Biochemical and Genetic Analyses of Acetoin Catabolism in *Alcaligenes eutrophus*

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In genetic studies on the catabolism of acetoin in *Alcaligenes eutrophus*, we used Tn5::mob-induced mutants which were impaired in the utilization of acetoin as the sole carbon source for growth. The transposon-harboring *EcoRI* restriction fragments from 17 acetoin-negative and slow-growing mutants (class 2a) and from six pleiotropic mutants of *A. eutrophus*, which were acetoin-negative and did not grow chemolithoautotrophically (class 2b), were cloned from pHCT9 gene banks. The insertions of Tn5 were mapped on four different chromosomal *EcoRI* restriction fragments (A, C, D, and E) in class 2a mutants. The native DNA fragments were cloned from a λ L47 or from a cosmid gene bank. Evidence is provided that fragments A (21 kilobase pairs [kb]) and C (7.7 kb) are closely linked in the genome; the insertions of Tn5 covered a region of approximately 5 kb. Physiological experiments revealed that this region encodes for acetoin:dichlorophenol-indophenol oxidoreductase, a fast-migrating protein, and probably for one additional protein that is as yet unknown. In mutants which were not completely impaired in growth on acetoin but which grew much slower and after a prolonged lag phase, fragments D (7.2 kb) and E (8.1 kb) were inactivated by insertion of Tn5::mob. No structural gene could be assigned to the D or E fragments. In class 2b mutants, insertions of Tn5 were mapped on fragment B (11.3 kb). This fragment complemented pleiotropic *hno* mutants in *trans*; these mutants were impaired in the formation of a *rpoN*-like protein. The expression of the gene cluster on fragments A and C seemed to be *rpoN* dependent.

Many species of bacteria degrade acetoin. The 2,3-butanediol cycle described by Juni and Heym (25) in *Acinetobacter calcoaceticus* involves 2,3-butanediol dehydrogenase and acetoin dehydrogenase (Fig. 1A). In contrast, other species, such as *Bacillus subtilis* (24, 31, 32) and *Pelobacter carbinolicus* (36, 41), degrade acetoin directly by an oxidative-thiolytic cleavage (Fig. 1C).

Alcaligenes eutrophus also degrades acetoin (50). Acetoin-grown cultures are devoid of both 2,3-butanediol dehydrogenase and acetoin dehydrogenase activities, and mutants lacking 2,3-butanediol dehydrogenase can still grow on acetoin. Thus, *A. eutrophus* also lacks the 2,3-butanediol cycle. During growth on acetoin, *A. eutrophus* induces the formation of at least three proteins: (i) an acetoin:dichlorophenol-indophenol oxidoreductase (Ao:DCPIP OR), which catalyzes the oxidative-hydrolytic cleavage of acetoin in vitro (Fig. 1B); (ii) a fast-migrating protein (FMP), which exhibits a high mobility toward amido black in polyacrylamide gels; and (iii) acetaldehyde dehydrogenase II (AcDH-II, EC 1.2.1.3), an enzyme which is also induced in ethanol-utilizing mutants during growth on ethanol. Although the function of FMP is still unknown, the presence of the other proteins is consistent with the hypothesis that in *A. eutrophus*, as in other bacteria, the catabolism of acetoin is initiated by oxidative cleavage into two C_2 compounds.

During investigations of the regulation of expression of the fermentative alcohol dehydrogenase in *A. eutrophus*, we isolated bundles of Tn5::mob-induced mutants which were impaired in the utilization of 2,3-butanediol because of the insertion of Tn5::mob into the *adh* gene (mutant class 1 [22, 29]). Mutants which were impaired in the utilization of 2,3-butanediol and acetoin appeared at a threefold-higher frequency (mutant class 2a [29]). Pleiotropic mutants which,

in addition to 2,3-butanediol and acetoin, did not grow chemolithoautotrophically on H_2 , CO_2 , and O_2 appeared less frequently (mutant class 2b [29]).

