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Acetoin Catabolic System of *Klebsiella pneumoniae* CG43: Sequence, Expression, and Organization of the *aco* Operon

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A cosmid clone which was capable of depleting acetoin in vivo was isolated from a library of *Klebsiella pneumoniae* CG43 cosmids. The smallest functional subclone contained a 3.9-kb DNA fragment of the cosmid clone. Sequencing of the DNA fragment revealed three open reading frames (ORFs A, B, and C) encoding polypeptides of 34, 36, and 52 kDa, respectively. The presence of these proteins was demonstrated by expression of the recombinant DNA clone in *Escherichia coli*. Considerable similarities between the deduced amino acid sequences of the ORFs and those of the following enzymes were found: acetoin dissimilation enzymes, pyruvate dehydrogenase complex, 2-oxoglutarate dehydrogenase complex, and branched-chain 2-oxo acid dehydrogenase complex of various origins. Activities of these enzymes, including acetoin-dependent dichlorophenolindophenol oxidoreductase and dihydrolipoamide acetyltransferase, were detected in the extracts of *E. coli* harboring the genes encoding products of the three ORFs. Although not required for acetoin depletion in vivo, a possible fourth ORF (ORF D), located 39 nucleotides downstream of ORF C, was also identified. The deduced N-terminal sequence of the ORF D product was highly homologous to the dihydrolipoamide dehydrogenases of several organisms. Primer extension analysis identified the transcriptional start of the operon as an A residue 72 nucleotides upstream of ORF A.

Acetoin (3-hydroxy-2-butanone) is a major fermentation product of many bacteria when they are grown on a medium with excess carbohydrates. This neutral product allows the bacteria to ferment large amounts of carbohydrates without self-inhibition. It also can be reutilized after the carbohydrates are exhausted and is therefore considered an energy-storing metabolite.

Many bacterial species are able to degrade acetoin, and some of the catabolic pathways have been elucidated. According to Juni and Heym (17), the dissimilation of acetoin by *Acinetobacter* spp. proceeds via a 2,3-butanediol cycle, in which the acetoin is an intermediate and is finally converted to acetate. *Bacillus subtilis* also degrades acetoin. The complete 2,3-butanediol cycle, however, is not essential for the dissimilation of acetoin in this bacterium (21). Instead, acetoin is catabolized by an oxidative cleavage (22) with acetaldehyde and acetyl coenzyme A as the end products, as in *Alcaligenes eutrophus* (10), *Streptococcus faecalis* (9), and *Pelobacter carbinolicus* (25).

The cleavage of acetoin in *A. eutrophus* is catalyzed by enzymes encoded by the *acoXABC* operon (32). *acoA* and *acoB* are the structural genes for the α and β subunits of acetoin:dichlorophenolindophenol oxidoreductase (Ao:DCPIP OR). The amino acid sequences deduced from *acoA* and *acoB* exhibit significant homology to the E1α and E1β sequences of various 2-oxo acid dehydrogenase complexes. Amino acid sequence similarities between the fast-migrating protein encoded by *acoC* and the dihydrolipoamide acetyltransferase (DHLAT) of *E. coli* have also been found. The function of the first gene product encoded by *acoX* has not been identified. Additional gene products, including the acetaldehyde dehydrogenase II which is encoded by *acoD* (33) and the regulator AcoR (19), are also required for acetoin catabolism in *A. eutrophus*. Most recently, the *P. carbinolicus aco* genes were also isolated (26). There are considerable similarities in the amino acid sequences corresponding to *acoA*, *acoB*, and *acoC* between the two acetoin dehydrogenase enzyme systems. Molecular organizations of the structural genes for E1α, E1β, and E2 components are also similar in the two bacteria. The *P. carbinolicus acoL* gene coding for dihydrolipoamide dehydrogenase (DHLDH), however, was not found in the *A. eutrophus aco* operon.

In *Klebsiella* spp., acetoin is an intermediate of the 2,3-butanediol biosynthetic pathway (16, 46). Three enzymes are involved in the pathway, α-acetolactate decarboxylase, α-acetolactate synthase, and acetoin (diacetyl) reductase. The genes encoding these enzymes compose the *budABC* operon (2). Acetoin reductase (EC 1.1.1.5) catalyzes the reversible conversion of acetoin into 2,3-butanediol. However, under normal physiological conditions, acetoin reductase only carries out the forward reaction, i.e., the synthesis of 2,3-butanediol. Although 2,3-butanediol is thought to be the end product of the pathway, a significant increase in the amount of acetoin parallel with the decrease in 2,3-butanediol concentration occurred after 14 h with batch cultures (16). Thus, acetoin is eventually accumulated in the culture containing excess carbohydrates. No evidence that acetoin could be metabolized by enzymes other than acetoin reductase in *Klebsiella* spp. has been reported. In this paper, we report the isolation of an operon from *Klebsiella pneumoniae* CG43 which is able to provide *E. coli* cells with an acetoin dissimilation activity. The results demonstrate the existence of an alternative route to the 2,3-butanediol pathway for acetoin metabolism in this organism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *K. pneumoniae* CG43, a K2 strain presenting a strong Voges-Proskauer reaction (29, 30), was the source for the genomic DNA preparation. *K. pneumoniae* ATCC 13883, a type strain,

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was obtained from the Culture Collection and Research Center, Hsin Chu, Taiwan; *K. pneumoniae* M5a1 was obtained from M. J. Merrick, Agriculture and Food Research Council Unit of Nitrogen Fixation, University of Sussex, Brighton, United Kingdom. *E. coli* DH1 [F^- *supE44 recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1*] was the host for cosmid library construction. *E. coli* JM109 [F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$ Δ(*lac-proAB*) *thi gyrA96 endA1 hsd17* ($r_K^-m_K^+$) *relA1 supE44 recA1*] was the recombinant DNA host. *E. coli* BL21 (DE3), a λ DE3 lysogen with a T7 RNA polymerase gene in an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible form (47), was used for in vivo protein analysis. Cosmid vector pHC79 was used for the construction of the *K. pneumoniae* cosmid library. Plasmid pGEM-1 was the vector for expression purposes, and pUC18 was routinely used as the cloning vector. Both *K. pneumoniae* and *E. coli* were grown at 37°C in Luria broth (LB) unless otherwise indicated. M9 (39) supplemented with 0.2% acetoin served as minimal medium.

