The Genes \(rubA\) and \(rubB\) for Alkane Degradation in \(Acinetobacter\) sp. Strain ADP1 Are in an Operon with \(estB\), Encoding an Esterase, and \(oxyR\)

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Alkanes are oxidized in \(Acinetobacter\) sp. strain ADP1 by a three-component alkane monoxygenase, composed of alkane hydroxylase, rubredoxin, and rubredoxin reductase. \(rubA\) and \(rubB\) encode rubredoxin and a NAD(P)H-dependent rubredoxin reductase. We demonstrate here that single base pair substitutions in \(rubA\) or \(rubB\) lead to defects in alkane degradation, showing that both genes are essential for alkane utilization. Differences in the degradation capacity for hexadecane and dodecane in these mutants are discussed. Two genes, \(estB\) and \(oxyR\), are located downstream of \(rubB\), but are not necessary for alkane degradation. \(estB\) encodes a functional esterase. \(oxyR\) encodes a LysR-type transcriptional regulator, conferring resistance to hydrogen peroxide. \(rubA\), \(rubB\), \(estB\), and \(oxyR\) constitute an operon, which is constitutively transcribed from a \(σ^{70}\) promoter, and an \(estB-oxyR\) containing message is also transcribed from an internal promoter.

Within gram-negative bacteria, \(Acinetobacter\) and \(Pseudomonas\) are the most important genera for the degradation of \(n\)-alkanes in the environment. \(Pseudomonas oleovorans\), which is able to use medium-chain alkanes ranging from hexane to dodecane as the sole source of carbon and energy (56), contains the \(alk\) genes necessary for the conversion of alkanes to acyl coenzyme A separated into two regions on the OCT plasmid. The \(alkBFGHJKL\) genes constitute an operon and encode the alkane hydroxylase, two rubredoxins, an aldehyde dehydrogenase, an alcohol dehydrogenase, an acyl coenzyme A synthetase, and an outer membrane protein of unknown function. The second locus contains \(alkS\) and \(alkT\), encoding a LuxR-UhpA-like regulator of \(alk\) operon transcription and rubredoxin reductase. In the initial degradation step, alkane is converted to the primary alcohol in \(P. oleovorans\) by a three-component alkane monoxygenase, composed of alkane hydroxylase, rubredoxin, and rubredoxin reductase. Several alkane oxidation pathways have been described for \(Acinetobacter\) sp. An alkane dioxygenase is involved in degradation of long-chain alkanes (\(C_{13}\) to \(C_{44}\)) in \(Acinetobacter\) sp. strain M-1 (24). The alkane hydroxylase in some \(Acinetobacter\) strains able to grow on medium-chain alkanes is a cytochrome P-450 (2), while a \(rubA\) neighboring gene, \(rubB\), encodes the rubredoxin reductase, as hypothesized on the basis of sequence similarity to NAD(P)H-dependent dehydrogenases (9).

In this paper, we describe sequence analysis of the DNA downstream of \(rubB\), revealing two open reading frames (ORFs), \(estB\) and \(oxyR\). We demonstrate that \(rubA\) and \(rubB\) are necessary for alkane degradation in ADP1 and that they form an operon together with \(estB\) and \(oxyR\). \(estB\) and \(oxyR\) encode a functional esterase and a peroxide response regulator, respectively, which are not necessary for alkane degradation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type \(Acinetobacter\) sp. strain ADP1 was formerly classified as \(A. calcoaceticus\) ADP1 and is synonymously called \(Acinetobacter\) sp. strain BD413 (17, 34).

**General methods.** \(Escherichia coli\) and \(Acinetobacter\) were transformed as described previously (13, 28) or electroporated with a Gene Pulser (Bio-Rad Laboratories, Munich, Germany). Total DNA was prepared according to the method of Ausubel et al. (4). Small-scale preparations of plasmids were made by the boiling lysis method (15); large-scale preparations were done with the Nucleobond kit (Macherey-Nagel, Düren, Germany). Total RNA was isolated with the RNasy Mini kit from Qiagen (Hilden, Germany).