In this study we analyzed these class 2a and class 2b mutants in detail and mapped the insertions of Tn5::mob. Our studies revealed that the gene loci that are essential for the utilization of acetoin are located on five different chromosomal *EcoRI* restriction fragments. The insertional inactivation of one gene locus in mutants belonging to class 2b caused a pleiotropic effect. DNA fragments which harbored the Tn5::mob-inactivated genes were cloned and provided homologous DNA probes for the isolation of the intact genes from the wild type. This investigation provides the basis for the identification and molecular characterization of those proteins that are involved in the catabolism of acetoin in *A. eutrophus*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *A. eutrophus* and *Escherichia coli*, as well as the plasmids and the bacteriophage used in this study, are listed in Table 1 and Fig. 2.

Growth of bacteria. *E. coli* was grown at 37°C in Luria-Bertani (LB) medium or in M9 mineral salts medium (33). *A. eutrophus* was grown either in a nutrient broth medium (0.8% [wt/vol]) or in a mineral salts medium (43) at 30°C.

Isolation of DNA. Total genomic and plasmid DNAs were isolated by standard procedures (33, 34). DNA restriction fragments were isolated from agarose gels by electroelution into a sodium acetate solution in an apparatus obtained from Biometra (Göttingen, Federal Republic of Germany).

λ DNA phage particles were prepared by the method of Maniatis et al. (33) from plates exhibiting confluent lysis of *E. coli* WL87. The phages were harvested by high-speed centrifugation ($48,000 \times g$ for 2 h at 4°C), suspended in SM

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FIG. 2. Pattern of DNA fragments isolated from acetoin-negative mutants of *A. eutrophus* (29) obtained by restriction of Tn5::mob-harboring *EcoRI* fragments with *SalI*. The fragments were separated on agarose or polyacrylamide gels and were characterized with respect to their sizes. An arrow at the respective fragment indicates that this *SalI* restriction fragment hybridized with a DNA probe specific for Tn5::mob. bp, Base pairs.

E. coli DH1 (cosmid DNA) or WL95 (λ L47 DNA) by the method of Hohn and Collins (15). For the propagation of λ L47 libraries, *E. coli* WL87 was used.

Hybridization experiments. DNA restriction fragments were separated electrophoretically in 0.8% (wt/vol) agarose gels as described above for the analysis of plasmid DNA. Conditions for the transfer of denatured DNA to nitrocellulose filters (pore size, 0.45 μ m; BA85; Schleicher and Schüll, Dassel, Federal Republic of Germany), for their hybridization with biotinylated probes (labeled with bio-11-dUTP), and for the detection of biotinylated DNA have been described previously (29). λ L47 libraries were screened on filters as described by the manufacturer (Bethesda Research Laboratories, Inc., Focus 7:11, 1985; Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

Transfer of DNA. For transformation, *E. coli* was grown aerobically in LB medium containing 20 mM $MgCl_2$ (9) at 37°C. Competent cells were prepared and transformed by using the calcium chloride procedure described by Maniatis et al. (33). Conjugations were performed on solidified nutrient broth medium as described by Friedrich et al. (8). After 20 h of incubation at 30°C, the cells were washed from the agar and plated onto medium that was suitable for isolation of the transconjugants.

Preparation of crude extracts and determination of enzyme activities. Crude extracts containing soluble protein were prepared from washed cells as described previously (50). Activities of Ao:DCPIP OR and AcDH-II were determined as described by Steinbüchel et al. (50) and Jendrossek et al. (21), respectively. Protein was routinely determined by the methods described by Beisenherz et al. (2).

Electrophoretic methods. Proteins were separated in gels containing 7.5% (wt/vol) polyacrylamide in Tris borate buffer (pH 8.9), as described by Stegemann et al. (49).

Protein was stained with Coomassie brilliant blue (53). To stain gels for AcDH-II or Ao:DCPIP OR, the procedures described by Steinbüchel et al. (50) were applied.

Immunological methods. Immunodiffusion (Ouchterlony) tests were carried out by using gels containing 1% (wt/vol) agarose in 50 mM diethylbarbiturate-acetate buffer (pH 8.2) as described by Oakley (35).

Analysis of metabolites. Acetoin, 2,3-butanediol, acetone, acetaldehyde, acetate, diacetyl, diacetylmethylcarbinol (DAMC), and methylacetoin were determined by gas chromatography, as described by Steinbüchel and Schlegel (51).

