

Two genes encoding proteins with similarities to rubredoxin and rubredoxin reductase are required for conversion of dodecane to lauric acid in *Acinetobacter calcoaceticus* ADP1

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Mutants of *Acinetobacter calcoaceticus* ADP1 unable to grow on dodecane, but retaining the ability to grow on lauric acid were isolated after ethylmethanesulphonate (EMS) treatment. This growth deficiency was complemented by a clone from a gene library constructed from chromosomal DNA of the wild-type strain. The complementing DNA mapped in a gene encoding a polypeptide with homology to rubredoxins. The deduced putative rubredoxin amino acid sequence is more similar to related proteins from Gram-positive bacteria than to the *Pseudomonas oleovorans* rubredoxin involved in alkane oxidation. An adjacent gene encodes a protein with similarity to rubredoxin reductase from *Pseudomonas oleovorans* and related NAD(P)-dependent reductases. Disruption of the rubredoxin-encoding gene by insertion of a *Km^r/lacZ* cassette rendered the resulting strain unable to grow on dodecane or hexadecane. This demonstrates that these genes are necessary for alkane degradation. Transcriptional fusion of *lacZ* to the rubredoxin-encoding gene led to low level constitutive β -galactosidase expression, whereas the fusion oriented in the opposite direction was not expressed.

Keywords: *Acinetobacter calcoaceticus*, alkane oxidation, rubredoxin reductase

INTRODUCTION

Many strains of *Acinetobacter calcoaceticus* widely distributed in natural habitats are able to utilize n-alkanes as sole carbon and energy source via the ω -oxidation pathway (Asperger & Kleber, 1991). The primary attack on the chemically inert alkane is achieved by hydroxylation involving either a cytochrome P450 as shown for *A. calcoaceticus* EB104 or a rubredoxin-dependent hydroxylase (Asperger & Kleber, 1991; Leahy & Colwell, 1990; Claus *et al.*, 1980). P450-dependent alkane hydroxylases also occur in *Candida tropicalis* (Sanglard & Loper, 1989), for example. The paradigm for a rubredoxin-dependent alkane hydroxylase is that in *Pseudomonas oleovorans* (van Beilen *et al.*, 1992; Eggink *et al.*, 1988, 1990).

We chose *A. calcoaceticus* ADP1, also known as BD413

(Juni & Janik, 1969; Patel *et al.*, 1975), to study alkane degradation, because it is a mutant of BD4 with a reduced capsule offering the possibility to study involvement of capsular material in alkane degradation in the future. Furthermore, the utilization of aromatic hydrocarbons has been well studied in that strain (DiMarco & Ornston, 1994; Shanley *et al.*, 1994) and has profited from the natural competence of the strain, making genetic constructions feasible which would require considerable technical developments in other strains.

In this article we describe the construction and complementation of an *A. calcoaceticus* ADP1 mutant deficient in dodecane utilization by a gene encoding a protein with high similarity to rubredoxins, which is located next to a gene with high similarity to NAD(P)-dependent reductases from various organisms. We prove by insertional inactivation that the isolated genes are essential for growth on dodecane and hexadecane.

METHODS

Bacterial strains and plasmids. *A. calcoaceticus* strains and plasmids used and constructed in this work are summarized in

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The GenBank accession number for the nucleotide sequence data reported in this paper is Z46863.

Table 1. *A. calcoaceticus* strains and plasmids used and constructed in this study

Strain/plasmid	Relevant genotype/properties	Source/reference
ADP1/BD413	<i>alk</i> ⁺	Juni & Janick (1969)
WH363	<i>alk</i>	This study
WH361	<i>alk::lacZ</i> , Km ^R	This study
WH362	<i>alk::lacZ</i> , Km ^R	This study
pWH1274	Shuttle vector	Hunger <i>et al.</i> (1989)
pWH1266	Shuttle vector	Hunger <i>et al.</i> (1989)
pWH660+ -	Shuttle vectors	This study
pKOK6.1	Source of <i>lacZ</i> /Km ^R cassette	Kokotek & Lotz (1989)
pWH964	<i>alk::lacZ</i> , Km ^R	This study
pWH965	<i>alk::lacZ</i> , Km ^R reverse orientation	This study
pWH1721	<i>alk</i> -complementing	This study
pWH979	<i>alk</i> -complementing	This study

Table 1. *A. calcoaceticus* ADP1 is able to grow on dodecane as sole carbon source. *Escherichia coli* XL1 blue (obtained from Stratagene) and DH5 α (Hanahan, 1983) were used as cloning hosts. pBluescript SKII[±] were obtained from Stratagene and used for isolating nested deletions and for sequencing. pKOK6.1 (Kokotek & Lotz, 1989) was kindly provided by W. Lotz, Erlangen, FRG.

