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Sequences and Expression of Pyruvate Dehydrogenase Genes from *Pseudomonas aeruginosa*

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A mutant of *Pseudomonas aeruginosa*, OT2100, which appeared to be defective in the production of the fluorescent yellow-green siderophore pyoverdine had been isolated previously following transposon mutagenesis (T. R. Merriman and I. L. Lamont, Gene 126:17–23, 1993). DNA from either side of the transposon insertion site was cloned, and the sequence was determined. The mutated gene had strong identity with the dihydrolipoamide acetyltransferase (E2) components of pyruvate dehydrogenase (PDH) from other bacterial species. Enzyme assays revealed that the mutant was defective in the E2 subunit of PDH, preventing assembly of a functional complex. PDH activity in OT2100 cell extracts was restored when extract from an E1 mutant was added. On the basis of this evidence, OT2100 was identified as an *aceB* or E2 mutant. A second gene, *aceA*, which is likely to encode the E1 component of PDH, was identified upstream from *aceB*. Transcriptional analysis revealed that *aceA* and *aceB* are expressed as a 5-kb polycistronic transcript from a promoter upstream of *aceA*. An intergenic region of 146 bp was located between *aceA* and *aceB*, and a 2-kb *aceB* transcript that originated from a promoter in the intergenic region was identified. DNA fragments upstream of *aceA* and *aceB* were shown to have promoter activities in *P. aeruginosa*, although only the *aceA* promoter was active in *Escherichia coli*. It is likely that the apparent pyoverdine-deficient phenotype of mutant OT2100 is a consequence of acidification of the growth medium due to accumulation of pyruvic acid in the absence of functional PDH.

The pyruvate dehydrogenase (PDH) complex catalyzes the oxidative decarboxylation of pyruvate to form acetyl coenzyme A (acetyl-CoA):

$$\text{pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

The complex contains multiple copies of three enzymatic components: PDH (E1) (EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2) (EC 2.3.1.12), and lipoamide dehydrogenase (E3) (EC 1.8.1.4). In all gram-negative bacteria studied so far, the inner core of the complex consists of 24 E2 molecules arranged with octahedral symmetry (42). The peripheral E1 and E3 subunits are bound to the surface of the E2 core.

Genes encoding PDH have been characterized in *Escherichia coli*. The three components of the complex are encoded by a single operon that includes a regulatory gene (*pdhR*) and the *aceE* (E1), *aceF* (E2), and *lpd* (E3) structural genes. The genes are expressed as a 7.4-kb polycistronic transcript originating upstream of the *pdhR* gene at the pyruvate-inducible *pdh* promoter (51). In addition, a smaller *lpd* transcript is initiated at the independent *lpd* promoter upstream of the *lpd* gene (61). It has been demonstrated that the gene product of *pdhR* negatively regulates transcription by binding to the *pdh* promoter in the absence of pyruvate (50, 51).

The genes encoding PDH in *Pseudomonas aeruginosa* have not been characterized, but the enzyme has been purified and some of its properties have been determined (37). The purified complex was found to contain three major and one minor polypeptide components: E1 (M_r, 92,500), E2 (major component, M_r 76,000; minor component, M_r 77,800), and E3 (M_r, 58,000). The purified complex had a sedimentation coefficient of 48S and was intermediate in size between the complexes from *E. coli* (24) and *Azotobacter vinelandii* (9). In addition, several *ace* mutants which lacked PDH activity have been isolated from *P. aeruginosa*, and lesions were identified in their E1 and E2 components by enzyme assays (36, 37). Genetic mapping revealed that the *ace* mutations were closely linked (36), suggesting the existence of an *ace* operon containing *aceA* (E1) and *aceB* (E2) genes, similar to the *pdhR-aceEF-lpd* operon of *E. coli.*

P. aeruginosa secretes pyoverdine, a yellow-green fluorescent iron-binding compound which dominates the color of cultures grown under iron-limiting conditions. A mutant of *P. aeruginosa*, OT2100, which was apparently defective for pyoverdine synthesis had been identified previously by using transposon mutagenesis (44). Evidence presented here shows that in fact mutant OT2100 has a defect in the PDH complex. The complete nucleotide sequences of the *P. aeruginosa aceA* and *aceB* genes, which encode the E1 and E2 components, respectively, of the PDH complex, are presented along with an analysis of their expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *P. aeruginosa* and *E. coli* strains used in this study are described in Table 1. *P. aeruginosa* strains were grown in brain heart infusion broth (BHI; GIBCO BRL), King's B medium (38), or minimal medium (45) and were maintained on BHI agar. *E. coli* strains were grown in Luria-Bertani (LB) broth or BHI and were maintained on LB agar plates. M13 vector DNA was propagated in *E. coli* TG-1, which was maintained on M9 minimal medium plates at 37°C with glucose and cultured in 2× YT medium (58). All liquid cultures were aerated at 200 rpm and 37°C. Cultures were supplemented with antibiotics at the following concentrations: ampicillin, 100 μg/ml; carbenicillin, 300 μg/ml; chloramphenicol, 30 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*; kanamycin, 50 μg/ml; and tetracycline, 12.5 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa.*

Genetic methods. The $CaCl_2$ method (15) was used for transforming DNA into *E. coli* DH5-α. Competent *E. coli* TG-1 cells were prepared by the method of Nishimura et al. (48). Triparental conjugation of plasmid DNA (pMP190) from

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TABLE 1. Bacterial strains, plasmids, and bacteriophages used in this study

Strain or plasmid	Genotype[a] and phenotype[b]	Source or reference
P. aeruginosa		
OT11	*leu-1 pro-1* Sm^r Pvd^+	Laboratory stocks
OT684	*leu-1 lys-1 hsdR* Rf^r Pvd^+	49
OT2100	*leu-1 lys-1 hsdR* Rf^r *aceB*::Tn*5*-OT182 Tc^r Cb^r	44
PAO1	*chl-2* FP^-	34
PAO2853	*ace-3 chl-2* FP^-	36
E. coli		
DH5-α	*supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR*17	26
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	8
MC1061	Δ*lac*(IPOZYA) Sm^r *araD139* Δ(*ara leu*)*7697 galU galK hsr*	12
TG-1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36* $proAB^+$ $laqI^q$ *lacZ*ΔM15]	21
Plasmids		
pMP190	*lacZ* (Lac^-) *mob* Cm^r	60
pMP190::P_{ace}	*ace* promoter-bearing 1.2-kb *Bam*HI-*Sal*I fragment from *P. aeruginosa* in pMP190	This study
pMP190::P_{aceB}	*aceB* promoter-bearing 0.7-kb *Xho*I fragment from *P. aeruginosa* in pMP190	This study
pOT2100/*Eco*RI	*P. aeruginosa* PDH DNA fused to Tn*5*-OT182 DNA, Ap^r Tc^r	44
pOT2100/*Xho*I	*P. aeruginosa* PDH DNA fused to Tn*5*-OT182 DNA, Ap^r Tc^r	44
pRK2013	ColE1/pRF2 hybrid, *mob* Km^r	17
pUC19	$lacI^q$ *lacZ* (α fragment), Ap^r	74
Phages		
λEMBL3	λ*b*189 *ninL*44 LH54 *nin5 trpE* red^+ gam^+	19
λ*ace2*	*P. aeruginosa* PDH DNA in λEMBL3	This study
M13mp18	$lacI^q$ *lacZ* (α fragment)	74
M13mp19	$lacI^q$ *lacZ* (α fragment)	74

[a] Nomenclature of Bachmann (4).
[b] Antibiotic markers: Ap (ampicillin), Cb (carbenicillin), Cm (chloramphenicol), Km (kanamycin), Rf (rifampin), Sm (streptomycin), and Tc (tetracycline).