Media and growth conditions. \(E. coli\) was grown at 37°C and \(Acinetobacter\) was grown at 28°C. Ampicillin was used at 100 mg/liter for \(E. coli\) and 300 mg/liter for \(Acinetobacter\). Kanamycin and chloramphenicol were used for \(Acinetobacter\) at 5 and 10 mg/liter, respectively. Growth with alkanes as the carbon source was monitored on plates as described previously (29). Indicator plates for esterase activity contained 1.5% (vol/vol) tributyrin in Difco nutrient broth (NB) (Difco, Detroit, Mich.). Tributyrin was added as a 50% (vol/vol) emulsion in 5% (wt/vol) gum arabic (Sigma, Steinheim, Germany) after sonication with a Branson Sonifier B12 (Braun, Melsungen, Germany).

**DNA sequence analysis.** Nucleotide sequences on both strands were determined by the dideoxy chain termination method (33) with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) and [\(α^{-32}\)]PdATP. Successive deletions were done by using the double-stranded nested deletion kit (Pharmacia, Freiburg, Germany). Sequences were analyzed with the GCG software package (6). The GCG program Fasta was used to determine the similarity (percentage of identical amino acids) over the whole protein sequence. Database searches were done by using the Blast 2.0 software offered by the National Center for Biotechnology Information (26a).
The linear DNAs were used to transform within the vector sequence to prevent integration of the circular plasmids, and steps (Fig. 1 and Table 1). The integration plasmids were cut with oxyRble1. The 4.7-kbp resulting in pWH891SK3, pWH963

Plasmids

pAKA22

Ap′ Tc′; 11.6-kbp Sau3A fragment of Acinetobacter DNA containing estB in BclI site of pUN121

pBluescript II SK+

Ap′

Stratagene, La Jolla, Calif.

pKOK6.1

Ap′ Km′; promoterless lacZ

pKT210

Cm′

5

pWH891

Ap′; 10,855-bp Acinetobacter DNA

pWH891∆I

Ap′; pWH891, deletion of AflII fragment (4,348 bp) containing ORFX, rubA, rubB, and estB

pWH891∆NcoI

Ap′ oxyR

pWH963SK

Ap′

This study

pWH963SK3

Ap′; HindIII-PvuII fragment (3,079 bp) from pWH891 in EcoRV site of pBluescript II SK+

pWH963SK3K0RFX: lacZ

Ap′ Km′; ORFX(MscI): lacZ fusion

pWH963SK6 and SK6i

Ap′; NruI-EcoRV fragment (2,149 bp) from pWH891 in EcoRV site of pBluescript II SK+

pWH963SK7i

Ap′ Km′; estB(∆MscI-Styl): lacZ fusion

pWH963SK7i

Ap′ HindIII-BamHI fragment (4,727 bp) from pWH891 in EcoRV site of pBluescript II SK+

pWH963SK7oxyR: Cm′

Ap′ Km′; oxyR(∆AflII-NdeI): Cm′ fusion

pWH963SK7oxyR: lacZ

Ap′ Km′; oxyR(∆AflII-NdeI): lacZ fusion

pWH963AMH

Ap′; MscI-Oral fragment (1,382 bp) from pWH91 in EcoRV site of pBluescript II SK+

pWH963AMHrubB: lacZ

Ap′ Km′; rubB(∆MscI-NruI): lacZ fusion

Southern and Northern hybridization. Southern and Northern hybridization were performed as described below (31). Total RNA (15 μg) was incubated for 5 min at 80°C and hybridized for 5 min with the 5'-end-labeled primer (50 fmol) at 37°C. The reaction mixtures containing 9U ofavian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) were incubated for 45 min at 37°C. One-third of the reaction mixtures containing 9U ofavian myeloblastosis virus reverse transcriptase were prepared by PCR with [α-32P]dATP amplifying fragments from nucleotides -32P]dATP amplifying fragments from nucleotides

TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Acinetobacter</strong></td>
<td></td>
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</tr>
<tr>
<td>ADP1</td>
<td>Wild type</td>
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</tr>
<tr>
<td>WH362</td>
<td>rubA(ΔMscI-Th111I):lacZ Km′</td>
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<tr>
<td>WH363</td>
<td>EMS mutant, alk</td>
<td></td>
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<tr>
<td>WH380</td>
<td>ORFX(MscI): lacZ Km′</td>
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</tr>
<tr>
<td>WH382</td>
<td>rubB(ΔMscI-NruI): lacZ Km′</td>
<td></td>
</tr>
<tr>
<td>WH384</td>
<td>estB(ΔMscI-Styl): lacZ Km′</td>
<td></td>
</tr>
<tr>
<td>WH384oxyR</td>
<td>estB(ΔMscI-Styl): lacZ oxyR(ΔAflII-NdeI) Km′</td>
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</tr>
<tr>
<td>WH386</td>
<td>oxyR(ΔAflII-NdeI): lacZ Km′</td>
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</tr>
<tr>
<td>WH432</td>
<td>EMS mutant, alk</td>
<td></td>
</tr>
<tr>
<td>WH433</td>
<td>EMS mutant, alk</td>
<td></td>
</tr>
<tr>
<td>WH434</td>
<td>EMS mutant, alk</td>
<td></td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>recA1 endA1 supE44 thi-1 endA1 supE44 gyrA96 thi hsdR17 (rK− mK−) relA1 d80ΔlacZΔM15 (lacZYA-aargF)ΔU169</td>
<td></td>
</tr>
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<td>pBluescript II SK+</td>
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</tr>
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<td></td>
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<tr>
<td>pWH963AMH</td>
<td>Ap′; MscI-Oral fragment (1,382 bp) from pWH91 in EcoRV site of pBluescript II SK+</td>
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</tr>
<tr>
<td>pWH963AMHrubB: lacZ</td>
<td>Ap′ Km′; rubB(ΔMscI-NruI): lacZ fusion</td>
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</tbody>
</table>

**Primer extension.** Primer extension reactions were performed as described previously (37). Total RNA (15 μg) was incubated for 5 min at 80°C and hybridized for 5 min with the 5'-end-labeled primer (50 fmol) at 37°C. The reaction mixtures containing 9U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) were incubated for 45 min at 37°C. One-third of the volume was loaded onto a sequencing gel and analyzed with a PhosphorImager (Fujifilm; BAS-1500). The sequence of the rubA-specific primer is 5'-CTT GTGCCAGCTCTTGCC-3'.

**Chromosomal disruption of ORFX, rubB, estB, and oxyR.** For construction of WH362 (rubA-lacZ) see the article by Geißdörfer et al. (9). DNA fragments harboring the gene to be inactivated were excised from pWH891, filled in with Klenow polymerase, and cloned into the EcoRV site of pBluescript II SK+, resulting in pWH891SK3, pWH963AMH, pWH989SK6, and pWH891SK7. (Table 1). The 4.7-kbp lacZ-Km′ cassette was excised with BamHI from pKOK6.1, filled in with Klenow polymerase, and cloned into these plasmids to yield fusion vectors to ORFX, rubB, estB, and oxyR on the resulting integration plasmids pWH891SK3ORFX::lacZ, pWH963AMHrubBlacZ, pWH989SK6rubBlacZ, and pWH891SK7oxyR::lacZ, respectively (Table 1). In the case of rubB, estB, and oxyR, internal fragments of the genes have been deleted during the cloning steps (Fig. 1 and Table 1). The integration plasmids were cut with Apal and SacII within the vector sequence to prevent integration of the circular plasmids, and the linear DNAs were used to transform *Acinetobacter* sp. strain ADP1. Transformants were selected on Luria-Bertani (LB) plates with kanamycin, and correct integration of the cassettes was confirmed by Southern hybridization (data not shown). The chromosomal organization of the resulting strains, called WH380 (ORFX::lacZ), WH382 (rubB::lacZ), WH384 (estB::lacZ), and WH386 (oxyR::lacZ), is shown in Fig. 1.