Transformations. *E. coli* XL1 blue was transformed as described by Hanahan (1983) or by electroporation (Stratagene). *A. calcoaceticus* ADP1 was transformed as described by Hunger *et al.* (1989), making use of its natural competency.

Media and growth conditions. Unless noted otherwise, *A. calcoaceticus* and *E. coli* strains were grown in Luria Broth (LB; Sambrook *et al.*, 1989) at 28 °C and 37 °C, respectively. Selective media for ampicillin resistance contained 200 mg ampicillin l⁻¹. Selective growth of *A. calcoaceticus* was in minimal medium supplemented with 'metals 44' (Breuil *et al.*, 1978) solidified with 1.5 % (w/v) agar in Petri dishes, which were kept inverted with a 3 cm² Whatman filter paper soaked with 0.2 ml dodecane placed inside the lid, so that the substrate was provided by diffusion through the gas phase. β -Galactosidase activities expressed in different media, as detailed in the text, were determined from three independently grown cultures according to Miller (1972).

Mutagenesis of *A. calcoaceticus* ADP1. Fresh LB (20 ml) was inoculated with 20 μ l of an overnight culture and grown to an OD₆₀₀ of 0.5. Then 180 μ l ethylmethanesulphonate (EMS) was added. After initial mixing the culture was kept for 3 h at 28 °C without shaking. The viability count dropped to 60 % during treatment. A portion (50 μ l) of culture was used to inoculate 4 ml of fresh LB, which was grown to an OD₆₀₀ of 0.14. The bacteria were then spread on minimal medium plates with 350 mg lauric acid l⁻¹ and incubated for 3 d at 28 °C. After purification by restreaking, single colonies from these plates were screened for lack of growth on dodecane minimal plates.

Preparation of DNA. Small-scale isolation of plasmid DNA from *A. calcoaceticus* and *E. coli* was done by the boiling lysis method (Crouse *et al.*, 1983). Large-scale preparation of plasmid DNA from *E. coli* was performed by the Brij-Doc method (Ausubel *et al.*, 1989). Chromosomal DNA was isolated according to Sambrook *et al.* (1989).

Complementation of *A. calcoaceticus* WH363. Total DNA from *A. calcoaceticus* ADP1 was digested with 0.1 U *Sau*3AI μ g⁻¹

for 2 min at 37 °C and ligated with *Bam*HI-digested, dephosphorylated pWH1266 (Sambrook *et al.*, 1989). This ligation mixture was used to transform *A. calcoaceticus* WH363. Selection was on LB plates with ampicillin and colonies were replica-plated to minimal plates with dodecane and ampicillin. The plasmids obtained were used to transform *E. coli*. They were then reisolated and used to transform *A. calcoaceticus* WH363.

Southern blotting. Restricted total DNA (8 μ g) or restricted plasmid DNA (20 ng) were run on a 1 % agarose gel and blotted to positively charged nylon membrane (Southern, 1975). The probe was prepared using the nick translation kit for biotin-7-dATP (obtained from BRL). A PhotoGene kit (obtained from BRL) was used for detection of signals.

DNA sequencing. DNA sequencing of both strands was done using the nested deletion method on an ALF automated sequencer according to the recommendations of the manufacturer (Pharmacia). Sequences were analysed using the uwgCG software package (Devereux *et al.*, 1984).

RESULTS

Isolation of *A. calcoaceticus* ADP1 mutants deficient in alkane oxidation

Using the mutagenesis protocol described in the Methods section, 2650 candidates able to grow on minimal medium with lauric acid were obtained and screened for inability to grow on dodecane. The former property indicates that degradation of fatty acids by β -oxidation is not affected by the mutations. The screen yielded 27 candidates with a negative phenotype on solid agar plates. Candidate WH363 has a reversion frequency of approximately 10⁻⁷ indicating that the defect for growth on dodecane may be due to a point mutation. Therefore, this mutant was used for complementation by a gene library.

Complementation of *A. calcoaceticus* WH363 for growth on dodecane

Partially *Sau*3A-digested total DNA from *A. calcoaceticus* ADP1 was inserted into *Bam*HI-cleaved pWH1266 and used to transform *A. calcoaceticus* WH363. The trans-

formants were selected for growth on LB with ampicillin and replica-plated on minimal medium with ampicillin and dodecane. Four candidates forming large colonies on these plates were further analysed. After preparation and passage of their plasmids through *E. coli* and retransformation of WH363, one of these plasmids was found to confer the ability to grow on dodecane minimal plates. Preparation and restriction analysis of the recombinant plasmid, called pWH1721, revealed a 17 kb insertion. When compared to wild-type ADP1 transformed with the vector pWH1266, WH363 transformed with pWH1721 grew to give larger colonies on dodecane minimal plates, indicating that the insertion may have a positive gene dosage effect for growth on dodecane under these conditions.