Enzymes and chemicals. Restriction endonucleases and DNA-modifying enzymes, including avian myeloblastosis virus reverse transcriptase and T4 DNA ligase, were purchased from Promega and were used under the conditions recommended by the supplier. All chemicals were reagent grade and were obtained either from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, Mo.). Isotopes (including [α-^{32}P] dCTP and ^{35}S-dATP) were purchased from Amersham Corp. (Buckinghamshire, England).

Assay for acetoin. LB (pH 6.0) supplemented with 0.01% (vol/vol) acetoin was the culture medium for screening clones carrying acetoin dissimilation activity. The amount of acetoin left in the broth grown overnight was determined by the method of Krampitz (18), which is based on the specific reaction of acetoin with creatine and α-naphthol at an alkaline pH. The reaction produces a bright red product, which was measured spectrophotometrically at 540 nm.

Construction of cosmid library. Genomic DNA of *K. pneumoniae* CG43 was prepared by the method of Caswell et al. (6). The DNA was partially digested with *Sau*3AI, and the fragments were size fractionated and ligated to the *Bam*HI site of cosmid vector pHC79. The ligation mixture was packaged with λ-coat proteins by using an in vitro packaging kit (Promega). The resulting phage particles were transfected into *E. coli* DH1.

Recombinant DNA techniques and DNA sequencing. Plasmid DNA preparation and DNA manipulation were carried out essentially as described previously (39). DNA sequence determination was performed by the dideoxy chain termination method (40) with the Sequenase kits (U.S. Biochemical, Cleveland, Ohio). Both universal *M13* primer and synthetic oligonucleotides for primer-hopping strategy were used. The sites of compressions were resolved by using dITP, and the sequence was confirmed in both strands. The nucleotide and amino acid sequences were analyzed with the DNASTAR program (DNASTAR Inc., Madison, Wis.) on a Macintosh LC computer.

Expression of *acoA*, *acoB*, and *acoC* in *E. coli*. *E. coli* strain BL21 containing the recombinant plasmid was grown in LB supplemented with 100 μg of ampicillin per ml at 37°C with vigorous shaking until the optical density at 600 nm (OD_{600}) reached 0.4. IPTG was added to a final concentration of 1.0 mM, and the incubation was continued at 37°C for 120 min. The cell pellet was suspended in 2× Laemmli sample buffer and boiled before being loaded on a sodium dodecyl sulfate (SDS)-polyacrylamide gel as described by Laemmli (20). The protein profiles were visualized with Coomassie brilliant blue R-250.

Ao:DCPIP OR activity assay. Activity of Ao:DCPIP OR was determined by the method of Frund et al. (10) with slight modification. Bacteria grown overnight were harvested by centrifugation and suspended in 100 mM potassium phosphate buffer (pH 7.0) for Ao:DCPIP OR activity assay. The assay mixture contained 100 mM potassium phosphate (pH 7.0), 0.08 mM thiamine pyrophosphate, 0.5 mM magnesium chloride, 0.10 mM DCPIP, 0.4 mM acetoin, 50 μl of toluene, and the bacterial suspension in a final volume of 1.0 ml. The reaction mixture was incubated at 37°C, and the amount of clarified supernatant at OD_{578} was measured. The presence of toluene rendered the cells permeable and allowed the reaction to continue without detectable interference. We have also found that Ao:DCPIP OR activity obtained through this modification is higher than that obtained from the cell extract by sonication. One unit of DCPIP OR activity is defined as the reduction of 1 μmol of DCPIP per min. Specific activity is recorded in units per milligram of protein. The amount of protein is determined by assuming that 10^9 cells yield approximately 150 μg of protein as estimated according to reference 24.

DHLAT activity assay. DL-Dihydrolipoamide, the substrate for the DHLAT assay, was prepared by reduction of DL-lipoamide with potassium borohydride (35). DHLAT activity was determined by measuring the formation of the colored hydroxamate ferric complex according to the procedure of Reed and Willms (34) with slight modification. The assay mixture contained 100 mM Tris-HCl (pH 7.5), 10 mM acetyl phosphate, 4 mM DL-dihydrolipoamide, 0.13 mM coenzyme A, 10 U of phosphotransacetylase, 50 μl of toluene, and the bacterial suspension in a final volume of 1.0 ml. The reaction mixture was incubated at 30°C for 30 min, terminated by the addition of 0.1 ml of HCl (1 N), and heated at 100°C for 5 min. Hydroxylamine and $FeCl_3$ were then added to the supernatant, and its OD_{540} was measured. One specific unit of DHLAT activity is defined as the formation of 1 μmol of acetyl phosphate per min per mg of protein.