**FIG. 1.** Schematic drawing of the relevant DNA from *Acinetobacter* sp. strain ADP1 cloned on pWH891. Numbers indicate the kilobase pair scale of the sequence in the EMBL database (accession no. Z46863). Inverted repeats are indicated as stem-loop structures. In the lower part, the chromosomal characteristics of the mutants with inserted lacZ-Km′ cassettes (simplified as lacZ) are shown. Alk, phenotype on alkane (+, no growth); estB, esterase; oxyR, peroxide response regulator; hisS, hspy RNA synthetase; pkp, polyphosphate kinase; rubA, rubredoxin; rubB, rubredoxin reductase.
An additional strain (WH384::oxyR), defective in both estB and oxyR, was constructed by transformation of WH384 with pWH91SK7::oxyR::Cm, linearized with Apal and PstI, and by selection of transformants on LB plates with chloramphenicol. pWH91SK7::oxyR::Cm is equivalent to pWH91SK7::oxy::lacZ, but carries a 3.2-kbp Cm' cassette from pKT210 (PstI fragment, filled in with Klenow polymerase) instead of the lac-Z Km'- cassette. Chromosomal disruptions of estB and oxyR in WH384::oxyR were confirmed by Southern hybridization (data not shown). The strains were resistant to kanamycin and chloramphenicol.

**Killing zone assay.** For qualitative determination of sensitivity to hydrogen peroxide, the killing zone assay (22) was modified. Two hundred microliters of overnight cultures in LB medium were spread on LB plates. After incubation for 2 h, 5 μl of a hydrogen peroxide solution was spotted onto the plates, and the killing zones were measured after 1 day. Standard deviations obtained from three independent experiments were lower than 15% of the respective means.

**Measurement of EstB activity.** *E. coli* was transformed with plasmid pAKA22 containing estB and grown in nutrient broth with 50 mM K2HPO4 (pH 7.0) and ampicillin to early stationary phase. Cells were washed once in ice-cold 50 mM Tris·HCl (pH 8.0), concentrated twofold by centrifugation, and sonicated on ice at 75 W (duty cycle, 50%) for 3 min with a Branson 250 sonifier. The suspension was immediately used for determination of esterase activity as described previously (28).

**RESULTS**

Sequence analysis of *estB* and *oxyR* from *Acinetobacter* sp. strain ADP1. The 2.152-bp NruI-EcoRV fragment from pWH91 containing the DNA between *rubB* (formerly called ORF2 [9]) and *papl* (11) was cloned in both orientations into the EcoRV site of pBluescript SK II+, resulting in plasmids pWH91SK5 and pWH91SK6. Sequence analysis revealed two ORFs, *estB* and *oxyR*, in the same orientation as ORFX, *rubA*, and *rubB* (Fig. 1). Inverted repeats were found downstream of *rubA* (AAAAGACCATGT-N6-ACATGCTTTTTT) and *oxyR* (AAAAGGGACCTGTTTAAAGCTCCTTTT). The *estB-oxyR* locus was independently cloned on plasmid pAKA22 by transformation of a genomic library of *Acinetobacter* sp. strain ADP1 into *E. coli* and screening of the resulting colonies for halo formation on LB plates containing tributyrin. *estB* encodes a protein of 312 aa with similarity to a putative esterase (LipG [301 aa]) from *Mycobacterium tuberculosis* (50% identical amino acids [EMBL accession no. Z92772]), poly(3-hydroxylalkanoate) depolymerase (PhaB [283 aa]) (16) from *P. oleovorans* (30% identical amino acids [SwissProt accession no. P26495]), and β-ketoacylase enol-lactone hydrolase (PcaD [260 aa]) from *Bradyrhizobium japonicum* (28% identical amino acids [EMBL accession no. Y10223]). The EstB sequence contains the GXXG box (aa 136 to 140), which forms the catalytic triad together with aspartate and histidine residues in serine hydrolases like lipases, esterases, and proteases.