Deletion analysis of the complementing insert

We have constructed a new shuttle vector for *A. calcoaceticus* and *E. coli* to allow deletional analysis for complementation to be combined with the nested deletion approach for sequencing. For this purpose, pBluescript SKII[±] was partially digested with *Pvu*II and ligated with the *Pvu*II–*Hinc*II fragment from pWH1266 carrying the *Acinetobacter ori* (Hunger *et al.*, 1989). The resulting plasmids, called pWH660[±], contained the respective fragment inserted in the *Pvu*II site at position 529 in the *lacZ* reading frame. pWH660⁺ was used to insert a 12 kb *Bam*HI fragment from pWH1721 resulting in pWH979, which complemented strain WH363 for growth on dodecane. Unidirectional deletions of the insert with a mean spacing of 1 kb were generated and tested for complementation of WH363 for growth on dodecane as sole carbon source. The smallest fragment complementing WH363 for growth on dodecane minimal plates contained 3.2 kb.

Nucleotide sequence of the complementing DNA

Nested deletions of the second smallest complementing insert of 3.7 kb were obtained, and the DNA was sequenced on both strands. Analysis of the sequence revealed two ORFs with similarities to genes involved in alkane degradation of *Ps. oleovorans* (see below). The genetic organization of that region is shown in Fig. 1. Two of the nested deletions mapped in this region are also shown in Fig. 1. Deletion of the right-hand 600 bp still yielded a complementing fragment, whereas deletion of the right-hand 1430 bp did not. Thus, the ability to complement the dodecane growth defect is encoded in the left half of the respective DNA. The nucleotide sequence of the DNA between the *Fsp*I and *Hae*II sites is shown in Fig. 2, along with the deduced amino acid sequences of two ORFs. The ORFs scored positively for elevated protein coding probability in the TESTCODE program and in a codon preference analysis using the codon usage table derived from *Acinetobacter* genes (Fickett, 1982; Pearson & Lipman, 1988; White *et al.*, 1991).

Restriction sites deduced from the sequence are shown in Fig. 1 and were used to confirm the origin of the complementing DNA from the chromosomal ADP1

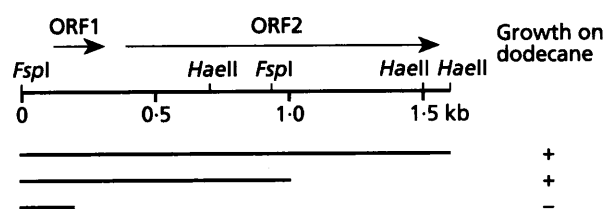


Fig. 1. Restriction map, genetic organization and complementation capacity of 1530 bp *Acinetobacter* DNA. ORF1 and ORF2 are indicated by arrows. Relevant restriction sites are shown. Fragments obtained after nested deletions of the originally cloned DNA are indicated by lines below the map (see Results for details). + indicates the ability of strain WH363 transformed with plasmids containing the DNA indicated by the respective lines to grow on dodecane.

DNA by Southern blotting using the *Fsp*I fragment as a probe (data not shown). DNA between the *Fsp*I sites and an 800 bp *Hae*II fragment corresponded with fragments of the same size in the chromosomal DNA. Thus, the region between the *Fsp*I and *Hae*II sites represents a contiguous piece of the ADP1 DNA (Fig. 1). Probing with *Bst*NI and *Sau*96I fragments from pWH979, including the DNA shown in Fig. 1, yielded fragments of different size from the plasmid than from the chromosome. This result indicates that disruptions of the insert must have occurred within 1200 bp upstream and 500 bp downstream of the region shown in Fig. 1.

Both ORFs on the complementing DNA are preceded by putative ribosome binding sites, as judged by sequence comparison with other *Acinetobacter* genes. ORF1 contains the sequence GGAG (commencing at position 113 in Fig. 2) spaced 6 nucleotides from the putative start codon ATG. ORF2 does not contain a putative ribosome binding site in front of the first ATG (commencing at position 312 in Fig. 2), but instead, the sequence GGAG precedes the second ATG (commencing at position 372 in Fig. 2) with a spacing of 7 nucleotides.