Primer extension assay. RNA was isolated (48) from *K. pneumoniae* grown in acetoin-containing minimal medium. The oligonucleotides were obtained from TIB MOLBIOL, Berlin, Germany. To ensure the accuracy of the results, two different primers were used independently. Primers CH61 (5′-GCCTGTTTGCTGAGCATCGT-3′) and CH62 (5′-TCT GATCTCCCGCATCTT-3′) are the reverse complements of positions 327 to 346 and 366 to 383 of the nucleotide sequence, respectively. The assay mixture for primer extension contained 10 pmol of either CH61 or CH62 synthetic oligonucleotide, 10 μg of cellular RNA, 0.2 mM (each) dATP, dTTP, and dGTP, 10 μCi of [α-^{32}P]dCTP (111 TBq/mmol; New England Nuclear), 5 U of RNasin, and 5 U of avian myeloblastosis virus reverse transcriptase. The reaction was performed at 42°C for 15 min. Excess cold dCTP was added, and the reaction was continued for another 10 min. The primer extension product was analyzed on a sequencing gel by using the sequence ladder generated by the same primer.

Nucleotide sequence accession number. The nucleotide sequence has been submitted to GenBank, and accession number U00985 has been assigned.

RESULTS

Acetoin catabolism in *K. pneumoniae*. To determine if *K. pneumoniae* CG43 is able to utilize acetoin as its sole carbon source, the bacteria were inoculated to an OD_{600} of 0.1 into M9 minimal medium supplemented with varied concentrations of acetoin and grown at 37°C with vigorous shaking. The

growth of bacteria at OD_{600} was monitored. A doubling time of approximately 8 h was observed for *K. pneumoniae* CG43 in the presence of 0.2% acetoin. The growth of the bacteria was accompanied by a decrease in the acetoin concentration. *E. coli*, however, did not grow under the same conditions, and no significant amount of acetoin in the medium was lost during incubation. Very little growth was observed for *K. pneumoniae* CG43 when 2,3-butanediol was used as the carbon source. Thus, the utilization of acetoin by *K. pneumoniae* does not adopt 2,3-butanediol as an intermediate. The presence of an alternative pathway in *K. pneumoniae* for the dissimilation of acetoin was therefore investigated.

Isolation of the genes responsible for acetoin catabolism. Since *E. coli* does not have the ability to utilize acetoin, it may be used to screen for genes which code for the acetoin utilization activity. To screen for the presence of genes involved in acetoin catabolism, about 1,000 *K. pneumoniae* CG43 cosmid clones in *E. coli* DH1 were transferred individually to 96-well plates containing LB (pH 6.0) and 0.01% acetoin. After overnight incubation at 37°C, the amounts of acetoin remaining in the medium were determined. Two positive cosmid clones with acetoin-dissimilating capability, as demonstrated by their inability to produce the bright red color in the screening reaction, were obtained. The two clones were found to contain DNA fragments overlapping each other, and hence only one of the clones, designated pHP654, was further characterized. A functional subclone (designated pHP679; see Fig. 5A) containing a 3.9-kb DNA fragment was obtained by serial deletions of pHP654 and confirmed by acetoin depletion activity assay.

Since the depletion of acetoin in the cell culture grown overnight could also be due to the conversion of acetoin into 2,3-butanediol by acetoin reductase which was encoded by the *budC* gene (2), acetoin conversion in vivo by acetoin reductase was examined by using cosmid clone pCD64, which contains the entire *bud* operon of *K. pneumoniae* (29). *E. coli* DH1 (pCD64), however, exhibited a much weaker acetoin-depleting activity than *E. coli* DH1(pHP654). In addition, no cross-hybridization between the inserts of pCD64 and pHP654 was observed, which indicates that these two clones are unrelated. These results further supported the postulate that there is an alternative to the 2,3-butanediol pathway for acetoin catabolism in *K. pneumoniae*.

Nucleotide sequence of the DNA involved in acetoin catabolism. The nucleotide sequence of the 3.9-kb insert of the smallest functional clone, pHP679, was determined and is shown in Fig. 1. Analysis of the nucleotide sequence revealed three major open reading frames (ORFs) (designated A, B, and C) which are capable of encoding polypeptides of 319, 339, and 493 amino acid residues with calculated molecular weights of 34,021, 36,561, and 52,216, respectively. There are 11 bases between ORF A and ORF B and only 3 bases between ORF B and ORF C. A possible fourth ORF (designated ORF D), located 39 bp downstream of ORF C, was also observed. The translation start codons of all four ORFs are preceded by Shine-Dalgarno sequences.

Amino acid sequence comparison. The deduced amino acid sequences of the ORFs were used to search for homologous data files in GenBank. Striking similarities to the following enzymes were observed: *A. eutrophus aco* operon (32)-encoding enzymes, *P. carbinolicus* acetoin dehydrogenase (26), pyruvate dehydrogenase (PDH) complex (13–15, 27, 43–45), 2-oxoglutarate dehydrogenase (OGDH) complex (36, 42, 49), and branched-chain 2-oxo acid dehydrogenase (BCODH) complex (3, 4, 41) of various origins (Fig. 2 through 4). The greatest amino acid homologies were found to be those between the ORF A product and *A. eutrophus* Ao:DCPIP OR subunit α (59.4%) (32), between the ORF B product and *A. eutrophus* Ao:DCPIP OR subunit β (56.8%) (32), and between the ORF C product and *E. coli* PDH E2 (28.7%) (44).