E. coli into *P. aeruginosa* was carried out by using the helper strain HB101(pRK2013) (17).

Recombinant DNA methods. A library of *P. aeruginosa* OT11 DNA that had been partially digested with *Sau*3A and packaged in λEMBL3 was a gift from M. Merriman. Infection and growth on plates of λ bacteriophage in *E. coli* MC1061, plaque lifts onto Hybond N^+ membrane, prehybridization and hybridization of filters to radiolabelled DNA probes, and preparation of λ bacteriophage DNA were all performed by using standard methods (58). DNA subcloning methods and large-scale preparations of double-stranded plasmid DNA were performed as described by Sambrook et al. (58). The sequencing strategy involved cloning of DNA fragments into pUC19 and M13, which were then sequenced in both directions with a combination of subcloning, oligonucleotide primers, and nested deletions. M13 recombinant subclones containing progressive unidirectional deletions were generated by using the Erase-a-Base system (Promega). Single-stranded recombinant M13 DNA for sequencing was prepared by the method of Bankier et al. (5). For manual sequencing of pUC19 clones, DNA was prepared by the method of Yie et al. (75). pUC19 DNA for automated sequencing was prepared with the Wizard 373 DNA purification system (Promega), using the protocol recommended by the manufacturer. Double-stranded and single-stranded DNA was manually sequenced by using the Hot Tub sequencing system (Amersham) as described by the manufacturer. The reaction mixture was electrophoresed on a 6% (wt/vol) polyacrylamide gel, using a Sequi-Gen nucleic acid sequencing cell apparatus (Bio-Rad). Automated sequencing was performed by the Centre for Gene Research, University of Otago, on an ABI model 373A DNA sequencer, using dye terminator chemistry. Sequence was analyzed by using the programs of the Genetics Computer Group sequence analysis software package (20).

Northern and Southern analyses. Total RNA for Northern analysis was isolated from *P. aeruginosa* cultures grown in 25 ml of BHI to early logarithmic phase, using the method of von Gabain et al. (68). RNA or DNA for Southern analysis was transferred to Hybond N^+ and hybridized to DNA probes as described by Sambrook et al. (58). Double-stranded DNA fragments to be used as probes were radiolabelled by oligonucleotide priming based on the method of Feinberg and Vogelstein (16).

Pyruvate assay, lactate assay, and enzyme assays. Culture supernatants were prepared by centrifugation of 10-ml *P. aeruginosa* overnight cultures grown in King's B medium. Pyruvate content of culture supernatants was measured by the method described by Czok and Lamprecht (14). Lactate content of culture supernatants was determined by the method of Gutmann and Wahlefeld (25).

Cell extracts for enzyme assays were prepared from 1.5-ml samples of overnight cultures of *P. aeruginosa* grown in BHI, using a modified version of the method of Jeyaseelan and Guest (36). Cultures were centrifuged at 13,000 × *g* for 20 s, and the bacterial pellets were washed in 40 mM K_3PO_4 buffer (pH 7.0) and placed on ice. Ultrasonic extracts were prepared by resuspending the bacteria in 0.5 to 1.5 ml of buffer and treating the suspensions for two 30-s periods (with an interval for cooling on ice) in an MSE ultrasonic cell disintegrator. The suspensions were clarified by centrifuging at 13,000 × *g* for 10 min at 4°C. The resulting supernatants were used as cell extracts, and their protein contents were determined by the bicinchoninic assay (59), using the microtiter plate method described by Redinbaugh and Turley (52). Enzyme assays were carried out on at least two independent cultures of each strain and were performed in triplicate for each sample, using a range of protein concentrations. Enzyme specific activities were determined from the region of proportionality between initial velocity and protein concentration and were calculated as micromoles of substrate transformed per milligram of protein per minute. PDH activity was determined as described previously (37) by monitoring the pyruvate-dependent reduction of 3-acetylpyridine adenine dinucleotide (APAD) to APADH at 366 nm. PDH reconstitution experiments were performed as described by Guest and Creaghan (23). E2 enzyme activity was determined by the method of Reed and Willms (53), which is based on a colorimetric determination of *S*-acetyldihydrolipoamide as a ferric acethydroxamate complex. E3 enzyme activity in crude cell extracts was determined as described by Jeyaseelan et al. (37) by monitoring the dihydrolipoamide-dependent reduction of APAD to APADH in the presence of E3 enzyme. β-Galactosidase (LacZ) assays were performed in duplicate as described by Miller (46). Duplicate cultures were grown in 5 ml of minimal medium (*P. aeruginosa*) or LB (*E. coli*) to mid-log phase prior to assaying for LacZ activity.

Nucleotide sequence accession number. The nucleotide and amino acid sequence of the *aceA* and *aceB* genes reported in this study has been submitted to GenBank under accession no. U47920.

RESULTS

Cloning and sequencing of DNA adjacent to the transposon insertion. Mutant OT2100 was isolated following insertion of transposon Tn*5*-OT182 (44), a Tn*5* derivative, into the chromosome of *P. aeruginosa* OT684 (Fig. 1). This mutant was apparently defective for production of the siderophore pyoverdine. DNA flanking the transposon had been cloned previously (44). To characterize this DNA, the sequences of the two flanking fragments, a 0.8-kb *Xho*I-*Bam*HI fragment (XB0.8)

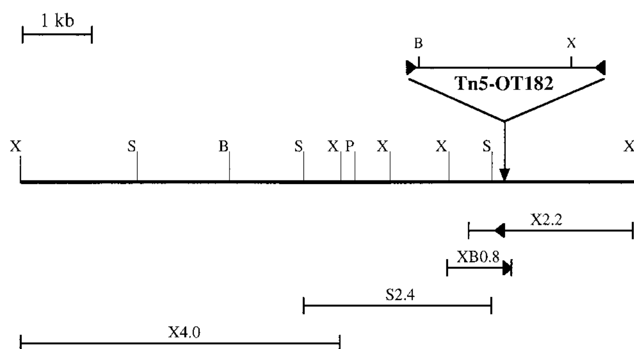

FIG. 1. Physical map and subclones of the DNA adjacent to the transposon insertion site in OT2100. Tn*5*-OT182 (10.9 kb) (44) is not shown to scale. The transposon inverted repeats are represented with converging arrows. Relevant restriction sites: B, *Bam*HI; P, *Pst*I; S, *Sal*I; X, *Xho*I.

and a 2.2-kb *Xho*I fragment (X2.2) (Fig. 1), were determined. Sequence analysis (see below) revealed the likely presence of a PDH gene. To clone the remainder of this gene along with adjacent DNA, a genomic library of *P. aeruginosa* OT11 DNA packaged in λEMBL3 was screened, and a clone which hybridized with probe XB0.8 was isolated and named λ*ace*1. DNA from this clone was digested with restriction enzymes, and Southern analysis was performed with the XB0.8 fragment as a probe; a 2.4-kb *Sal*I fragment (S2.4 [Fig. 1]) was found to hybridize to the probe (data not shown). This fragment, along with an overlapping 4-kb *Xho*I fragment (X4.0 [Fig. 1]), was cloned into pUC19, and the DNA sequences were determined.

Nucleotide sequences of *aceA* and *aceB*. The 4,859 bp of nucleotide sequence obtained from both strands of X4.0, S2.4, XB0.8, and X2.2 (Fig. 1) is presented in Fig. 2. Two complete ORFs (open reading frames) were identified within the sequence. The first ORF was 1,660 bp in length; translated sequence from this ORF showed high identity with PDH (E1) components of the PDH complex from other bacterial species (Table 2). The ORF was subsequently named *aceA*, consistent with the nomenclature used previously for PDH genes of *P. aeruginosa* (36). The deduced amino acid sequence from the second ORF (2,642 bp) showed very high identity with the dihydrolipoamide acetyltransferase (E2) components of the PDH complexes in several bacterial species (Table 2). The second ORF was subsequently referred to as *aceB*. This result indicated that the genes encoding E1 and E2 of PDH are adjacent in *P. aeruginosa* (Fig. 3). The site of the transposon insertion was found to be within the *aceB* gene (Fig. 3). Comparison of wild-type and transposon-flanking DNA revealed a 9-bp duplication (nucleotides 4863–4871 [Fig. 2]) which is characteristic of Tn*5* insertions (3). The sequence was determined for a further 500 bp of DNA downstream from *aceB*, but no significant ORFs were detected (data not shown). A partial ORF was detected upstream of, and on the opposite DNA strand to, *aceA*. The upstream portion of translated sequence from the ORF had significant identity with GlnE from *E. coli* (30). GlnE functions as adenylyltransferase in the glutamine synthetase adenylylation cascade. The ORF was named ORFG (Fig. 2 and 3) and has not been further investigated.