Similarities of the amino acid sequences encoded by ORF1 and ORF2 with known proteins

Sequence similarities were searched in the GenBank, EMBL and Swissprot databases. The protein encoded by ORF1 is homologous to all rubredoxins present in these databases. A sequence alignment is shown in Fig. 3. The highest similarity was found with a rubredoxin from *Clostridium thermosaccharolyticum* (Meyer *et al.*, 1990) with 63% identical residues. An identity of 60% was found for rubredoxin from *Chlorobium thiosulfatophilum* (Wooley & Meyer, 1987) and 57% identity with the one from the archaeon *Pyrococcus furiosus* (Blake *et al.*, 1991). These results reiterate the view that rubredoxins are highly conserved proteins. A rubredoxin involved in alkane oxidation has been characterized in *Ps. oleovorans* (Kok *et al.*, 1989). It has twice the molecular mass of the other rubredoxins, resulting probably from a gene duplication. The functional part is located in the C-terminal half, which has 59% identity with the product of ORF1.

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1  TGC G C A A A A T C G A A G C C A A A T A T G A G C A C T G A T G A T G T T A T A T T C A G A G T C G T T A T T A C A T T A T A T G G A A T T G T T T T A C T G A A A G G C A
91  G C T G A A G C G A A C T T A T C A G G T T G G A G A A T C A A A T G A A A A G T A T C A A T G T A T C G T T T G T G G T T G A T T A T G A T G A A G C C G A A G C T G G C
      M K K Y Q C I V C G W I Y D E A E G W P
181  C A C A A G A C G G C A T T G C A C C A G G G A C A A A A T G G G A A G A T A T T C C T G A T G A C T G G A C T T G C C C T G A C T G C G G C G T T C A A A G T T G A T T T T G
      Q D G I A P G T K W E D I P D D W T C P D C G V S K V D F E
271  A A A T G A T C G A A G T T T A A T C G G T A T T T C A A A G A C C A T G T C C A T G A A A C A T G G T C T T T T A A T T T A A T C T T A G T T T T A T T A C C T T A C T A C
      M I E V *
361  G G A G A A T A A T A T G C A C C C A A T C G T C A T T A T C G G A T C A G G A A T G G C A G T T A T A C C C T T G C T C G T G A A T T C G C A A G C T T A A T C C T G A A C
      M H P I V I I G S G M A G Y T L A R E F R K L N P E H
451  A T G A A C T T G T C A T G A T T T G T G C T G A C G A T G C A G T G A A T T A T G C A A A C C A A C C T T A T C T A A T G C G C T T T C T G G C A A C A A G C A C C A G A A C
      E L V M I C A D D A V N Y A K P T L S N A L S G N K A P E Q
541  A G A T T C C A T T A G G T G A T G C A G A A A A A T G A G T A C C C A G C T C A A G T G C A A A T T C G T C A G A C A C T T G G G T A A A G C C A T T A A T C C A G A A
      I P L G D A E K M S T Q L K L Q I S S D T W V K A I N P E T
631  C T C A T G A A T T A A A A T T A G A A A A A T G G T C A G A A A C G A T T C A G C C C T A T T C C A A A C T G G T T C T G G C T G T A G C G C C A A T C C A A C C G T T
      H E L K L E K N G Q E T I Q P Y S K L V L A V G A N P T R L
721  T A G C C A T T G C C G T G A T G G T A G T G A T A T T C A T G T G G T G A A C T C T C T T A T C G A T T A T C G C G C C T T T C G T G A A A A T C T T G C A A A C G T C
      A I A G D G S D D I H V V N S L I D Y R A F R E N L A K R Q
811  A A G A T A A G C G T G T T G T C A T T C T A G G T G C A G G T C T T A T T G G T T G T G A A T T T G C C A A T G A T T A C A G C A T A C T G G G C A T C A G G T G A C A G T C A
      D K R V V I L G A G L I G C E F A N D L Q H T G H Q V T V I
901  T C G A C T T G T C T C C T C G C C C A C T G G G T C G T C A T T A C C T G C G C A T A T T G C A G A T G C T T T T C A G A A A A T C T G G A G A A G C G T A T T C A C T T T G
      D L S P R P L G R L L P A H I A D A F Q K I W R K R I H F V
991  T A C T T T C A A C C A C A G T A G A A A A G T A T C T A A A A T T A C G A T G G T C A A G A T T A T G C T G T C A C A C T G G C A A A T G G T C A G A C G T A G T G G C C G
      L S T T V E K V S K I N D G Q D Y A V T L A N G Q T L V A D
1081  A C A T T G T T T A T C T G C A A T C G G A T T A C A A C C A A C A T T G A T C T G G C A A A C A C G C A G G C G T G C A T A C C A G T C G A G G T A T T T T G A C T A A T A
      I V L S A I G L Q P N I D L A K H A G V H T S R G I L T N S
1171  G C C T C C T A G A A A C C A A C C T T G A A G A C A T C T A T G C A A T A G G C G A C T G T G C T G A A G T T A A T G G A A C C T T G C T C C C T A C G T G A T G C C G A T C A
      L L E T N L E D I Y A I G D C A E V N G T L L P Y V M P I M
1261  T G C A G C A G G C C C G C G C C C T C G C G A A A A C C T T G A G T G G T G A A A C A C A C A T G T T C A C T A T C C T G C C A T G C A G T T G C T G T A A A A C G C C T G
      Q Q A R A L A K T L S G E T T H V H Y P A M P V A V K T P A
1351  C A G C C C C G C T C A C C G T A C T T C C T G T A C C T G T G G A T G T A G A T G T A A A C T G G G A A C A G A A G A A T T T G A A G A T G G T A T G C T T G C C A A G C T A
      A P L T V L P V P V D V D V N W E T E E F E D G M L A K A I
1441  T C G A C A A T A C C G A T A C A C T C A G A G G T T T T G T A C T A T T A G G T G C T A C G G C G G T A A A C A G C G C C T G A C A C T C A C C A A A C T G G T T C C A G A C T
      D N T D T L R G F V L L G A T A G K Q R L T L T K L V P D L
1531  T G A T T C C C G C A C A G C T T T A A G G T T T A A A T A G C A A G G A C A T T C A T C T G A G T G T C C T T T T A G G A A C G G A T C A T A A A T G A A T A G C G C T
      I P A Q L *