A putative thiamine pyrophosphate binding motif, GDG-n_{7-8}-E-n_4-A-n_{5-6}-P-n_6-NN (12), was identified in the deduced amino acid sequence of ORF A but not in that of ORF B (Fig. 2). As with *E. coli* PDH E2 (1, 44), a lipoyl-binding site and the E3-binding sequence were also noted in the gene product of ORF C (Fig. 3A and B). The C-terminal region of the ORF B product, which represents the E1-binding sequence and the inner core domain, however, exhibited a greater homology (Fig. 3C) to *E. coli* OGDH E2 (42) and to OGDH E2 of other origins (4, 36, 49). In the partial amino acid sequence of the putative ORF D product, a conserved sequence representing the flavine adenine dinucleotide (FAD)-binding domain I (7) in *Pseudomonas* lipoamide dehydrogenase, *P. carbinolicus* AcoL (26), and PDH E3 of *E. coli* (45) and other organisms (5, 14, 28) was identified (Fig. 4).

These homology studies suggest that the enzymes encoded by ORFs A, B, C, and D work together in a way similar to that of enzymes in the acetoin dehydrogenase systems of *A. eutrophus* and *P. carbinolicus* and that of 2-oxo acid dehydrogenase multienzyme complexes of various organisms. The gene cluster that codes for the ORFs is therefore designated *acoABCD*.

Expression of the *K. pneumoniae aco* genes in *E. coli*. In order to identify the *aco* gene products encoded by the insert DNA of pHP679, plasmid pHP713 (Fig. 5A) was constructed by inserting the 3.9-kb *Eco*RI-*Hin*dIII fragment of pHP679 into the expression vector, pGEM-1. Examination of the proteins synthesized in vivo following induction with IPTG indicated that pHP713 was capable of synthesizing three proteins. As shown in Fig. 5B, the proteins could be easily identified even in the absence of IPTG. The sizes of these three proteins, as determined by SDS-polyacrylamide gel electrophoresis (PAGE), are approximately 34, 36, and 52 kDa, which agree well with those of the deduced proteins of the ORFs. To assign each of the in vivo-synthesized proteins to the corresponding gene, several deletion subclones of pHP713 were constructed (Fig. 5A). Plasmid pHP719 was derived from pHP713 with a deletion of an *Nco*I-*Stu*I fragment containing part of the ORF C sequence. Plasmid pHP720 was generated by deleting an *Nru*I-*Stu*I fragment which contains parts of both the ORF B sequence and the ORF C sequence of pHP713. The protein profiles of bacteria containing pHP719 and pHP720 were then analyzed in parallel with those of the cells harboring the parental plasmid, pHP713. As shown in Fig. 5B, two smaller proteins (A and B) from pHP719 were identified, and the 34-kDa protein was the only protein from pHP720 that was identified. Therefore, the 52-kDa protein was designated AcoC, and the 36-kDa protein was called the *acoB* gene product. The sizes of the proteins are also consistent with the molecular weights calculated from the deduced amino acid sequences of the corresponding gene products.

Enzymatic activities of the *K. pneumoniae aco* genes. In addition to *K. pneumoniae* CG43, the *K. pneumoniae* type strain (ATCC 13883) and M5a1 were also grown in M9 supplemented with 0.1% Casamino Acids and 0.2% acetoin. Comparable Ao:DCPIP OR activities in the extracts of these *K. pneumoniae* strains were detected, which further supports the presence of an acetoin utilization system in *K. pneumoniae*.

On the basis of sequence analysis, ORF A and ORF B most likely encode an enzyme with Ao:DCPIP OR activity, whereas the ORF C protein should possess DHLAT activity. To verify this theory, the enzymatic activities in the extracts of *K.*

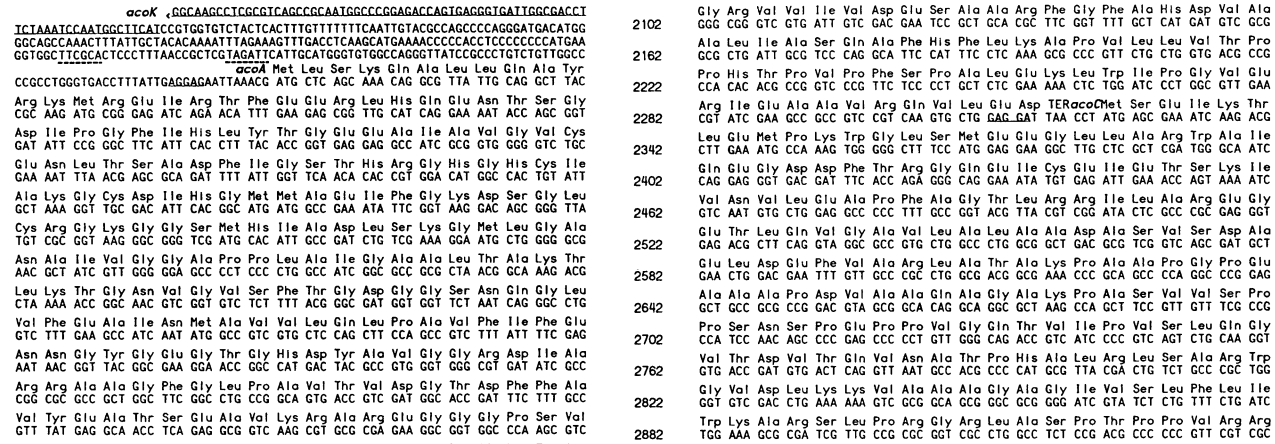

FIG. 1. Nucleotide sequence of the *K. pneumoniae* CG43 *aco* operon. The amino acids encoded by the ORFs are shown above the nucleotide sequence. The putative Shine-Dalgarno sequences are underlined. The potential −10 and −35 sequences are indicated with dashed lines. The position and direction of ORF K (*acoK*) are also indicated.

