite direction.

Southern blot detection of *P. aeruginosa* PAO1 *alkB1/alkB2* and *P. putida* GPo1 *alkB* in environmental and clinical *P. aeruginosa* strains

To study the presence of *alkB* genes in environmental and clinical isolates of *P. aeruginosa*, we carried out Southern blots using the *P. aeruginosa* PG201 *alkB1* probe and a *P. putida* GPo1 *alkBFGH'* probe. Chromosomal DNA was isolated according to standard procedures, digested with *Bam*HI, and blotted onto positively charged nylon membranes (Roche Diagnostics, Rotkreuz, Switzerland). With the GPo1 *alkBFGH'*-probe, homologous DNA fragments were detected only in the four environmental isolates able to grow on *n*-octane, in accordance with previous results (van Beilen et al. 1998), while PAO1, PG201, and the clinical strains did not show any signal with this probe (data not shown). The PG201 *alkB1* probe hybridized with two fragments in all *P. aeruginosa* strains, including the strains that did not grow on long-chain alkanes (Figure 2). There was no correlation between the fragment sizes and the ability of the isolates to grow on alkanes (CPA1 and CPA5, which grew on C_{12}-C_{16} alkanes, possessed the same fragments as CPA2–4, which did not grow on the same alkanes).

Discussion

Most clinical *P. aeruginosa* isolates are able to grow on long-chain *n*-alkanes, in accordance with literature data (Alonso et al. 1999). However, the clinical strains did not grow as well on long-chain alkanes as the environmental strains, perhaps because certain factors necessary for growth on these substrates (for example putative uptake proteins, alkane-solubilizing factors such as PraA and rhamnolipids, or enzymes inolved downstream-metabolism) are not optimally expressed in these strains. All other (environmental) strains were isolated with *n*-alkanes as the carbon- and energy-source, and thus were preselected for their ability to grow well on these substrates. Some of the clinical strains did not grow on alkanes, but still contain both alkane hydroxylase genes. It is possible that these strains have mutations in the *alkB* genes or in other genes that are directly or indirectly involved in the initial alkane oxidation. For example, mutations in the rhamnolipid biosynthesis pathway can reduce or abolish growth on *n*-alkanes (Koch et al. 1991; Ochsner et al. 1994). One strain (CPA3) did not grow on E2 medium with 0.2% citrate, and may have an auxotrophy.

The organisation of genes involved in alkane oxidation in *P. aeruginosa* PAO1 is different from that in other *Pseudomonas* species. *P. putida* strains GPo1 and P1 possess two operons, which contain all genes involved in alkane degradation and are located on a (putative or defective) transposon (van Beilen et al. 2001), while *P. fluorescens* CHA0 contains an operon encoding the alkane hydroxylase, two proteins (homologs of *P. aeruginosa* PraA) that may be involved in alkane solubilization, and a putative outer membrane protein (Smits et al. 2002). The two alkane hydroxylase genes in *P. aeruginosa* PAO1 are not in close proximity of the genes coding for the electron transfer proteins rubredoxin and rubredoxin reductase (*rubA1A2B*) on the genome of *P. aeruginosa* PAO1 (Stover 2000). This gene organisation most closely resembles that of *Acinetobacter* spp. ADP1 and M1. In these strains, the *rubAB* operon is constitutively expressed, whereas the alkane hydroxylases are induced by alkanes (Ratajczak et al. 1998; Geißdörfer et al. 1999; Tani et al. 2001). Interestingly, the genome sequences of *P. putida* KT2440 and *P. fluorescens* Pf-01 both contain a gene cluster encoding a rubredoxin and a rubredoxin reductase that are very similar to the *P. aeruginosa* proteins, although alkane hydroxylases are not present in the genome. Thus, it is possibly that rubredoxins and rubredoxin reductases have additional functions other than electron transfer in alkane hydroxylation, also in *P. aeruginosa* PAO1.

The ability of clinical *P. aeruginosa* strains to grow on long-chain alkanes may be explained by the fact that *P. aeruginosa* strains are not committed to a

pathogenic lifestyle: patients may be infected by clinical strains as well as bacteria picked up from soil or water. Apparently, *P. aeruginosa* frequently encounters alkanes as a carbon-source, and normally retains genes required for alkane degradation. In this respect, *P. aeruginosa* is similar to the pathogens *Burkholderia pseudomallei* K96243 and *Legionella pneumophilia* Philadelphia-1, which are also common soil or water organisms containing alkane hydroxylase homologs (Smits et al. 2002). Interestingly, even the important obligate pathogen *Mycobacterium tuberculosis* and its close relative *M. avium* are able to grow (slowly) on alkanes (Lukins and Foster 1963) and contain functional alkane hydroxylase and rubredoxin genes (Smits et al. 2002; van Beilen et al. 2002). *P. aeruginosa* strains isolated from gasoline spills with C_6 or C_8 as sole carbon source often contain *alk*-genes that are (nearly) identical to the *P. putida* GPo1 *alk*-system (Sotsky et al. 1994; van Beilen et al. 1998; Belhaj et al. 2002). These genes were not present in clinical isolates.

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