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Fig. 2. Nucleotide and deduced amino acid sequence of genes encoding rubredoxin and rubredoxin reductase of *A. calcoaceticus* ADP1. The encoded amino acid sequences are shown using one letter abbreviations under the nucleotide sequence. Putative ribosome binding sites are indicated in underlined bold print.

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Ac  MKKYCCIVCGIYDEAEQGPQDGTAFGTRFEDIIPDDWICPDGCVSKVDFEMIEV
Cs  MEKWCCIVCGIYDPEVGDPTQNIFFGTRFEDLPDDWVCPDCCVGGKQDFEKI
Ct  MOKYVCSVCGIYDEADGDEDDIIDGTFEDLPDDWVCPVCGVDDKDLFEPES
Po  YLKWICITCGIYDEADGDEAEQFIFGTRFEDIIPDDWICPDGCAATKEDYVLYBEK
Pf  AKKWKICGIIYDEADGDDNGLISGTRFEDLPDDWVCPICCAKSEFEKLED

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Fig. 3. Homology of rubredoxin sequences from various micro-organisms. When at least three identical amino acid residues occur at a given position they are shown in reversed-out print. Abbreviations: Ac, *A. calcoaceticus* (this work); Cs, *Clostridium thermosaccharolyticum* (Meyer *et al.*, 1990); Ct, *Chlorobium thiosulfatophilum* (Woolley & Meyer, 1987); Po, *Ps. oleovorans* (C-terminal 55 amino acids are shown) (Kok *et al.*, 1989); Pf, *Py. furiosus* (Blake *et al.*, 1991).

The protein encoded by ORF2 is homologous to four oxidoreductases involved in the degradation of hydrocarbons. The sequence alignment is shown in Fig. 4. The identities of residues are: 28% with terpredoxin reductase from *Pseudomonas* spp. involved in α -terpineol degradation (Peterson *et al.*, 1992); 27% with rubredoxin reductase from *Ps. oleovorans* involved in alkane degradation (Eggink *et al.*, 1990); 26% with putidaredoxin reductase from *Ps. putida* involved in camphor degradation (Peterson *et al.*, 1990); and 25% with the ferredoxin reductase of the toluene dioxygenase system from *Ps. putida* (Zylstra *et al.*, 1989). Thus, we propose that ORF2 encodes the rubredoxin reductase involved in alkane degradation in *A. calcoaceticus* ADP1.