pneumoniae CG43 and of several *E. coli* strains containing different derivatives of pHP654 were analyzed. Both Ao:DCPIP OR and DHLAT activities could be demonstrated in the extracts of *E. coli* DH1(pHP654) and *K. pneumoniae* CG43 cells grown on acetoin. The addition of acetoin to the culture medium greatly enhanced Ao:DCPIP OR activity in *E. coli* DH1(pHP654). The Ao:DCPIP OR activity in the crude extract of the cells induced with 0.01% acetoin for 60 min increased approximately fourfold compared with that of uninduced cells. The results indicated that acetoin could act as an inducer. We also found that acetoin was the most appropriate substrate for the DCPIP OR activity. The enzymatic activities could also be detected by using diacetyl but neither 2,3-butanediol nor pyruvate as the substrate (Table 1). The results suggest that an acetyl group on a four-carbon compound is crucial for the interaction with DCPIP OR.

As shown in Fig. 5A, both Ao:DCPIP OR and DHLAT activities were detected in the cell extracts of *E. coli* JM109(pHP679) and *E. coli* BL21(pHP713). Even without acetoin induction, the enzyme activities in these cell extracts were comparable to those of *K. pneumoniae* CG43 and *E. coli* DH1(pHP654), suggesting that a regulatory gene in the original cosmid clone for the *aco* operon was missing. On the basis of sequence analysis, the Ao:DCPIP OR activity can only be detected in the extracts of recombinant *E. coli* clones that contain intact *acoA* and *acoB* sequences. On the other hand, DHLAT activity should be observed only in bacteria possessing a complete *acoC* gene. Thus, comparative studies of the enzymatic activities of *E. coli* BL21 harboring pHP713 containing the three *aco* genes, pHP719 containing complete *acoA* and *acoB* sequences, and pHP720 containing only the *acoA* gene were carried out. In the crude extract of *E. coli* BL21(pHP720), which contained only the *acoA* gene product, neither Ao:DCPIP OR nor DHLAT activity was demonstrable. The extract of *E. coli* BL21(pHP719), which contained both *acoA* and *acoB* gene products, exhibited Ao:DCPIP OR activity but no DHLAT activity. Only the strain carrying pHP713 showed both Ao:DCPIP OR and DHLAT activities. These results indicated that proteins encoded by *acoA* and *acoB* indeed code for the Ao:DCPIP OR activity and that the presence of the *acoC* gene product is necessary for DHLAT activity. These results were further supported by the finding that purified Ao:DCPIP OR from DH1(pHP654) was composed of two subunits of 34 and 36 kDa (data not shown).

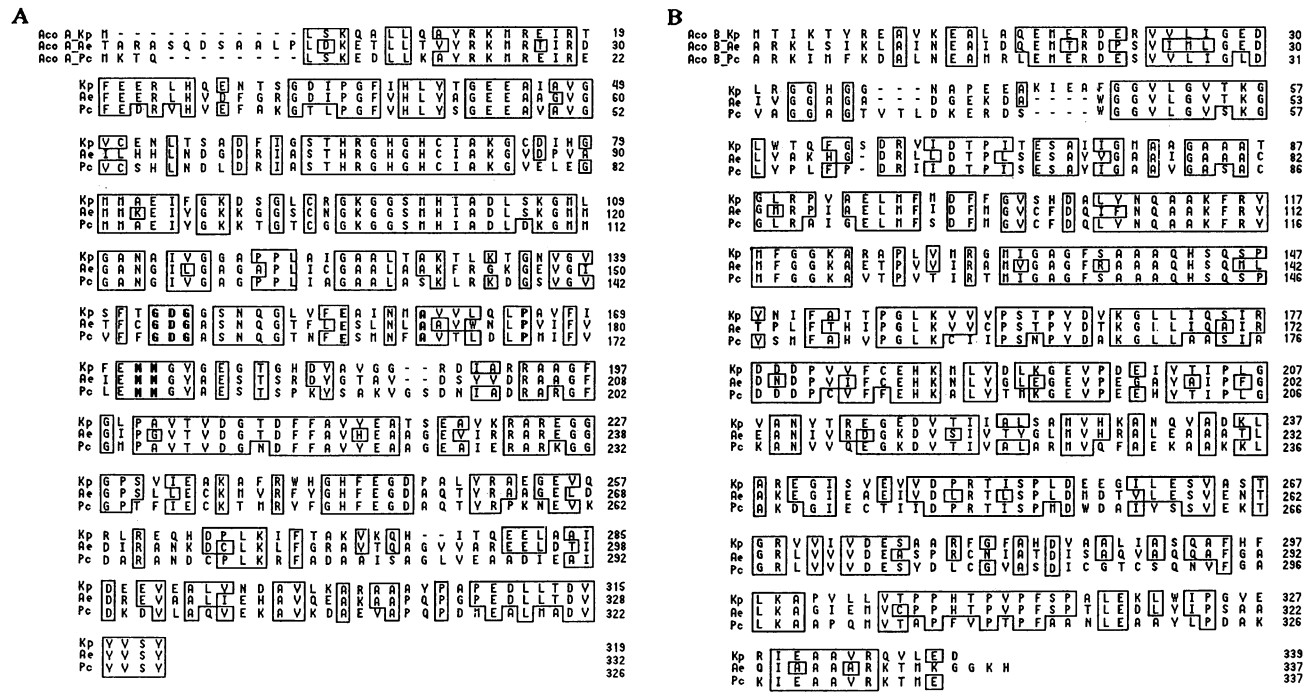

FIG. 2. (A) Amino acid sequence comparison of *K. pneumoniae* (Kp) AcoA, *A. eutrophus* (Ae) AcoA, and *P. carbinolicus* (Pc) AcoA. The exact matches are boxed, and the putative thiamine pyrophosphate binding consensus sequence is indicated by bold letters. (B) Amino acid sequence comparison of *K. pneumoniae* AcoB, *A. eutrophus* AcoB, and *P. carbinolicus* AcoB. The matched residues are boxed.