The *oxyR*-encoded protein (301 aa) is homologous to the functionally characterized OxyR from *Xanthomonas campestris* (313 aa [35% identical amino acids]) (26), *E. coli* (305 aa [34% identical aa]) (38), *Mycobacterium marinum* (311 aa [34% identical amino acids]) (27), *Haemophilus influenzae* (301 aa [33% identical amino acids]) (23), and *Mycobacterium leprae* (311 aa [32% identical amino acids]) (7); to the putative OxyR from *Erwinia carotovora* (302 aa [33% identical amino acids]) (GenBank accession no. U74302) and *M. avium* (311 aa [31% identical amino acids]) (SwissProt accession no. P52677); and to other members of the LysR family of transcriptional regulators (28 to 24% identical amino acids). OxyR from *Acinetobacter* shows the highly conserved N-terminal α-helix–turn–α-helix motif (aa 22 to 41) and matches a new signature of 18 aa (OxyR box [Fig. 2]), which specifically identifies OxyR sequences within the databases.

**rubA and rubB are necessary for alkane degradation in *Acinetobacter* sp. strain ADP1.** To examine the function of the ORFs, we inserted a lacZ Km'- cassette into ORFX, *rubB*, and *oxyR* on the chromosome of *Acinetobacter* sp. strain ADP1 (see Materials and Methods) and tested the resulting strains for growth on minimal medium plates with dodecane or hexadecane as the sole carbon source (Fig. 1). WH380 (ORFX::lacZ), WH384 (estB::lacZ) and WH386 (oxyR::lacZ) are able to use these alkanes as the sole source of carbon, demonstrating that ORFX, *estB*, and *oxyR* are not necessary for alkane degradation in *Acinetobacter* sp. strain ADP1. The alkane-negative phenotypes of WH362 (*rubA::lacZ*) and WH382 (*rubB::lacZ*) indicate that *rubA* and *rubB* are necessary for alkane degradation.

For further characterization, we used a gap repair strategy (12) to determine the mutations conferring the alkane-negative phenotype in strains WH363, WH342, WH433, and WH434 generated by ethyl methanesulfonate (EMS) mutagenesis of *Acinetobacter* sp. strain ADP1 (9). The 4.3-kbp *AlfIII* fragment containing ORFX, *rubA*, *rubB*, and *estB* was deleted on plasmid pWH91 by restriction with *AlfII* and religation. The resulting plasmid (pWH91Δ*AlfII*) was linearized with *AlfII* and was used to transform *Acinetobacter* sp. strains ADP1, WH363, WH432, WH433, and WH434 via natural competence. Transformants were selected on LB plates with ampicillin. After passage through *E. coli* cells, the plasmids were digested with *AlfII*, showing the presence of the respective 4.3-kbp *AlfII* fragments of the transformed strains, and the sequences between ORFX and *rubB* were determined (nucleotides 4980 to 7295 under accession no. Z46863). The nucleotide sequence of the ADP1 wild type revealed five errors in the published DNA sequence (9): aa 75 (S→L), 77 (D→E), and 199 to 204 (IWRK→NLEESG) must be corrected in the published RubB alignment (9). The sequences obtained from WH363, WH342, WH433, and WH434 revealed the mutations listed in Table 2. The defects in alkane utilization of WH342 and WH434 are caused by G→D exchanges in RubA and RubB, respectively. Since these mutations have no polar effect, this shows unambiguously that *rubA* and *rubB* are necessary for alkane degradation in *Acinetobacter* sp. strain ADP1. Mutations in *rubB* present in WH363 and WH434 lead to growth
defects with dodecane as the sole carbon source, whereas growth on hexadecane is still possible.

**rubA, rubB, estB, and oxyR constitute an operon that is not regulated by alkanes.** We performed primer extension analysis with a primer hybridizing within the coding region of rubA (Fig. 3). Two major products, P1 and P2 (Fig. 3 [top panel]), indicate transcription from two σ^70 promoters (Fig. 3 [bottom panel]). The results obtained with RNAs from Acinetobacter sp. strain ADP1 grown in various media (Fig. 3 [top panel, lanes 1 to 6]) confirm that rubA expression is not induced by alkanes, as was also shown before by β-galactosidase expression from a rubA::lacZ fusion in WH362 (9). No change in rubA transcription is detectable, even when ADP1 was grown on hexadecane as a carbon source (lane 7). However, rubA expression is increased when present in multiple copies (lane 8). Disruption of ORFX or oxyR does not change the efficiency of rubA transcription (lanes 9 and 10).