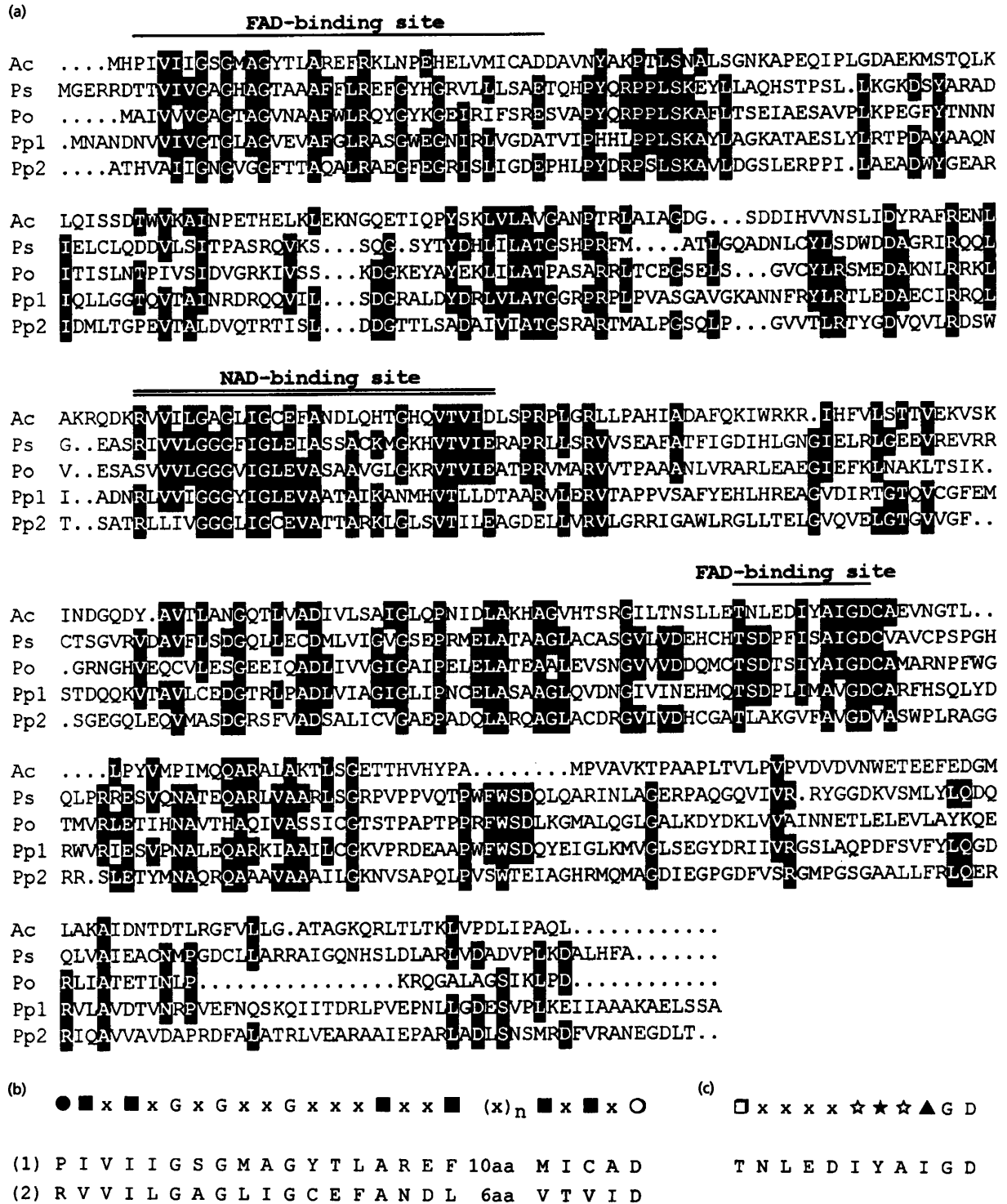


Fig. 4. Homology of NAD(P)-dependent reductases from different bacteria. (a) Five primary structures are aligned to give maximal identity of the residues. Three or more identical residues per position are given in reversed-out print. The two consensus sequences for the FAD-binding sites and the motif for the NAD-binding site are indicated above the sequences. Abbreviations: Ac, *A. calcoaceticus* (this work); Ps, *Pseudomonas* spp. (Peterson *et al.*, 1992); Po, *Ps. oleovorans* (Eggink *et al.*, 1990); Pp1, 2 = *Ps. putida* camphor- and toluene-specific reductases, respectively (Peterson *et al.*, 1990; Zylstra *et al.*, 1989). (b) Consensus sequence of the dinucleotide binding site (Wierenga *et al.*, 1986). The sequence elements for (1) FAD- and (2) NAD-binding are shown below the consensus sequence. Symbols: x, any amino acid; ●, K, R, H, S, T, Q or N; ■, A, I, L, V, M or C; ○, D or E. (c) Sequence of the motif found only in FAD-binding proteins (Eggink *et al.*, 1990). The corresponding sequence of the ORF2-encoded protein is shown below the consensus sequence. Symbols: x, any amino acid; □, T or M; ☆, I, V, A or L; ★, Y, W or F; ▲, I, V or A.

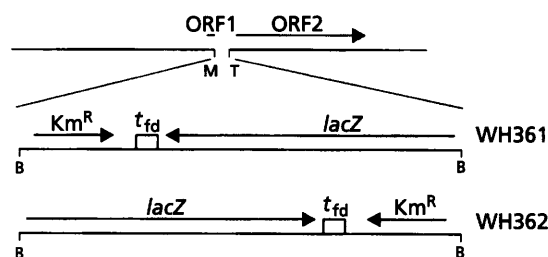


Fig. 5. Chromosomal organization of the insertion strains *A. calcoaceticus* WH361 and WH362. For details of construction, see Results. The *lacZ* cassette of pKOK6.1 used here is the same as that described by Kokotek & Lotz (1989), but *lacZ* is preceded by stop codons in each reading frame. M, T and B indicate the restriction sites of *MscI*, *Tth111I* and *BamHI*, respectively.