The overall G+C content of the gene cluster was 65.5%, which is in good agreement with the estimate of 67.2% for the *P. aeruginosa* genome (71). The G+C contents of *aceA* and *aceB* were 64.2 and 67.5%, respectively. ORFG, *aceA*, and *aceB* showed codon preferences typical of genes from *P. aeruginosa* (71). Putative translational start codons were identified in ORFG (position 715), *aceA* (position 997), and *aceB* (position 3783) and were preceded by likely Shine-Dalgarno sequences (Fig. 2).

A possible promoter sequence which had similarities with the enterobacterial σ^{70}-type promoter consensus sequence (29) was identified in the region 5′ to *aceA* (Fig. 2). A relatively large intergenic region (IGR) of 142 bp (nucleotides 3640 to 3782 [Fig. 2]) is between *aceA* and *aceB*. This region contains a large inverted repeat with a free energy calculated to be −23.1 kcal/mol (76). Downstream of *aceB*, another inverted repeat was identified (Fig. 2). The structure, which has a predicted free energy of −13 kcal/mol, is followed by several U residues and is a likely transcriptional termination signal.

***aceA* and *aceB* gene products.** The *aceA* gene encodes a polypeptide (E1) with a calculated molecular mass of 99,362 Da, which is in good agreement with the estimate of 92,500 Da for the *P. aeruginosa* E1 polypeptide obtained from sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis (37). The amino acid sequence showed a high degree of similarity with PDHs from other species (Table 2), and an alignment is shown in Fig. 4. The *P. aeruginosa* E1 polypeptide contains a sequence (residues 226 to 255 [Fig. 4]) which matches a putative thiamine pyrophosphate (TPP)-binding motif that has been observed in a variety of TPP-binding enzymes from various species (28).

The gene product of *aceB* (E2) has a predicted molecular mass of 56,683 Da. This estimate is significantly lower than those for the major and minor *P. aeruginosa* E2 components (76,000 and 77,800 Da, respectively) determined by SDS-polyacrylamide gel electrophoresis (37). However, anomalies in the migration behavior of dihydrolipoamide acetyltransferases on SDS-polyacrylamide gels have been reported previously and are evidently caused by distorted lipoyl domains (7, 27, 31, 65). Consistent with this observation, two 74-amino-acid repeating units (residues 5 to 79 and 122 to 196 [Fig. 5]) with 69% identity to each other were identified in the N-terminal portion of *P. aeruginosa* E2 by using DIAGON (63). These repeats showed significant similarity to the lipoyl domains of other species, including the presence of putative lipoyl-binding lysines in the center of each domain (Fig. 5). Flanking the repeats are alanine/proline-rich sequences, which are characteristically found either side of lipoyl-binding domains observed in the E2 polypeptides of *A. vinelandii*, *E. coli*, and other organisms (27, 65). These regions are thought to act as flexible linkers permitting different active sites on the complex to interact (54).

P. aeruginosa E2 shows high similarity to E2 polypeptides from other species (Table 2 and Fig. 5), particularly in the region of the C-terminal catalytic domain which is highly conserved. The *P. aeruginosa* putative E2 catalytic domain contains the proposed active-site serine and histidine residues (positions 467 and 519, respectively [Fig. 5]) identified in *A. vinelandii* (42) and shown to be essential for E2 activity in a number of species (22, 56, 57).

PDH enzyme assays. PDH enzyme assays were performed with cell extracts from mutant OT2100, wild-type strain OT684, and strain PAO2853, which is mutant for the E1 subunit of PDH (36), to determine if the PDH complex was inactive in OT2100 (Table 3).

PDH activity was not detectable in extracts from OT2100 or the E1 mutant PAO2853 (Table 3), indicating at least one of the PDH enzyme components was defective in OT2100. Subsequent assays showed that the E3 subunit is functional in mutant OT2100. However, E2 activity was not detected within the limits of the assay. The activity of the E1 subunit was not directly assayed, but addition of OT2100 cell extract to that of

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                                           ORFG <-       RBS
 670  CGGGCAGCAGGGTGGCCGGCAGATTCGCCAGCGAAGGCAGGCTCATGGTCTATCCTGTTTGGGCTTCCGACCGAAGACCACAGGCTCGAC   759
      A  P  L  L  T  A  P  L  N  A  L  S  P  L  S  M

 760  GCACAACGTCGAAAAGGCTCTACATCGGGCCTTATAGCCTGACAGCGACAATCGAAATGTAGTTTTACTACTAAAAATTGTATCCAGACG   849
                                      -35                   -10

 850  GCTGTTTCTCAGACTGATCTGTAGTAAAACTACAAGTCGCCGGGTACACCCCCGGAGAACATCCAAGAATTAGCTGCGGTCGCCCACGAG   939

                                                   RBS      -> aceA
 940  GCCGGCCGCTCACCTATGGCATCCGATTCTGGAAGCCTTTCCGCCCTGGAGCAAGCCATGCAAGACCTCGATCCCGTCGAAACCCAGGAA  1029
   1                                                          M  Q  D  L  D  P  V  E  T  Q  E     11

1030  TGGCTGGACGCCCTGGAGTCCGTACTCGACCGCGAAGGTGAAGACCGCGCTCATTACCTGATGACCCGCATGGGCGAGCTGGCCAGCCGT  1119
  12  W  L  D  A  L  E  S  V  L  D  R  E  G  E  D  R  A  H  Y  L  M  T  R  M  G  E  L  A  S  R     41

1120  AGCGGCACCCAACTGCCCTACGCGATCACCACGCCCTATCGCAACACCATTCCGGTAACCCACGAAGCACGCATGCCCGGCGACCTGTTC  1209
  42  S  G  T  Q  L  P  Y  A  I  T  T  P  Y  R  N  T  I  P  V  T  H  E  A  R  M  P  G  D  L  F     71
                  BamHI
1210  ATGGAACGCCGGGATCCGCTGCCGCTGGTGCGCTGGAACGCCCTGGCGATGGTGATGCGGGCGAACAAGCACGACCCGGACCTGGGCGGC  1299
  72  M  E  R  R  D  P  L  P  L  V  R  W  N  A  L  A  M  V  M  R  A  N  K  H  D  P  D  L  G  G    101

1300  CACATCTCCACCTTCGCCTCCTCGGCGACCCTCTACGACATCGGGTTCAACTATTTCTTCCAGGCTCCCACCGACGAACACGGGGGCGAC  1389
 102  H  I  S  T  F  A  S  S  A  T  L  Y  D  I  G  F  N  Y  F  F  Q  A  P  T  D  E  H  G  G  D    131

1390  CTGGTGTTCTTCCAGGGCCACGCGTCCCCCGGGGTCTACGCCCGGGCCTTCCTCGGAAGGCCGCATCAGCGGAGGAACAAACTGGAGAAC  1479
 132  L  V  F  F  Q  G  H  A  S  P  G  V  Y  A  R  A  F  L  G  R  P  H  Q  R  R  N  K  L  E  N    161

1480  TTCCGCCAGGAAGTGGACGGCACCGCCTGTCCTCCTATCCGCACCCCCTGGCTGATGCCAGACTTCTGGCAGTTCCCCACCGTATCCATG  1569
 162  F  R  Q  E  V  D  G  T  A  C  P  P  I  R  T  P  W  L  M  P  D  F  W  Q  F  P  T  V  S  M    191

1570  GGCCTTGGCCCGATCCAGGCGATCTACCAGGCACGCTTCATGAAGTACCTGGAAAGCCGCGGCTTCATCCCCGCCGGCAAGCAGAAGGTC  1659
 192  G  L  G  P  I  Q  A  I  Y  Q  A  R  F  M  K  Y  L  E  S  R  G  F  I  P  A  G  K  Q  K  V    221

1660  TGGTGCTTCATGGGCGACGGCGAGTGCGACGAGCCGGAATCCCTCGGCGCGATCTCCCTGGCCGGCCGCGAGAAACTCGACAACCTGATC  1749
 222  W  C  F  M  G  D  G  E  C  D  E  P  E  S  L  G  A  I  S  L  A  G  R  E  K  L  D  N  L  I    251