Primer extension analysis of the *K. pneumoniae aco* operon. To determine the precise initiation site of transcription in the *acoABCD* operon, primer extension was performed by using the RNA prepared from *K. pneumoniae* CG43 under acetoin induction. By using two different primers, the primer extension products were mapped to the same adenyl residue 72 bp upstream of *acoA*. The data obtained with one of the primers are shown in Fig. 6. The control region for the *K. pneumoniae aco* operon resembled the σ^{70} consensus elements in *E. coli* (38). Upstream of the transcription initiation site, a potential −10 region, TAGATT, was identified. A putative −35 site, TTCGCA (Fig. 1), which is 17 bp away from the −10 site, was also observed.

DISCUSSION

The sequencing data indicate that the gene organization of the *K. pneumoniae* CG43 *acoABCD* operon is similar to the operons of the *A. eutrophus* and *P. carbinolicus* acetoin utilization systems and to the 2-oxo acid dehydrogenase multienzyme complexes of several bacteria. It is conceivable that the biochemical properties of these proteins are very similar. Thus, acetoin is likely to be cleaved into two C_2 compounds in the acetoin utilization pathway of *K. pneumoniae*. The acetyl group of one of the C_2 compounds is transferred to a coenzyme A to form acetyl coenzyme A by the catalysis of the acetoin-dependent DCPIP OR, DHLAT, and DHLDH encoded by the *acoABCD* operon. The other C_2 compound, presumably an acetaldehyde, is probably captured by an acetoin-dependent acetaldehyde dehydrogenase.

A significant homology was observed among the *acoABCD* operon of *K. pneumoniae* CG43, the *acoXABC* operon of *A. eutrophus* (32), and the *aco* genes of *P. carbinolicus* (26). The very high degree of similarity in the deduced amino acid sequences corresponding to the two subunits of E1 (Fig. 2) suggests that the E1 activity of the acetoin dehydrogenase enzyme system is acetoin specific. The dependence of E1 on acetoin is shown in Table 1. E1α may catalyze the initial step of a nucleophilic attack on acetoin, as suggested by Oppermann and Steinbuchel (26). E1β is required either for correct assembly of the E1 heterotetramers or for binding of E1 to the E2 core (8, 13, 50). It also likely interacts directly with acetoin in the reaction.

However, several noteworthy differences among the three acetoin dehydrogenase enzyme systems were also demonstrated. *K. pneumoniae* AcoC is a protein composed of 493 amino acid residues which is 119 amino acids longer than the corresponding fast-moving protein in *A. eutrophus*. The size of *K. pneumoniae* AcoC is, however, comparable to those of *P. carbinolicus* AcoC and most E2 components of various 2-oxo acid dehydrogenase multienzyme complexes identified so far, whose molecular masses range from 46 to 52 kDa (4, 15, 36, 42). In contrast to the two lipoyl domains of the *P. carbinolicus* AcoC, there is only one lipoyl domain in the E2s of *K. pneumoniae* and *A. eutrophus*. A single lipoyl domain, however, is sufficient for the dehydrogenase complex activity (31). A truncated ORF (ORF D) of 25 codons in length (Fig. 1) has been identified in the nucleotide sequence of the insert of pHP679. The deduced amino acid sequence has significant homology (Fig. 4) with the N-terminal sequences of many enzymes with DHLDH activity, including *P. carbinolicus* AcoL (5, 14, 26, 28, 45). In contrast, analysis of the nucleotide sequence of the *A. eutrophus acoXABC* operon did not identify a gene which encodes a DHLDH (32). An identical E3 component is usually shared by the various multienzyme complexes of a given organism. Two exceptions found so far are *Pseudomonas putida*, which has three different genes encoding respective E3s with DHLDH activity, and the re-