Chemicals. Restriction endonucleases, biotin-11-dUTP, the nick-translation kit, the DNA detection kit, T4 DNA ligase, λ DNA, and substrates used in the enzyme assays were obtained from C. F. Boehringer & Soehne (Mannheim, Federal Republic of Germany) or from GIBCO/BRL-Bethesda Research Laboratories GmbH (Eggenstein, Federal Republic of Germany).

RESULTS

Optimization of growth on acetoin and growth characteristics. Growth of *A. eutrophus* on acetoin varied, depending on the concentration and the age of the carbon source. If acetoin was prepared freshly and was used to a final concentration of 0.3% (wt/vol), growth proceeded with a doubling time of approximately 3.5 to 4.0 h (Fig. 3). At higher concentrations the doubling time increased; e.g., it exceeded 5 h with 0.5% (wt/vol) acetoin. These effects were probably due to the presence of diacetyl in aqueous solutions of acetoin. Diacetyl appears as an autoxidation product of acetoin and exerts an inhibitory effect on the growth of bacteria (20) because of the reaction with arginyl residues of proteins (18). Growth of *A. eutrophus* on acetoin was not

FIG. 3. Growth of *A. eutrophus* on acetoin. *A. eutrophus* wild-type strain H16 (□) and acetoin-negative mutants H1098 (●; subclass A), H1094 (○; subclass E), and H1074 (△; subclass D) were grown aerobically at 30°C. Mineral salts medium containing 0.2% (wt/vol) acetoin was inoculated with cells that were grown on fructose. Growth was monitored with a Klett-Summerson photometer.

stimulated by supplementing the medium with 100 μ g of thiamine per ml or 200 nM pyrroloquinoline quinone or by increasing the concentration of iron 10-fold.

In a previous study (29), we described the isolation of 32 transposon-induced mutants of *A. eutrophus*, which did not grow on acetoin (mutant class 2a), and of eight pleiotropic mutants, which did not grow on acetoin or chemolithoautotrophically (mutant class 2b). An examination of various mutants for their ability to use different alcohols revealed that class 2b mutants also did not grow on 1-butanol. All class 2b mutants and most of the class 2a mutants were completely unable to use acetoin; only a few class 2a mutant strains (H1050, H1068, H1074, H1094) formed faint colonies after a period of prolonged incubation on agar plates containing 0.3% (wt/vol) acetoin. In order to describe the growth of these mutant strains on acetoin more precisely, growth was monitored in liquid cultures by recording the increase in absorbance (Fig. 3). Growth of mutant strains H1050 and H1094 only started after a long lag phase (20 to 25 h); the doubling time was approximately 6 h. With mutant strains H1068 and H1074 the doubling time was 28 h (Fig. 3).

Growth on methylacetoin. In order to discriminate between the cleavage of acetoin and that of diacetyl, we tested 2-hydroxy-2-methyl-butanone-3 (methylacetoin) as a substrate for growth. Cells of *A. eutrophus* were able to use methylacetoin as the sole carbon source; however, growth was rather slow (doubling time, 12.5 h). Concomitant with the utilization of methylacetoin, equimolar amounts of acetone appeared in the medium. Methylacetoin is probably oxidatively-hydrolytically cleaved into acetate and acetone by Ao:DCPIP OR (Fig. 1D [36]; see below). As methylacetoin cannot be oxidized to the corresponding ketone compound, this experiment demonstrates that, in addition to the butanediol cycle, at least one different catabolic pathway for the utilization of acetoin must exist in *A. eutrophus*, if the 2,3-butanediol cycle exists at all.

Further evidence against the existence of the 2,3-butanediol cycle was provided by the fact that mutants which were defective in the fermentative alcohol dehydrogenase that enables the cells to utilize 2,3-butanediol were still able to grow on acetoin (29) and did not excrete relevant amounts of

2,3-butanediol (data not shown). Participation of the cycle should have resulted in the accumulation of half equimolar amounts of 2,3-butanediol in the medium during the utilization of acetoin.