1750  TTCGTCATCAACTGCAACCTGCAGCGCCTCGACGGCCCGGTCCGCGGCAACGCCAAGATCATCCAGGAACTGGAAGGCGTGTTCCGCGGC  1839
 252  F  V  I  N  C  N  L  Q  R  L  D  G  P  V  R  G  N  A  K  I  I  Q  E  L  E  G  V  F  R  G    281

1840  GCCGAGTGGAACGTCAACAAGGTCATCTGGGGTCGCTTCTGGGACCCGCTGTTCGCCAAGGACACCGCCGGCCTGCTGCAGCAGCGCATG  1929
 282  A  E  W  N  V  N  K  V  I  W  G  R  F  W  D  P  L  F  A  K  D  T  A  G  L  L  Q  Q  R  M    311

1930  GACGAGGTCATCGACGGCGAATACCAGAACTACAAGGCGAAAGACGGCGCCTACGTGCGCGAGCACTTCTTCGGCGCGCGTCCGGAACTG  2019
 312  D  E  V  I  D  G  E  Y  Q  N  Y  K  A  K  D  G  A  Y  V  R  E  H  F  F  G  A  R  P  E  L    341

2020  CTGGAAATGGTCAAGGACCTTTCCGACGAGGAAATCTGGAAGCTCAACCGCGGCGGCCACGACCCCTACAAGGTCTATGCGGCCTACCAC  2109
 342  L  E  M  V  K  D  L  S  D  E  E  I  W  K  L  N  R  G  G  H  D  P  Y  K  V  Y  A  A  Y  H    371

2110  CAGGCGGTCAACCACAAGGGCCACCCGACCGTCATCCTGGCCAAGACCATCAAGGTCTACGGCACCGCCAGCGGCGAAGCGAAGAACATC  2199
 372  Q  A  V  N  H  K  G  H  P  T  V  I  L  A  K  T  I  K  V  Y  G  T  A  S  G  E  A  K  N  I    401
                         SalI
2200  GCGCACAACGTGAAGAAGGTCGACAGCCTGCGCGCGTTCCGCGACAAGTTCGACATCCCGGTCAAGGACGCCGACCTGGAGAAGCTGCCG  2289
 402  A  H  N  V  K  K  V  D  S  L  R  A  F  R  D  K  F  D  I  P  V  K  D  A  D  L  E  K  L  P    431

2290  TTCTACAAGCCTGAAGAAGGCAGCGCCGAGGCCAAGTACCTGGCCGAGCGCCGCGCCGCGCTGGGCGGCTTCATGCCGGTGCGCCGGCAG  2379
 432  F  Y  K  P  E  E  G  S  A  E  A  K  Y  L  A  E  R  R  A  A  L  G  G  F  M  P  V  R  R  Q    461

 380  AAGAGCATGAGCGTGCCGGTTCCGCCGCTGGAAACCCTGAAGGCCATGCTCGACGGCTCCGGCGATCGCGAGATCTCCACCACCATGGCC  2469
 462  K  S  M  S  V  P  V  P  P  L  E  T  L  K  A  M  L  D  G  S  G  D  R  E  I  S  T  T  M  A    491

2470  TTCGTGCGGATCATCTCGCAGCTGGTCAAGGACAAGGAACTCGGCCCGCGCATCGTCCCGATCGTCCCGGACGAGGGCCGTACCTTCGGC  2559
 492  F  V  R  I  I  S  Q  L  V  K  D  K  E  L  G  P  R  I  V  P  I  V  P  D  E  G  R  T  F  G    521

2560  ATGGAAGGCATGTTCCGCCAGCTCGGCATCTACTCCTCGGTCGGCCAGCTCTACGAGCCGGTGGACAAGGACCAGGTGATGTTCTACCGC  2649
 522  M  E  G  M  F  R  Q  L  G  I  Y  S  S  V  G  Q  L  Y  E  P  V  D  K  D  Q  V  M  F  Y  R    551
                            XhoI
2650  GAGGACAAGAAGGGCCAGATCCTCGAGGAAGGCATCAACGAGGCCGGCGCCATGTCCAGCTGGATTGCCGCCGGTACCTCCTACAGCACC  2739
 552  E  D  K  K  G  Q  I  L  E  E  G  I  N  E  A  G  A  M  S  S  W  I  A  A  G  T  S  Y  S  T    581

2740  CACAACCAGCCGATGCTGCCGTTCTACATCTTCTATTCGATGTTCGGCTTCCAGCGCATCGGCGACCTGGCCTGGGCCGCCGGCGACAGC  2829
 582  H  N  Q  P  M  L  P  F  Y  I  F  Y  S  M  F  G  F  Q  R  I  G  D  L  A  W  A  A  G  D  S    611
                                                                         PstI
2830  CGCGCGCACGGCTTCCTGATCGGCGGCACCGCCGGTCGCACCACGCTGAACGGCGAAGGCCTGCAGCACGAGGACGGCCACAGCCACCTG  2919
 612  R  A  H  G  F  L  I  G  G  T  A  G  R  T  T  L  N  G  E  G  L  Q  H  E  D  G  H  S  H  L    641

2920  CTGGCCTCGACCATCCCGAACTGCCGCACCTACGATCCGACCTACGCCTACGAACTGGCGGTGATCATCCGCGAAGGCTCGCGGCAGATC  3009
 642  L  A  S  T  I  P  N  C  R  T  Y  D  P  T  Y  A  Y  E  L  A  V  I  I  R  E  G  S  R  Q  I    671

3010  GAAGAGCAGCAGGACATCTTCTACTACATCACCGTGATGAACGAGAACTACGTCCAGCCGGCCATGCCGAAGGGCGCAGAGGAAGGCATC  3099
 672  E  E  Q  Q  D  I  F  Y  Y  I  T  V  M  N  E  N  Y  V  Q  P  A  M  P  K  G  A  E  E  G  I    701

3100  ATCAAGGGCATGTACCTCCTCGAGGAGGACAAGAAGGAAGCCGCTCACCACGTACAACTGCTGGGCTCCGGCACCATCCTGCGCGAAGTC  3189
 702  I  K  G  M  Y  L  L  E  E  D  K  K  E  A  A  H  H  V  Q  L  L  G  S  G  T  I  L  R  E  V    731

3190  GAGGAAGCGGCCAAGCTGCTGCACAACGACTTCGACATCGTCGCCGACGTCTGGAGCGTGCCGAGCTTCAACGAACTCCGTCACGACGAC  3279
 732  E  E  A  A  K  L  L  H  N  D  F  D  I  V  A  D  V  W  S  V  P  S  F  N  E  L  R  H  D  D    761
           XhoI
3280  CTTACCCTCGAGCGCTGGAACCGCCTGCATCCGGGCCAGAAGCCGAAGCAGAGCTACGTCGAGGAGTGCCTGGGCGCGCCGTCGCGGCCG  3369
 762  L  T  L  E  R  W  N  R  L  H  P  G  Q  K  P  K  Q  S  Y  V  E  E  C  L  G  A  P  S  R  P    791
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3370  GTGATCGCCTCCACCGACTACATGAAGCTCTACGCCGAGCAGATCCGCCAGTGGGTTCCCTCCAAGGAGTACAAGGTGCTCGGCGACGGC  3459
 792  V  I  A  S  T  D  Y  M  K  L  Y  A  E  Q  I  R  Q  W  V  P  S  K  E  Y  K  V  L  G  D  G   821

3460  TTCGGCCGCAGCGACAGCCGCAAGAAGCTGCGCAACTTCTTCGAAGTGGATCGTCACTGGGTGGTGCTGGCCGCGCTCGAAGCGCTGGCC  3549
 822  F  G  R  S  D  S  R  K  K  L  R  N  F  F  E  V  D  R  H  W  V  V  L  A  A  L  E  A  L  A   851

3550  GATCGCGGCGATATCGAGCCGAAAGTGGTGGCCGAGGCCATCGCCAAGTACGGTATCGATCCGGAGAAGCGCAACCCGCTGGATTGCTAA  3639
 852  D  R  G  D  I  E  P  K  V  V  A  E  A  I  A  K  Y  G  I  D  P  E  K  R  N  P  L  D  C  *   881

3640  TTCGCTCCATACTCCCCTCGGCAGCGAGGGGTTTCGAGCACGCCGGCGCCGCCAACGGCGGCGCCGGCTCACAACGAACCAAGGACCAAA  3729
                                                 ---------->     <----------