Chromosomal disruption of ORF1 and ORF2

We have inactivated ORF1 and ORF2 on the ADP1 chromosome by insertion of a Km^R cassette. For that purpose, the 2.1 kb *DraI* fragment from pWH1721 containing ORF1 and ORF2 was cloned into pBluescript SKII+, resulting in pWH963. This plasmid was cleaved with *MscI* and *Tth111I*, and the Km^R cassette was excised with *BamHI* from pKOK6.1 (Kokotek & Lotz, 1989). The protruding ends of both fragments were filled in using Klenow polymerase and ligated to yield pWH964 and pWH965, which differ with respect to the orientation of the insertion. The inserts from both plasmids were excised with *SacI* and *ApaI* and transformed into *A. calcoaceticus* ADP1 using natural competency. Transformants were selected on LB plates supplemented with 15 mg kanamycin l^{-1} . Integration of the cassettes into ORF1 and ORF2 was confirmed by Southern blotting. The resulting strains were called WH361 and WH362. The gene disruption is shown in Fig. 5.

Alkane utilization phenotypes of the mutant *A. calcoaceticus* strains

The mutant strain WH363 showed clear phenotypes when grown on solid media. We consistently observed no growth on dodecane, but growth like that of the wild-type on hexadecane and lauric acid. In liquid media, however, growth on dodecane was about the same for WH363 and wild-type (see Fig. 6). WH361 and WH362 containing the gene disruptions, in contrast, had a clear growth defect on both dodecane and hexadecane in liquid media, while growth on lauric acid was not affected. The same result was obtained on solid growth medium and establishes clearly that the genes isolated in this study are necessary for consumption of long chain length alkanes.

Regulation of expression of the rubredoxin gene-*lacZ* transcriptional fusion

The β -galactosidase activities determined in strains ADP1, WH361 and WH362 grown in minimal medium with 10 mM succinate and in the same medium with 0.25 mM

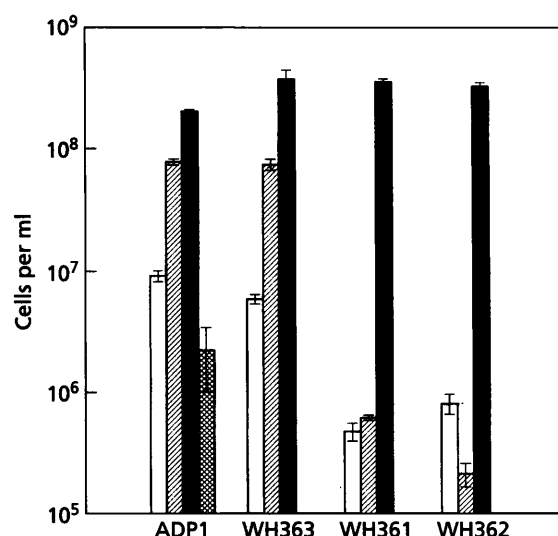


Fig. 6. Growth of *A. calcoaceticus* strains ADP1, WH363, WH361 and WH362 on 0.67 mM dodecane (\square), 0.5 mM hexadecane (\square) and 0.67 mM lauric acid (\blacksquare) as sole carbon source. \square , no addition. The cultures (50 ml) were incubated at 28 °C and shaken at 180 r.p.m. Alkanes were added to the minimal medium in emulsified form after sonification. Titres of the cultures grown to saturation were determined by plating diluted aliquots on LB plates. Cultures were inoculated to a titre of 3×10^6 cells ml^{-1} and reached stationary phase after about 12 h. Standard deviations were calculated from three titres determined after 27, 31 and 50 h of incubation.

hexadecane are shown in Table 2. No *lacZ* expression was found in ADP1 and WH361, in which the reporter gene is oriented in the opposite direction to the rubredoxin gene. A low level of constitutive expression was found in WH362. A similar result was obtained when succinate was replaced by ethanol as a potentially non-regulative carbon source (not shown).