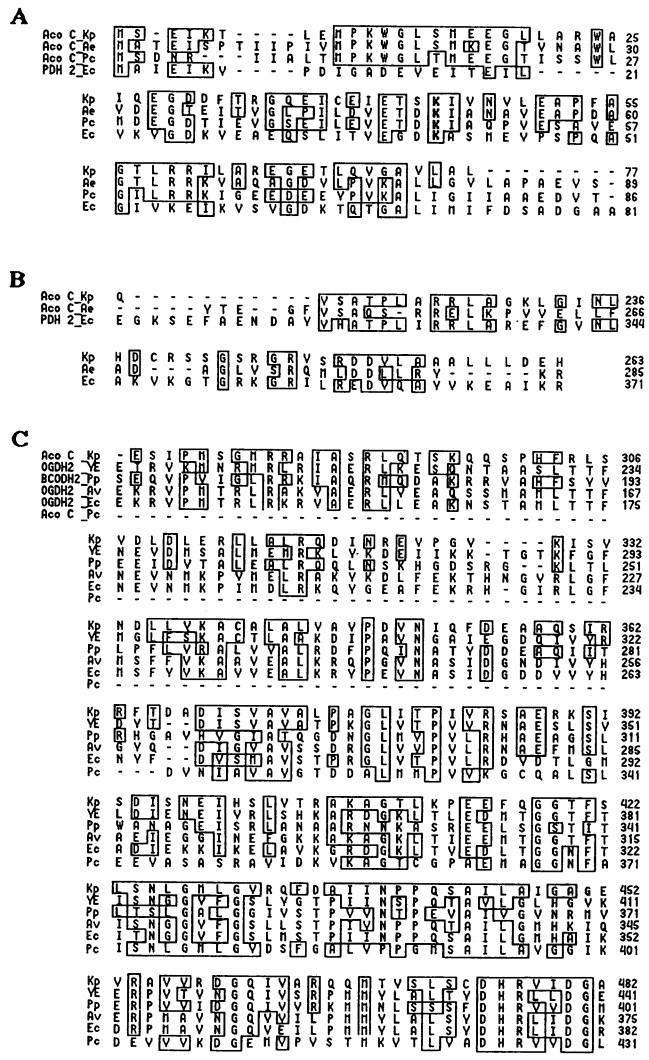

FIG. 3. (A) Comparison of the N-terminal sequences of proteins AcoC and PDH E2. The putative lipoyl-binding residue, Lys, is indicated by bold letters. *K. pneumoniae* CG43 (Kp), *A. eutrophus* (Ae), *P. carbinolicus* (Pc), and *E. coli* (Ec) were the sources of the sequences. (B) Comparison of the putative E3 binding domains of proteins AcoC and PDH E2. (C) Sequence alignment of the E1-binding sequences and inner core sequences of proteins AcoC, OGDH E2, and BCODH. Yeast (*Saccharomyces cerevisiae*) (YE), *P. putida* (Pp), and *Azotobacter vinelandii* (Av) were the sources of the sequences.

cently isolated AcoL in *P. carbinolicus* (26). The discovery of a specific E3 component of the acetoin dehydrogenase enzyme system in *K. pneumoniae* may add one more example to these exceptional situations. The absence of a transcriptional terminator downstream of AcoC and the presence of only a 39-bp intervening sequence suggest that ORF D is likely part of the *K. pneumoniae aco* operon. The structural genes encoding E1α, E1β, E2, and E3 are also clustered in an operon in the *P. putida* BCODH complex (41). The *P. carbinolicus acoL* that codes for E3, however, is not included in the operon in which the structural genes for the E1α, E1β, and E2 are organized (26). Determination of the complete nucleotide sequence of the *K. pneumoniae acoD* and characterization of the gene product are being carried out to confirm the above-described notion.

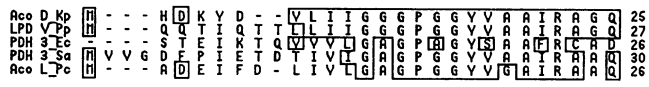

FIG. 4. Sequence alignment of the N-terminal sequences of *K. pneumoniae* AcoD and the homologous proteins. *P. carbinolicus* (Pc), *P. putida* (Pp), *E. coli* (Ec), and *Staphylococcus aureus* (Sa) were the sources of the sequences.

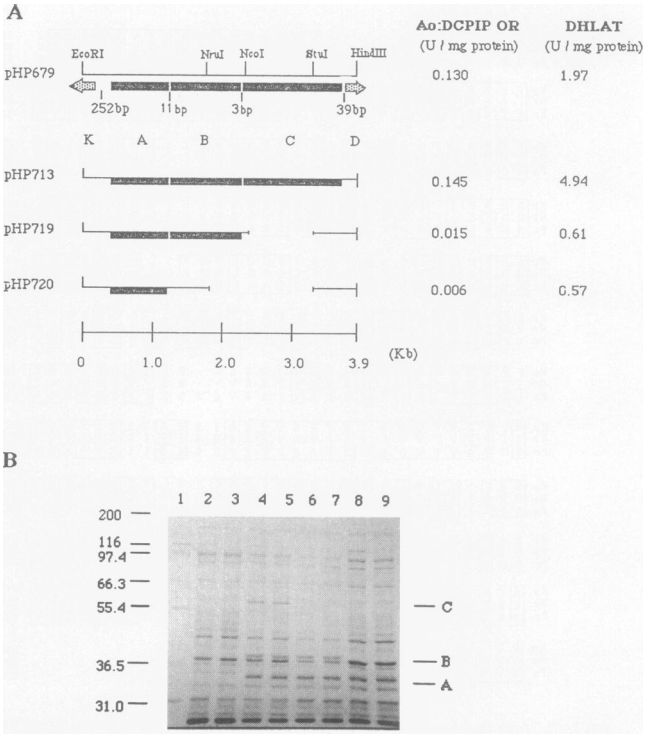

FIG. 5. (A) Restriction map of the *acoA*, *acoB*, and *acoC* genes of *K. pneumoniae* CG43. The locations of the coding regions are indicated. The intergenic distances and the restriction sites for deletion purposes are also shown. Ao:DCPIP OR and DHLAT activities in the toluenized extracts of the recombinant clones grown in LB with 0.01% acetoin are shown at the right. (B) Heterologous expression of the *K. pneumoniae aco* operon in *E. coli*. *E. coli* BL21 containing the recombinant plasmid was grown in LB supplemented with 100 μg of ampicillin per ml at 37°C with vigorous shaking until an OD_{600} of 0.4 was obtained. IPTG was then added to a final concentration of 1 mM, and the incubation was continued at 37°C for 120 min. Total cellular proteins were prepared and resolved by SDS-PAGE. Lanes 2, 4, 6, and 8 contain whole-cell proteins from IPTG-induced cells carrying pGEM-1 and its derivatives. Lanes 3, 5, 7, and 9 contain proteins from the samples without IPTG induction. Lanes: 1, molecular markers; 2 and 3, BL21(pGEM-1); 4 and 5, BL21(pHP713); 6 and 7, BL21(pHP719); 8 and 9, BL21(pHP720). The overexpressed products of *acoA*, *acoB*, and *acoC* are marked at the right by A, B, and C, respectively.