                                                    RBS      -> aceB
3730  AGTCTTTGTTTTGCGCGCCGCACAGCGGTGGTGGCACGTAAAAGGAGAGGAATGTGAGCGAACTCATTCGCGTACCCGACATCGGCAACG  3819
   1                                                          M  S  E  L  I  R  V  P  D  I  G  N  G   13

3820  GTGAGGGTGAAGTCATCGAGCTGCTGGTCAAGCCCGGCGACAAGGTCGAGGCCGATCAGAGCCTGCTGACCCTGGAATCCGACAAGGCCA  3909
  14   E  G  E  V  I  E  L  L  V  K  P  G  D  K  V  E  A  D  Q  S  L  L  T  L  E  S  D  K  A  S  43
                                                                                         XhoI
3910  GCATGGAAATCCCCAGTCCCAAGGCCGGGGTAGTGAAAAGCATCAAGGCGAAGGTCGGCGACACCTTGAAAGAAGGTGACGAAATCCTCG  3999
  44   M  E  I  P  S  P  K  A  G  V  V  K  S  I  K  A  K  V  G  D  T  L  K  E  G  D  E  I  L  E  73

4000  AGCTGGAAGTGGAAGGCGGCGAACAGCCTGCCGAAGCCAAGGCCGAGGCAGCGCCCGCCCAACCGGAAGCGCCGAAAGCCGAAGCGCCTG  4089
  74   L  E  V  E  G  G  E  Q  P  A  E  A  K  A  E  A  A  P  A  Q  P  E  A  P  K  A  E  A  P  A  103

4090  CTCCCGCCCCGAGCGAGAGCAAGCCGGCCGCCCCCGCCGCGGCCAGCGTCCAGGACATCAAGGTCCCGGACATCGGCTCGGCCGGCAAGG  4179
 104   P  A  P  S  E  S  K  P  A  A  P  A  A  A  S  V  Q  D  I  K  V  P  D  I  G  S  A  G  K  A  133

4180  CCAACGTCATCGAAGTGATGGTCAAGGCCGGCGACACGGTCGAGGCCGACCAGTCGCTGATCACCCTGGAATCCGACAAGGCCAGCATGG  4269
 134   N  V  I  E  V  M  V  K  A  G  D  T  V  E  A  D  Q  S  L  I  T  L  E  S  D  K  A  S  M  E  163

4270  AGATCCCCTCGCCGGCCTCCGGGGTGGTGGAAAGCGTCTCGATCAAGGTCGGTGACGAAGTCGGCACCGGCGACCTGATCCTCAAGCTGA  4359
 164   I  P  S  P  A  S  G  V  V  E  S  V  S  I  K  V  G  D  E  V  G  T  G  D  L  I  L  K  L  K  193

4360  AGGTGGAAGGCGCCGCTCCGGCAGCCGAAGAGCAACCGGCAGCCGCTCCGGCCCAGGCCGCGGCGCCCGCCGCCGAGCAGAAGCCCGCCG  4449
 194   V  E  G  A  A  P  A  A  E  E  Q  P  A  A  A  P  A  Q  A  A  A  P  A  A  E  Q  K  P  A  A  223

4450  CGGCGGTCCCTGCGCCAGCCAAGGCCGATACCCCGGCTCCGGTCGGCGCACCCAGCCGCGACGGCGCCAAGGTCCACGCCGGCCCGGCGG  4539
 224   A  V  P  A  P  A  K  A  D  T  P  A  P  V  G  A  P  S  R  D  G  A  K  V  H  A  G  P  A  V  253

4540  TGCGCATGCTGGCGCGCGAGTTCGGCGTCGAGCTGAGCGAAGTGAAAGCCAGCGGTCCCAAGGGTCGCATCCTCAAGGAAGACGTCCAGG  4629
 254   R  M  L  A  R  E  F  G  V  E  L  S  E  V  K  A  S  G  P  K  G  R  I  L  K  E  D  V  Q  V  283
                                                                                     SalI
4630  TCTTCGTCAAGGAGCAACTGCAGCGCGCCAAGTCGGCGGTGCCGGCGCCACGCGGAGCCGGCATCCCGCCGATCCCGGAAGTCGACTTCA  4719
 284   F  V  K  E  Q  L  Q  R  A  K  S  A  V  P  A  P  R  G  A  G  I  P  P  I  P  E  V  D  F  S  313

4720  GCAAGTTCGGCGAAGTGGAAGAAGTGGCGATGACCCGCCTGATCGAGGTCGGCGCCGCCAACCTGCATCGCAGCTGGCTGAACGTGCCGC  4809
 314   K  F  G  E  V  E  E  V  A  M  T  R  L  I  E  V  G  A  A  N  L  H  R  S  W  L  N  V  P  H  343
                                                       Tn insertion site
4810  ACGTGACCCAGTTCGACCAGTCGGACATCACCGACATGGAAGCCTTCCGCGTTGCCCAGAAGGCCGCGGCGGAGAAGGCCGGGGTCAAGC  4899
 344   V  T  Q  F  D  Q  S  D  I  T  D  M  E  A  F  R  V  A  Q  K  A  A  A  E  K  A  G  V  K  L  373

4900  TGACCGTACTGCCGATCCTGCTCAAGGCCTGCGCCCACCTGCTCAAGGAACTGCCGGACTTCAACAGTTCGCTGGCCCCCAGCGGCAAGG  4989
 374   T  V  L  P  I  L  L  K  A  C  A  H  L  L  K  E  L  P  D  F  N  S  S  L  A  P  S  G  K  A  403

4990  CGCTGATCCGCAAGAAGTACGTACACATCGGCTTCGCCGTGGACACTCCGGACGGCCTGCTGGTCCCGGTGATCCGCGATGTCGACCGGA  5079
 404   L  I  R  K  K  Y  V  H  I  G  F  A  V  D  T  P  D  G  L  L  V  P  V  I  R  D  V  D  R  K  433

5080  AGAGCCTCCTGCAACTGGCCGCCGAGGCCGCCGACCTGGCCGACAAGGCCCGCAACAAGAAGCTCTCGGCCGATGCCATGCAGGGCGCCT  5169
 434   S  L  L  Q  L  A  A  E  A  A  D  L  A  D  K  A  R  N  K  K  L  S  A  D  A  M  Q  G  A  C  463

5170  GCTTCACCATCTCCAGTCTCGGCCACATCGGCGGCACCGGCTTCACGCCGATCGTCAACGCGCCGGAAGTGGCGATCCTCGGTGTGTCCA  5259
 464   F  T  I  S  S  L  G  H  I  G  G  T  G  F  T  P  I  V  N  A  P  E  V  A  I  L  G  V  S  K  493

5260  AGGCGACCATGCAGCCGGTATGGGACGGCAAGGCCTTCCAGCCGCGCCTGATGCTGCCGCTGTCGCTGTCCTACGACCATCGCGTGATCA  534
 494   A  T  M  Q  P  V  W  D  G  K  A  F  Q  P  R  L  M  L  P  L  S  L  S  Y  D  H  R  V  I  N  523

5350  ACGGTGCCGCCGCGGCGCGCTTCACCAAGCGCCTGGGCGAGCTGCTGGCGGACATCCGCACCCTGCTCCTGTAATACCTGCGCGCTCCCC  5439
 524   G  A  A  A  A  R  F  T  K  R  L  G  E  L  L  A  D  I  R  T  L  L  L  *                   553

5440  ACGGAGCGGCCAGCCCCTCTCGCGAGCATGCCACGCTCGTCTCTCGGCCCCACCGGATGGTGGGGCTTTTTTTGCCCGTGACGGCTCGGC  5529
                                                           ------->     <-------          .
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FIG. 2. Nucleotide sequence of the *ace* genes of *P. aeruginosa*. The deduced amino acid sequences are shown below the nucleotide sequence. Putative ribosome-binding sites (RBS), a potential promoter sequence upstream of *aceA* (−35 to −10), relevant restriction sites, and the site of the transposon insertion are underlined, potential stem-loop structures are indicated with converging arrows, and termination codons are represented with asterisks. The nucleotide sequence is numbered from the leftmost *Sal*I site in Fig. 1, which corresponds to position 1 (not shown).

the E1 mutant PAO2853 resulted in restoration of active PDH, indicating that functional E1 subunit is present in OT2100 cell extracts.