DISCUSSION

It has been reported that some strains of *A. calcoaceticus* can grow on linear alkanes of variable length (Asperger & Kleber, 1991). The strain ADP1 combines the ability to grow on dodecane with natural competency (Juni & Janik, 1969), which seemed to be of advantage for identification of genes by complementation. The Southern analysis indicates consistency of restriction fragment lengths only between the *FspI* and *HaeII* sites (Fig. 1). Thus, only about 1600 bp out of the 17 kb of the original insertion in the complementing plasmid turned out to be a continuous piece from the chromosome. This is most probably related to the mechanism of DNA uptake leading to the formation of mixed plasmids when gene libraries are transformed (for a detailed discussion, see Palmen *et al.*, 1993). It is, therefore, advisable to use electroporation for DNA transfer, as has been described for non-competent *A. calcoaceticus* strains (Ehrt *et al.*, 1994). Nucleotide sequencing of the insertion revealed two *HaeII* sites close together (positions 1498 and 1612 in

Table 2. Expression of β -galactosidase in different *A. calcoaceticus* strains

Strain	β -Galactosidase activity (Miller Units) after growth in minimal media supplemented with:	
	Succinic acid (10 mM)	Succinic acid (10 mM) + hexadecane (0.25 mM)*
ADP1	-0.2 ± 0.3	-0.2 ± 0.3
WH361	-0.1 ± 0.3	0.0 ± 0.3
WH362	15.6 ± 0.8	14.2 ± 0.8

* The medium was sonicated after the addition of hexadecane.

Fig. 2). The small *Hae*II fragment contains the last 16 codons of ORF2. Its presence in the chromosome has not been formally confirmed. However, the last 16 amino acids do not considerably influence the sequence analysis of ORF2.

The deletional analysis (Fig. 1) suggests that ORF1 complements the mutation in WH363 indicating that a putative rubredoxin is involved in dodecane oxidation. The first rubredoxin involved in alkane oxidation by *A. calcoaceticus* was found in a culture grown on a mixture of different long chain alkanes as sole carbon source (Aurich *et al.*, 1976). Subsequently, the corresponding rubredoxin reductase was isolated (Claus *et al.*, 1979). These results agree well with our finding that genes encoding proteins with similarity to both enzymes are necessary for growth on long chain alkanes. Their functional requirement is also reflected by their proximity on the chromosome.

The lack of apparent promoter and transcriptional terminator structures flanking these genes suggests that they may be part of a larger operon. This has also been found for the alkane utilization genes in *Ps. oleovorans*, which are organized in two operons. However, the genetic organization of genes for alkane degradation must be different in ADP1, because the putative rubredoxin and rubredoxin reductase genes are located in different operons in *Ps. oleovorans*, whereas they are adjacent and appear to be in the same operon in ADP1 (von Beilen *et al.*, 1992; Kok *et al.*, 1989). Furthermore, the *Ps. oleovorans alkG* gene, encoding rubredoxin, contains an apparent gene duplication leading to a rubredoxin with twice the molecular mass of the one from *A. calcoaceticus*. The *Ps. oleovorans* genes are located on the OCT plasmid, whereas the DNA analysed in this study from *A. calcoaceticus* ADP1 appears to be located on the chromosome (A. Ratajczak & W. Hillen, unpublished). Thus, the similarities of the primary structures (see Figs 3 and 4) are not reflected in the genetic organization of these genes in both organisms. It is also remarkable to note that the rubredoxin with highest similarity to the one from *A. calcoaceticus* originates from *Clostridium thermosaccharolyticum*.

Many reductases, five of which are shown in Fig. 4(a), have cosubstrate binding sites in common. These are indicated in Fig. 4(b) and correspond to dinucleotide

binding consensus elements typical for NAD⁺ and FAD binding sites. They have been determined on the basis of sequence (Wierenga *et al.*, 1986; Eggink *et al.*, 1990) and structural (Karplus & Schulz, 1987; Schierbeek *et al.*, 1989) comparisons.

At present we do not have an explanation for the different phenotypes of WH363 on agar plates and in liquid media. We speculate that the toxicity of the alkanes (Asperger & Kleber, 1991) may have different effects on growth in solid and liquid media. This is supported by the fact that only dodecane, the more toxic alkane compared to hexadecane, leads to the growth deficiency on solid medium. The important experimental difference may be that the alkanes are provided through the gaseous phase for growth on solid media, while they are mixed by sonication with the liquid media. Thus, it may be speculated that *A. calcoaceticus* has a more efficient defence mechanism in liquid than on solid medium. This would imply that the presumed point mutation in the rubredoxin gene of WH363 leads to a reduced activity of the encoded product. Therefore, the insertions were generated and, indeed, resulted in clear and consistent phenotypes on both media. However, these mutants may have a polar effect on the expression of any genes which may be located downstream from the sequenced region in the operon.

The insertion strains indicate clearly, that the putative rubredoxin gene is expressed, as the β -galactosidase activity in the insertion with the parallel orientation was at least 50-fold higher than in the opposite orientation. Under the conditions tested, transcription of the rubredoxin gene was constitutive and leads to a low level of mRNA as indicated by the low level of β -galactosidase.

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