We detected a 3.7-kb RNA in ADP1 and WH380 in Northern blot analyses with an oxyR-specific probe (Fig. 4 [top panel, lanes 1 and 2]). This is in good agreement with an assumed transcript of 3.55 kb extending from the rubA promoter to the putative transcriptional termination sequence downstream of oxyR. This 3.7-kb RNA is not detectable in strains carrying a lacZ cassette integrated into rubA, rubB, estB, or oxyR (lanes 3 to 6). In ADP1 and WH380, an additional 2.1-kb RNA is detectable (lanes 1 and 2). Because this signal is also present in WH362 and WH382 (lanes 3 and 4), where the 3.7-kb RNA is not found, it cannot be explained by degradation of the 3.7-kb RNA, but indicates the presence of an internal promoter for transcription of an estB-oxyR-containing message. Signals cor-

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**TABLE 2. Mutations determined for alkane mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype^a</th>
<th>Plasmid</th>
<th>Gene</th>
<th>Codon</th>
<th>Protein</th>
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</thead>
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<tr>
<td>WH363</td>
<td>DD – HD +</td>
<td>pWH891rubBL381F</td>
<td>rubB</td>
<td>CTC → TTC</td>
<td>L381F</td>
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<td>WH434</td>
<td>DD – HD +</td>
<td>pWH891rubBG204S</td>
<td>rubB</td>
<td>GGT → AGT</td>
<td>G204S</td>
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<tr>
<td>WH433</td>
<td>DD – HD +</td>
<td>pWH891rubBG263D</td>
<td>rubB</td>
<td>GGT → GAT</td>
<td>G263D</td>
</tr>
<tr>
<td>WH432</td>
<td>DD – HD +</td>
<td>pWH891rubAG182F</td>
<td>rubA</td>
<td>GGC → GAC</td>
<td>G182D</td>
</tr>
</tbody>
</table>

^a The EMS mutants were tested for growth with dodecane (DD) or hexadecane (HD) as the sole carbon source.

^b The mutated codons and the respective amino acid substitutions are listed.
responding to RNAs of 1.35 and 1.2 kb may be degradation products of the 3.7- or 2.1-kb RNA. Further Northern blot analyses using probes specific for estB and rubB are in agreement with the results obtained with the oxyR-specific probe (data not shown). No RNA is detectable with an ORFX-specific probe (data not shown). No RNA is detectable with an ORFX-specific probe (data not shown). An RNA of 310 bases was detected in a 2% agarose gel (data not shown). P1,2 and P3, respectively, indicate the promoters P1 and P2 determined by primer extension and promoter P3 proposed on the Northern blot shown in the top panel.

**FIG. 4.** Detection of oxyR transcripts. (Top) Northern blot hybridized with an oxyR-specific probe. Eleven micrograms of total RNA was run on each lane of a 1% agarose gel. RNA was prepared from the following cells growing exponentially in LB medium: ADP1 (lane 1), WH380 (lane 2), WH362 (lane 3), WH382 (lane 4), WH384 (lane 5), and WH386 (lane 6). On the left, the positions of RNA molecular size marker bands are given in kilobases. On the right, the RNA bands discussed in the text are marked. (Bottom) Interpretation. For explanation of the genomic situation depicted at the top see Fig. 1. In the bottom part, RNA species detected in Northern blot analyses are indicated as bars, with their sizes given in the right and left margins in kilobases. The rubA-specific 0.3-kb RNA was detected in a 2% agarose gel (data not shown). P1,2 and P3, respectively, indicate the promoters P1 and P2 determined by primer extension and promoter P3 proposed on the Northern blot shown in the top panel.