Expression of *aceA* and *aceB*. To determine if *aceA* and *aceB* were expressed individually and/or as a polycistronic transcript, RNA was prepared from cultures of *P. aeruginosa* and hybridized to gene probes specific for *aceA*, *aceB*, and the IGR (Fig. 6). A 5-kb transcript was detected with all probes. An additional 2-kb transcript was detected with the *aceB* probe (Fig. 6). Transcript amounts were not affected by the addition of pyruvate to the growth medium (result not shown).

DNA fragments containing the upstream portion of *aceA* and the IGR (P_{ace} and P_{aceB}, respectively [Fig. 3]) were cloned into the promoter-probe vector pMP190 (60) to generate transcriptional fusions in order to determine if these fragments had promoter activity and, if so, whether the promoters were reg-

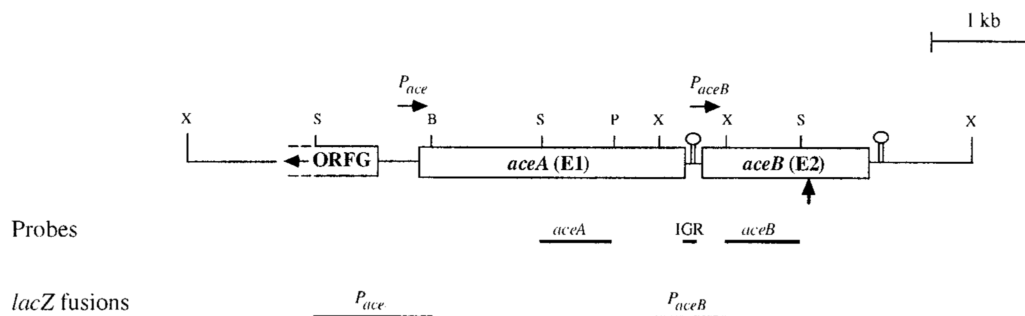

FIG. 3. *ace* operon of *P. aeruginosa* encoding the E1 and E2 components of the PDH enzyme complex. Genes are depicted as open boxes connected by IGRs (narrow lines). Known promoters are indicated by arrows, and regions of potential secondary structure are indicated with stem-loop structures. *P. aeruginosa* DNA fragments used as gene probes or fused to a promoterless *lacZ* gene in an expression vector are shown below the genes. The site of the transposon Tn*5*-OT182 insertion in the *aceB* gene is indicated with an arrow. Relevant restriction sites: B, *Bam*HI; P, *Pst*I; S, *Sal*I; X, *Xho*I.

ulated by pyruvate and/or other carbon sources. The constructs were introduced into *E. coli* and *P. aeruginosa*, and the promoter activities of the cloned fragments were compared by growing the bacteria in BHI or in minimal media (with a variety of carbon sources) (Table 4).

The P_{ace} fragment showed promoter activity in both bacterial species, and it is likely that this corresponds to the promoter of the *aceAB* operon. The promoter was active with all carbon sources tested, although activities were noticeably lower during growth on acetate (Table 4). The IGR (P_{aceB}) also showed constitutive promoter activity, though activity was not detected in *E. coli.*

Analysis of OT2100 pyoverdine. The data presented above show that *P. aeruginosa* OT2100 has a mutation in the *aceB* gene of PDH. However, this mutant was originally isolated because its phenotype suggested that it was defective in pyoverdine production (44). Since the absorbance spectra of noncomplexed pyoverdine is pH dependent between pH 3 and 10 (1), the pH was measured for cultures grown in King's B medium and found to differ considerably between OT2100 and wild-type OT684. When the pH of the mutant supernatant (pH 5.3) was adjusted to that of the wild-type (pH 8.0), it assumed the yellow-green appearance of wild-type pyoverdine. Correspondingly, the pyoverdine spectra of the wild type assumed the appearance of the mutant (pale yellow) when the pH was adjusted to 5.3. These observations were confirmed by spectrophotometric analysis (results not shown).

As OT2100 contains a mutation in a PDH gene, the low pH of the growth medium could be due to a buildup of pyruvic or lactic acid. To test this hypothesis, assays for pyruvate and lactate were performed on culture supernatants from OT684 and OT2100. Pyruvic acid was detected in OT2100 culture supernatants at concentrations averaging 86 ± 7 mM. Pyruvic acid was not detectable in culture supernatants of wild-type bacteria. Lactic acid was not detectable in culture supernatants of either strain.

TABLE 2. Amino acid identities between the E1 and E2 components of the PDH complex of *P. aeruginosa* and the equivalent components of 2-oxo acid dehydrogenase complexes from different sources[a]

Component	Source[b]	% Amino acid identity	Reference
E1	PDH, *Alcaligenes eutrophus* (*pdhA*)	61.9	31
	PDH, *Neisseria meningitidis*	61.3	2
	PDH, *Escherichia coli*	57.9	66
	PDH, *A. eutrophus* (*pdhE*)	56.6	32
	PDH, *Haemophilus influenzae*	56.5	18
E2	PDH, *Azotobacter vinelandii*	71.5	27
	PDH, *E. coli*	54.3	65
	PDH, *N. meningitidis*	52.4	2
	PDH, *A. eutrophus*	50.5	31
	PDH, *Acholeplasma laidlawii*	35.4	69
	PDH, *Bacillus stearothermophilus*	35.3	7
	PDH, *Bacillus subtilis*	35.2	33
	BCDH, *B. subtilis*	33.3	70
	KGDH, *A. vinelandii*	33.3	72
	BCDH, *Pseudomonas putida*	33.1	10
	KGDH, *B. subtilis*	32.5	11
	KGDH, *E. coli*	30.8	61

[a] Identities were determined by using TFASTA (20).
[b] BCDH, branched-chain 2-oxo acid dehydrogenase; KGDH, α-ketoglutarate dehydrogenase.

DISCUSSION

In this study, we have cloned and characterized the *aceA* and *aceB* genes, encoding the E1 and E2 enzyme components, respectively, of the PDH complex in *P. aeruginosa*. *aceA* and *aceB* comprise an operon (Fig. 3), and as with the PDH operon of *A. vinelandii* (27), there is no downstream E3 gene. Most other PDH operons, however, including the well-characterized *E. coli* PDH operon, do have an E3 gene immediately downstream of E1 and E2. The *P. aeruginosa* PDH operon lacks an equivalent to the *E. coli* upstream *pdhR* regulatory gene (51) and also differs from *E. coli* and most other species, including *A. vinelandii*, in having a large IGR between *aceA* and *aceB*. The cloned *aceAB* operon described here has been physically mapped to 3.1 to 7.1 min on the *P. aeruginosa* chromosome (35). This is the same position to which the *ace* mutants of Jeyaseelan and Guest had previously been mapped (36). Therefore, it is likely that the cloned DNA described here corresponds to the *aceA* and *aceB* genes identified previously (36, 37).

Enzyme assays showed that the PDH complex in mutant OT2100 is inactive and that this is a result of a nonfunctional E2 subunit. The other subunits (E1 and E3) appear to be fully functional. This result is consistent with the sequence data in which the site of the transposon insertion corresponds to residue 363 in the catalytic domain of *P. aeruginosa* E2 (Fig. 5), effectively truncating the protein at this point. As a result, most of the catalytic domain, including the conserved active-site residues, would be expected to be absent from OT2100 E2,