On the basis of sequence analysis, it is reasonable to propose that the *K. pneumoniae* acetoin dehydrogenase originally evolved from the PDH complex and was adopted for the cleavage of acetoin, whose structure is similar to that of pyruvate. Therefore, it is not surprising that the *K. pneumoniae* acetoin dehydrogenase does not utilize 2,3-butanediol, which has a structure distinct from that of pyruvate. The conversion

TABLE 1. Substrate specificity of DCPIP OR[a]

Substrate[b]	% Activity
Acetoin	100.0
Acetate	3.8
Acetaldehyde	6.7
Acetoacetate	1.9
2,3-Butanediol	3.8
Diacetyl	85.6
Ethyl alcohol	3.8
Glycerol	1.9
Pyruvate	3.8
None	3.8

[a] The DCPIP OR (89 μg) of *E. coli* (pHP654), partially purified by chromatography on DEAE-agarose (Bio-Rad), was incubated at 37°C in 1 ml of 100 mM potassium phosphate buffer (pH 7.0) in the presence of 0.10 mM DCPIP, 0.08 mM thymine PP_i, 0.5 mM $MgCl_2$, and 0.4 mM substrate.

[b] The concentration of each substrate was 0.4 mM.

of 2,3-butanediol to acetoin in *K. pneumoniae* CG43 is quite sluggish (data not shown), which may explain the poor growth of *K. pneumoniae* CG43 on 2,3-butanediol. When *K. pneumoniae* CG43 was grown in M9 medium containing 0.2% acetoin, a doubling time of approximately 8 h was recorded. Although an acetoin depleting activity could be demonstrated in vivo for *E. coli* DH1(pHP654), the strain grew poorly with acetoin as the sole carbon source. This result is consistent with the finding that *E. coli* cells carrying the entire *aco* operon of *A. eutrophus* do not grow when acetoin is used as the sole carbon source (32). With *A. eutrophus* H16, at least an additional locus encoding the acetoin-dependent acetaldehyde dehydrogenase is required for the growth of the organism on acetoin (33). It is possible that the energy derived from the dissimilation of acetoin is produced sluggishly in the presence of only *acoABCD* and may not be sufficient to sustain the growth of bacteria. Since a similar acetoin catabolic pathway may be adopted from *A. eutrophus*, an acetoin-dependent acetaldehyde dehydrogenase is probably also required for the growth of *K. pneumoniae* CG43 on acetoin. Two closely linked ORFs with unknown biochemical functions which are required for the utilization of acetoin in *B. subtilis* have recently been reported (11). No homology between those two gene products and the *acoABCD* products was observed. The likelihood of the presence of similar genes in *K. pneumoniae* involved in acetoin catabolism is still to be determined.

The *aco* operon of *K. pneumoniae* CG43 is highly active in *E. coli* BL21, even in the absence of an inducer. We believe that the expression of the *K. pneumoniae aco* operon contained in pHP713 is under the control of its own potent promoter. In contrast to the σ^{54}-dependent transcription suggested for *A. eutrophus acoXABC* (32), the transcription start site of *K. pneumoniae acoABCD* is preceded by a σ^{70}-type promoter. It is likely that the *K. pneumoniae acoABCD* operon resembles the *P. putida* BCODH operon, which does not require the *rpoN* sigma factor for expression (23).

Although many of its features are homologous to those of the *A. eutrophus aco* operon, *acoX* could not be found in the *K. pneumoniae acoABCD* operon. On the other hand, we found an ORF (*acoK*; Fig. 1) of at least 475 amino acids in length running in the opposite direction of the *acoABCD* operon. Thus, *acoK* is divergently transcribed from a promoter which probably overlaps the promoter of the *acoABCD* operon. Analysis of the partial AcoK sequence revealed a significant homology with the regulatory protein MalT of the *E. coli mal* regulon (37). Our preliminary result has shown that the *acoK* gene is essential for the acetoin-induced expression of the *acoABCD* operon and that deletion of the gene results in a constitutive expression. It is likely that the AcoK protein plays a regulatory role in the acetoin-inducible expression of the *K. pneumoniae aco* operon.

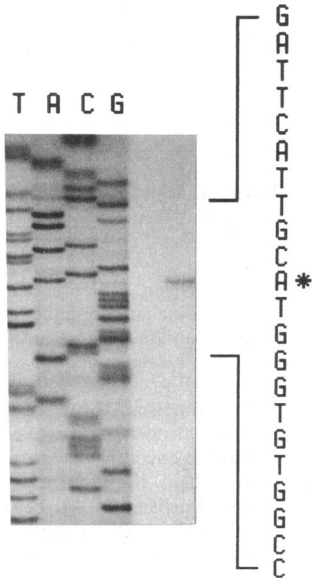

FIG. 6. Primer extension analysis of the *K. pneumoniae aco* operon. The transcription start site was mapped by extension of the primer, a reverse complement to sequence positions 327 to 346 in Fig. 1, with reverse transcriptase, and the product was analyzed on a 6% polyacrylamide-urea sequencing gel. The dideoxy sequencing ladder was generated by using the same primer. Lanes T, A, C, and G show the sequencing reaction products. The primer extension product and the relevant region of the sequence are shown together with the start site (*).

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