**FIG. 5.** EstB activity in E. coli. (Top) Mapping of the DNA conferring esterase activity. For explanation of the genomic situation depicted in the upper part, see Fig. 1. Plasmids with successive deletions were derived from pWH891SK6i and pWH896k and analyzed by sequencing. The plasmids were transformed into E. coli, and transformants were analyzed for esterase activity after 7 days at 37°C on indicator plates (–, no halo detectable; + and ++, intensity of halo formation). The fragments present on the deletion plasmids are indicated as bars, with the name of the plasmids on the right indicating the number of base pairs that have been deleted. (Bottom) Acyl chain length specificity of EstB. Activity was measured with crude extracts of E. coli cells expressing estB from pAKA22. No activity was found with pNP esters with chain lengths of 14, 16, and 18 carbons or in the vector controls.

**oxyR encodes a peroxide response regulator.** Sequence analysis indicates that oxyR may regulate the peroxide stress response in Acinetobacter sp. strain ADP1. The substrate specificity of EstB was studied with crude extracts of E. coli, expressing estB from pAKA22, with pNP esters used as substrates. EstB showed activity with pNP esters with acyl chain lengths from 2 to 12 carbon atoms, with a preference around 6 and 8 carbon atoms (Fig. 5 [bottom panel]).

**estB encodes a functional esterase.** Colonies of E. coli cells, transformed with pWH891SK6 and pWH891SK6i, form clear halos on turbid NB plates with tributyrin after incubation at 37°C for 7 and 5 days, respectively. The different level in esterase activity may be due to the plasmid-borne lac promoter, which is in the same orientation as the estB gene in pWH891SK6i. Transformation of plasmids obtained from nested deletion reactions mapped the DNA relevant for this phenotype into the estB gene (Fig. 5 [top panel]). This shows that estB encodes a functional esterase from Acinetobacter sp. strain ADP1. The substrate specificity of EstB was studied with crude extracts of E. coli, expressing estB from pAKA22, with pNP esters used as substrates. EstB showed activity with pNP esters with acyl chain lengths from 2 to 12 carbon atoms, with a preference around 6 and 8 carbon atoms (Fig. 5 [bottom panel]).
for WH384oxyRpWH391Δncol; 15 mm for WH362, WH382, and WH380; and 14 mm for ADP1. Thus, the sensitivity of mutants with mutations in ORFX, rubA, and rubB to hydrogen peroxide was the same as that of the wild-type, whereas it was clearly increased in the estB mutant and strongly increased in the oxyR mutant. The increased sensitivity to hydrogen peroxide in WH386 is complemented by oxyR; the effect of the estB disruption (WH384) is further increased by additional deletion of oxyR (WH384ΔoxyR) and also is complemented by oxyR. We conclude that absence of estB does not lead to increased sensitivity to hydrogen peroxide and that the effect seen in WH384 results from a polar effect of estB disruption on oxyR expression. The phenotype of oxyR mutants indicates that the OxyR protein regulates the peroxide stress response, as known from other bacteria (8).

**DISCUSSION**

Previous studies did not prove that rubA and rubB are involved in alkane degradation in *Acinetobacter* sp. strain ADP1 (9), since the insertion of the lacZ-Km cassette into rubA may affect expression of rubB. The single mutations in WH432 and WH433 lead to exchanges of highly conserved glycine to aspartic acid residues in rubredoxin and rubredoxin reductase. This proves the necessity of both proteins for alkane degradation in *Acinetobacter* sp. strain ADP1. Rubredoxin reductase (AKT) in *P. oleovorans* is not essential for alkane degradation, because it can be substituted for by an unknown reductase probably encoded on the chromosome (36). In contrast, ADP1 seems to have only one gene encoding rubredoxin reductase. WH363 and WH343 contain mutations in rubB. A conserved glycine is replaced by a serine in WH363, and a leucine, located in the variable C terminus, is replaced by phenylalanine in WH434. The rubredoxin reductase in both mutants is not completely inactive, because WH363 and WH434 are able to grow on hexadecane. Because rubredoxin reductase serves as an electron transporter for the alkane hydroxylase, it probably does not directly interact with alkanes, and, therefore, a mutation in rubredoxin reductase should not change the utilization spectrum of alkanes. Because the wild type grows faster with hexadecane as the sole carbon source than with dodecane, the latter is a poorer substrate, probably for alkane monooxygenase. Therefore, a rate-limiting rubredoxin reductase activity could reduce alkane turnover below the level necessary for growth on dodecane, whereas growth on hexadecane is still possible. A chain-length-dependent toxicity of alkanes has been postulated for *A. calcoaceticus* 69-V (1). The reduced turnover could lead to an accumulation of alkanes in the cell, which may be less well tolerated for the more toxic dodecane.