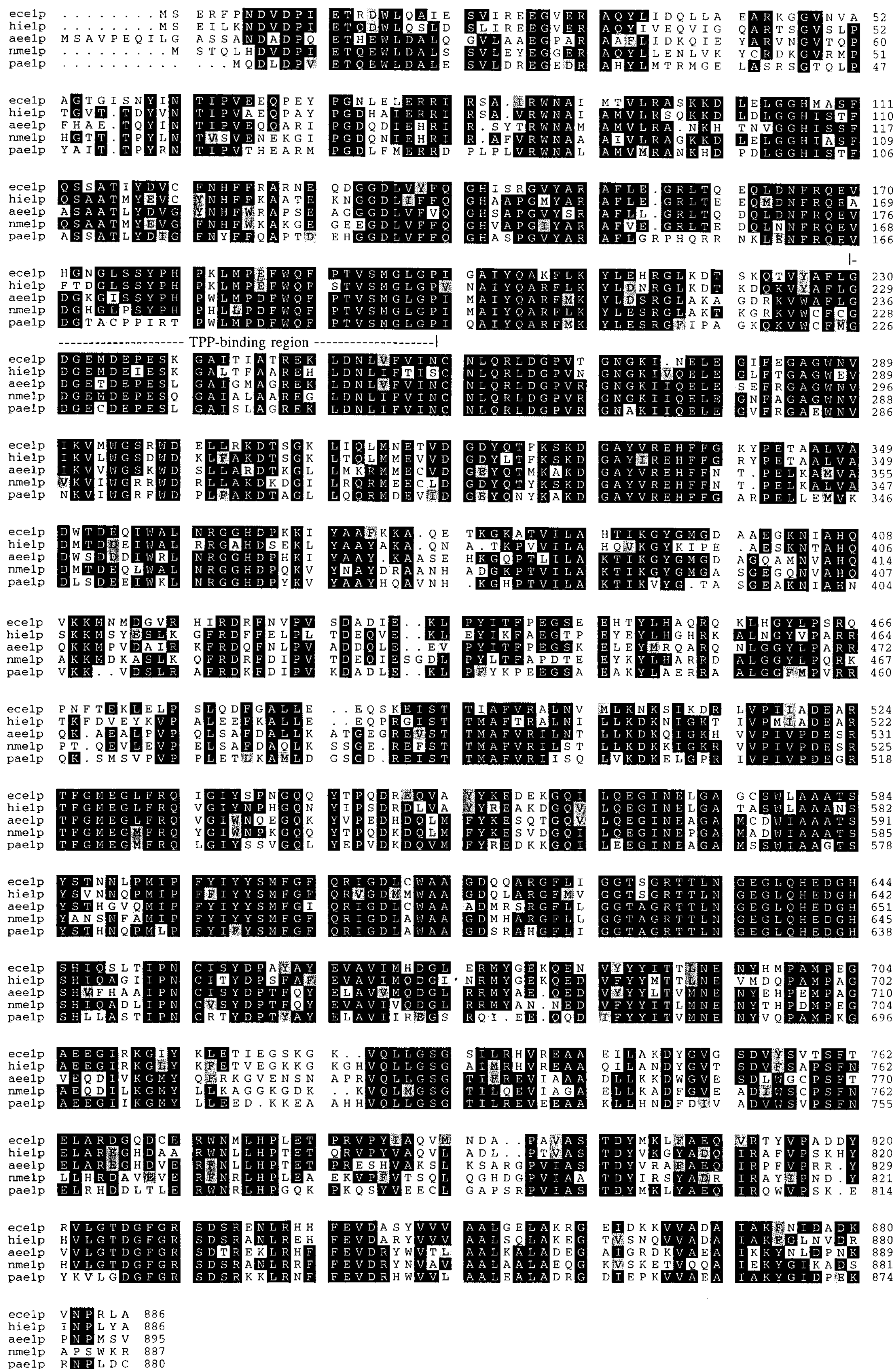

FIG. 4. Alignment of the amino acid sequences of the E1 components of PDHs from *E. coli* (ece1p), *Haemophilus influenzae* (hie1p), *Alcaligenes eutrophus* (aee1p), *Neisseria meningitidis* (nme1p), and *P. aeruginosa* (pae1p). The position of the putative TPP-binding motif is indicated above the sequence. The sequences were aligned by using PILEUP (20) and were displayed by using PRETTYBOX (20), which presents the alignments as shaded boxes. The degree of shading indicates the extent of similarity with the sequence alignment.

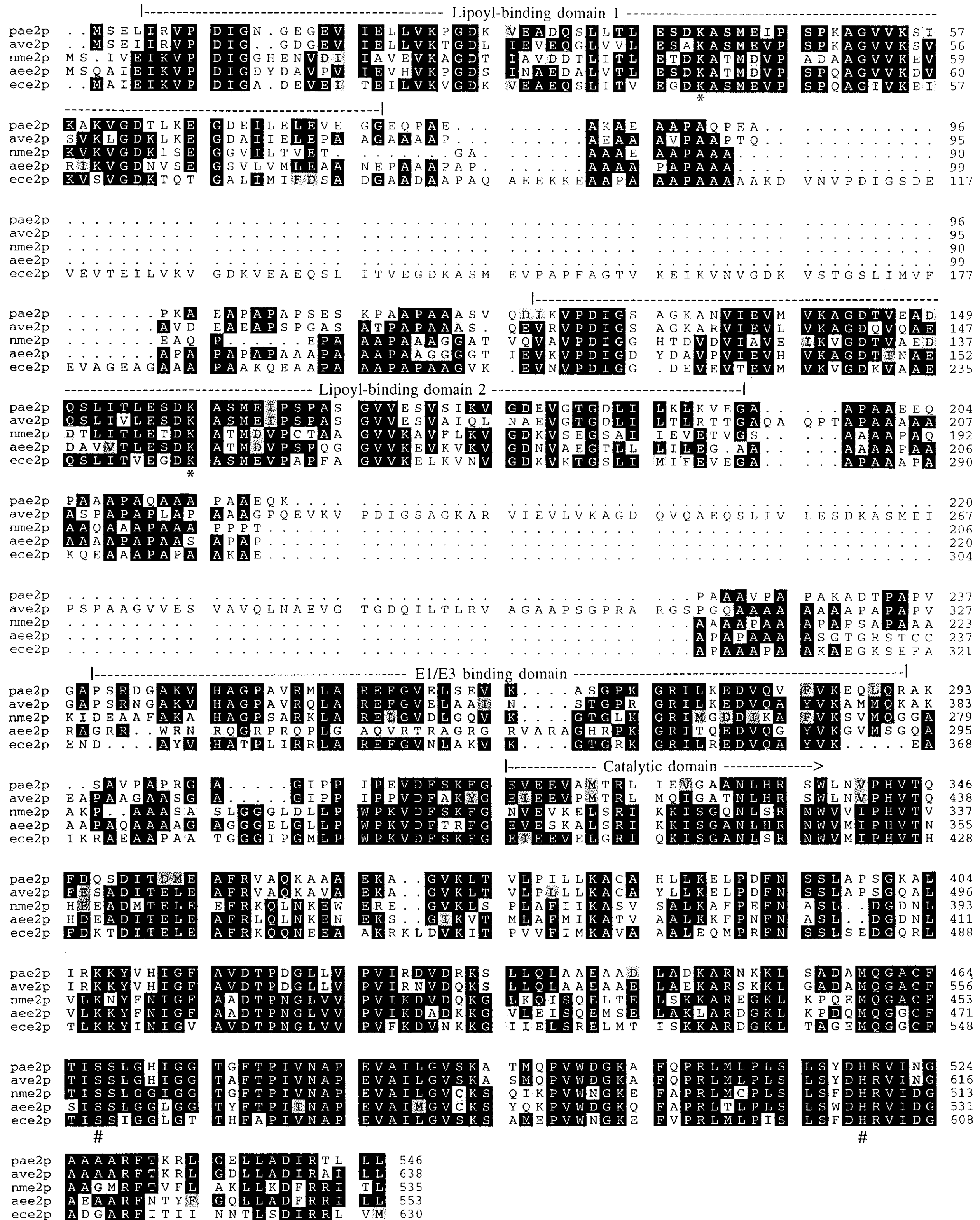

FIG. 5. Alignment of the amino acid sequences of the E2 components of PDHs from *P. aeruginosa* (pae2p), *A. vinelandii* (ave2p), *Neisseria meningitidis* (nme2p), *Alcaligenes eutrophus* (aee2p), and *E. coli* (ece2p). Putative lipoyl-binding lysines (*) and conserved active site residues (#) in the acetyltransferase active site (42) are indicated below the sequence. The positions of the domains for *P. aeruginosa* E2 are estimates based on sequence identity with other organisms. The sequences were aligned by using PILEUP (20) and were displayed by using PRETTYBOX (20), which presents the alignments as shaded boxes. The degree of shading indicates the extent of agreement with the sequence alignment.