Analysis of the sequence downstream of rubB revealed the genes estB and oxyR, encoding a hydrolase and a transcriptional regulator of the LysR family. We have demonstrated by Northern blot analyses that rubA and rubB constitute an operon together with estB and oxyR (Fig. 4 [top panel]). ORFX is not part of this operon, because insertion of a lacZ-Km cassette in ORFX has no polar effect on expression of the rubA-rubB-estB-oxyR operon, as shown by the phenotype of WH380 (ORFX::lacZ) on alkane plates (Fig. 1), primer extension (Fig. 3 [top panel]), and Northern blot analyses (Fig. 4 [top panel]). The presence of a rubA-specific 310-bp RNA indicates that a stem-loop structure downstream of rubA in vivo functions as a transcriptional termination signal or as a stabilizing element preventing degradation of RNA. Primer extension analyses suggest that rubA is transcribed by σ70 RNA polymerase and show that transcription is neither induced by alkane nor subject to repression by succinate or any compound present in LB medium. Taken together, rubA in *Acinetobacter* sp. strain ADP1 differs from the homologous alkG in *P. oleovorans* not only in genetic organization and size, but also in regulation, because transcription of alkG is induced by alkane (36). In *A. calcoaceticus* 69-V, like in *P. oleovorans*, an alkane-inducible rubredoxin has been found (3). Thus, alkane degradation is regulated differently even within the genus *Acinetobacter*, assuming the absence of posttranscriptional regulation.

Despite the fact that rubAB, estB, and oxyR are in one operon, there is no indication for a functional relationship. The activity of estB in *E. coli*, monitored on indicator plates with tributyrin, demonstrates that estB encodes a functional esterase, although tributyrin is probably not the physiological substrate in ADP1. There is no amino-terminal signal peptide in the EstB sequence, indicating that it is a cytoplasmic protein. Tributyrin is hardly internalized by *E. coli*, which explains that halo formation on indicator plates requires several days, because it depends on cell lysis. Aside from estB, two further genes, lipA and estA, encoding lipolytic enzymes have been cloned from *Acinetobacter* sp. strain ADP1 (19, 20). An additional esterase, named EstC, with activity for TWEEN 80 is secreted via the general secretory pathway (29). EstA and LipA differ from EstB in substrate specificity, since they show optimal activity with nPEST esters with acyl chains of 4 and 16 carbon atoms, respectively (18, 20). This may indicate that these enzymes have different functions in vivo.

oxyR is separated from its target genes (e.g., katG, aphCF, and gorA) (35), on the *E. coli* chromosome, whereas the *X. campestris*-encoded oxyR is located in an autoregulated aphFoxyR-orfX operon (25). The similarity of OxyR from ADP1 to other OxyR proteins (31 to 35% identical amino acids) is only a little higher than that to other members of the LysR family (28% identical amino acids). OxyR proteins, however, are distinguished from all other proteins in the databases by a stretch of amino acids which we called the OxyR box (Fig. 2). This box contains the two cysteine residues, which form reversible disulfide bridges in OxyR from *E. coli* upon induction by hydrogen peroxide (38). The presence of that sequence in OxyR from ADP1 agrees with its function in regulating the peroxide stress response. oxyR occurs in a unique genetic arrangement in ADP1, together with genes encoding apparently unrelated functions. This resembles the previous observation that genes needed for, e.g., tryptophan biosynthesis are scrambled on the chromosome (14). Thus, the genetic organization in *Acinetobacter* may be peculiar, because related genes are often apart, and functionally unrelated genes are linked. The consequences of such an arrangement for regulation are clearly seen for the genes encoding alkane degradation, in which only the monooxygenase gene alkM is regulated, whereas rubAB genes are constitutive, unlike the situation in *P. oleovorans*. It is surprising to conclude that such an arrangement is obviously stable in evolution.

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