TABLE 3. PDH enzyme activities in cell extracts of *P. aeruginosa*

Strain	Mean activity (U/mg of protein) ± SE[a]		
	PDH assay	E2 assay	E3 assay
OT684	0.180 ± 0.016	0.134 ± 0.013	1.30 ± 0.08
OT2100	NA[b]	NA	1.50 ± 0.12
PAO2853	NA	0.100 ± 0.005	1.55 ± 0.08
OT2100 + PAO2853[c]	0.073 ± 0.009	ND[d]	ND

[a] The detectable limits were 0.001 (PDH), 0.03 (E2), 0.01 (E3) U/mg of protein.
[b] NA, no activity detected.
[c] The reconstitution experiment was performed as described previously (23).
[d] ND, not determined.

TABLE 4. P_{ace} and P_{aceB} promoter activities in *P. aeruginosa* and *E. coli*[a]

Plasmid	Mean β-galactosidase (U) ± SD				
	P. aeruginosa				*E. coli*
	Glucose medium	Pyruvate medium	Succinate medium	Acetate medium	BHI medium
pMP190::P_{ace}	2,394 ± 266	1,507 ± 98	1,692 ± 160	825 ± 71	928 ± 103
pMP190::P_{aceB}	1,161 ± 67	709 ± 39	1,185 ± 37	970 ± 46	27 ± 4
pMP190 (control)	51 ± 9	52 ± 9	57 ± 4	52 ± 8	17 ± 3

[a] Media contained chloramphenicol and the carbon source shown (0.1%).

abolishing its catalytic activity. The absence of PDH activity explains the earlier observation that OT2100 does not give rise to normally colored (yellow-green) pyoverdine on King's B medium. An accumulation of pyruvic acid causes a reduction in culture pH, which alters the absorbance properties of the pyoverdine secreted by *aceB* mutant OT2100.

The predicted products of *aceA* and *aceB* show substantial similarities to the E1 and E2 components, respectively, of PDH and other 2-oxo acid dehydrogenases from a variety of sources (Table 2). The *P. aeruginosa* E2 component in particular has striking similarities to other E2 enzymes, especially in the region of the highly conserved C-terminal catalytic domain, where there is 88.7% identity with the putative catalytic domain of *A. vinelandii* (27). This result reflects the many similarities between the closely related *Pseudomonas* and *Azotobacter* genera (64). The number of lipoyl-binding domains in the N-terminal portion of E2 enzymes is variable, and there is no obvious correlation with the phylogenetic source of the enzyme (42). *P. aeruginosa* E2 has two such domains, as do *Alcaligenes eutrophus*, *Acholeplasma laidlawii*, and *Neisseria meningitidis* (2, 31, 69), while E2 from *A. vinelandii* and *E. coli* each possess three domains (27, 65). The *P. aeruginosa* E1 enzyme contains the conserved TPP-binding motif of E1-type enzymes and is monomeric, as are the E1 components from the species listed in Table 2. There was no significant identity with the heteromeric E1 components of 2-oxo acid dehydrogenases.

Northern analysis (Fig. 6) showed that the *aceA* and *aceB* genes are expressed as an operon, most likely extending from the putative *ace* promoter to the putative transcriptional terminator downstream of *aceB* (Fig. 2 and 3). Consistent with the results from Northern analysis, LacZ assays (Table 4) showed

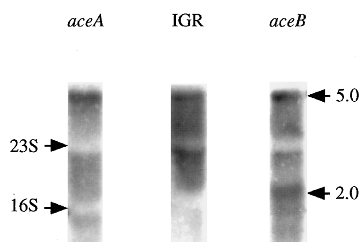

FIG. 6. Northern analysis of *P. aeruginosa* RNA with PDH-specific probes. RNA was prepared from *P. aeruginosa* PAO1, transferred to a nylon membrane, and hybridized with probes specific for *aceA*, the IGR, and *aceB* (Fig. 3). The sizes of the hybridizing transcripts, 5.0 and 2.0 kb, are indicated. 23S and 16S represent rRNAs. The presence of the rRNA species creates distinct clear patches in the pattern of mRNA smearing on the blots. This is due to the rRNAs saturating potential RNA-binding sites at their positions on the membrane (47). The shadows observed directly next to the ribosomal bands, particularly the 23S band, are likely to be mRNA degradation products entrapped by the abundant rRNA species (39).

there was promoter activity upstream of *aceA* (P_{ace}). The *ace* promoter was active in both *E. coli* and *P. aeruginosa* and may possibly originate from the *E. coli*-type promoter consensus identified in Fig. 2. P_{ace} activity was partially downregulated by acetate. This is consistent with a PDH-type promoter, as the glyoxylate pathway (which is employed to utilize acetate and permits the growth of *ace* mutants) bypasses the requirement for PDH. This result is also consistent with the observation of Jeyaseelan and Guest (36) that less PDH activity was detected during growth on acetate.

The *P. aeruginosa ace* promoter produced significantly more units of activity in response to glucose as a carbon source rather than pyruvate (Table 4). This is in contrast with the *E. coli pdh* promoter, where three- to fourfold-higher activity was observed in minimal medium containing pyruvate than in minimal medium containing glucose or succinate (51). This is probably a reflection of the fact that *E. coli* and *P. aeruginosa* use very different systems for the uptake and utilization of glucose and pyruvate. Pyruvate is not a preferred substrate for *P. aeruginosa* (40), which in this study grew poorly in minimal medium supplemented with pyruvate. It has also been shown that there is active transport of pyruvate across the cytoplasmic membrane in *E. coli* but not in *Pseudomonas* (41). Glucose, in contrast, is taken up very efficiently by *P. aeruginosa* (13, 67, 73). In *E. coli*, expression of PDH genes is regulated by a pyruvate-sensitive repressor, PdhR (50, 51). The mechanisms regulating expression of the *P. aeruginosa aceAB* operon are unknown, and regulation of the PDH genes in other bacterial species has not been studied.

An IGR of 143 bp is present between *aceA* and *aceB* (nucleotides 3640 to 3782 [Fig. 2]) and contains a potentially very stable stem-loop structure. A transcript of approximately 2 kb in size was identified with an *aceB* probe by using Northern analysis (Fig. 6), and its size corresponds to that expected for an independent *aceB* transcript (1.8 kb) originating in the IGR. Consistent with this result, a promoter (P_{aceB}) was identified in the IGR upstream of *aceB* in assays using a *lacZ* reporter gene expression system (Table 4). P_{aceB} was active in *P. aeruginosa* but not *E. coli*, as expected from the lack of an identifiable *E. coli* promoter consensus sequence in the IGR. It is possible the IGR inverted repeat is involved with promoter function; for instance, it may serve as a site for DNA-binding proteins which may repress or upregulate the putative *aceB* promoter in response to the demands of the cell. An independent transcript corresponding to *aceB* suggests that E2 may have another role in cells of *P. aeruginosa* in addition to its involvement in the PDH complex.

IGRs (of unknown function) similar to that described here have also been observed between the PDH E1 and E2 genes in *Alcaligenes eutrophus* and *N. meningitidis* (2, 31). However, the IGRs in these species do not have any significant sequence

similarity with each other or with the *P. aeruginosa* IGR. Like the IGR, they do not contain any recognizable promoter consensus sequence; to the best of our knowledge, they have not been analyzed for possible promoter activity.

The lack of a hybridizing band corresponding to the expected size for an *aceA* transcript (2.7 kb) indicates that the intergenic stem-loop does not appear to function as a transcriptional terminator or a site for RNA processing (6). As the subunit molar ratio E1 to E2 is estimated to be 3:1 in *P. aeruginosa* (37), this would imply that regulation of the subunit ratios may occur at the level of translation.

From the foregoing data, we conclude that mutant OT2100 contains a transposon insertion in the *aceB* gene of the PDH complex from *P. aeruginosa*, thereby rendering the enzyme complex inactive and causing an increase in pyruvate concentration and acidity of the culture medium. The deduced primary structures of the E1 and E2 polypeptides indicate very strong similarity to the corresponding components from other bacterial species. Lastly, we have shown that the *aceA* and *aceB* genes are expressed as an operon, with expression being regulated very differently from that of *E. coli*; in addition, the *aceB* gene can be expressed independently from a second promoter in an IGR different from that found in any other characterized PDH operon.

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