Properties of Ao:DCPIP OR. Crude extracts of acetoin-grown cells of *A. eutrophus* H16 exhibited Ao:DCPIP OR (Fig. 1B), diacetyl:DCPIP oxidoreductase (Di:DCPIP OR; Fig. 1E), and methylacetoin:DCPIP oxidoreductase (MeAo:DCPIP OR; Fig. 1D) activity. All three activities were completely lost by dialysis. Activity was restored by the addition of thiamine pyrophosphate (TPP) and magnesium ions. The apparent K_m values for TPP and Mg^{2+} were 14.5 and 50 μ M, respectively. Upon heat inactivation (5 min, 95°C) approximately 25% of the activity remained. In polyacrylamide gels Ao:DCPIP OR, Di:DCPIP OR, and MeAo:DCPIP OR were localized at the same position and exhibited a relative mobility of 0.42 toward amido black. As shown by activity staining, the protein which most likely catalyzed all three reactions migrated slightly more slowly than did the fermentative alcohol dehydrogenase, which exhibited a relative mobility of 0.46 toward the tracking dye.

A five- to sixfold-enriched, partially purified preparation of Ao:DCPIP OR was obtained by chromatography of dialyzed crude extract on anion-exchanger DEAE-Sephacel which was equilibrated with 20 mM potassium phosphate buffer (pH 7.2). The enzyme was coeluted with Di:DCPIP OR and MeAo:DCPIP OR from the column at 170 mM KCl.

This preparation was incubated in 100 mM potassium phosphate buffer (pH 7.2) in the presence of 0.5 mM DCPIP, 0.08 mM TPP, 0.5 mM Mg^{2+} , and 2 mM acetoin. As revealed by gas chromatographic analysis, acetaldehyde and acetate appeared in equimolar amounts to the same extent as acetoin disappeared. If acetoin was replaced by methylacetoin, equimolar amounts of acetate and acetone appeared. These reactions were strictly dependent on the presence of DCPIP. With diacetyl as the substrate, DAMC and acetate were formed (see below).

As these reactions were TPP dependent, and as acetaldehyde or acetone were released from acetoin and methylacetoin, respectively, hydroxyethyl-thiamine pyrophosphate was probably one intermediate of this reaction. In vitro this intermediate was hydrolyzed to acetate and TPP if an oxidant was present (17). This oxidative-hydrolytic cleavage may be an artificial reaction in vitro, e.g., the oxidation of a carbanionic enzyme substrate intermediate, and may result in the formation of products different from those formed in vivo (6, 11).

Formation of DAMC and DAMC reductase activity. One key step in the 2,3-butanediol cycle (Fig. 1A) is the formation of DAMC. If crude extracts were incubated in 100 mM potassium phosphate buffer (pH 7.2)–0.08 mM TPP–0.5 mM $MgCl_2$ –3 mM diacetyl, diacetyl was converted to acetate and DAMC at a rate of 0.03 μ mol/min $\cdot$ mg of protein (Fig. 4). The stoichiometry of the conversion was consistent with the reaction catalyzed by DAMC synthase (step 3 in Fig. 1A). Although the formation of the characteristic intermediate of the cycle could be demonstrated, the rate was far too low to account for the rate of acetoin consumption during the growth of *A. eutrophus* on this substrate. The rate was fourfold higher if an eluate from DEAE-Sephacel enriched for Ao:DCPIP OR was used. The formation of DAMC did not occur with acetoin.

As shown previously, enzymes forming hydroxyethyl-TPP (HETPP) as an intermediate can catalyze the condensation reaction of HETPP with various aldoses or with acetaldehyde, resulting in the formation of 1-deoxyketoses

FIG. 4. Formation of DAMC by partially purified Ao:DCPIP OR. The Ao:DCPIP OR (10 mg) of *A. eutrophus*, which was partially purified by chromatography on DEAE-Sephacel, was incubated at 30°C in 10 ml of potassium phosphate buffer (pH 7.2) in the presence of 0.08 mM TPP, 0.5 mM $MgCl_2$, and 3 mM diacetyl. Samples were taken and analyzed by gas chromatography for the presence of diacetyl (□), DAMC (●), and acetate (■).

or acetoin (1, 54), respectively. Therefore, the formation of DAMC may be not dependent on the presence of a distinct DAMC synthase. A low rate of DAMC formation, as was observed with *A. eutrophus*, may result from the condensation of diacetyl with HETPP, which probably occurs as an intermediate of the diacetyl cleavage reaction, and may be catalyzed by Ao:DCPIP OR.

Another prerequisite for the 2,3-butanediol cycle was the presence of DAMC reductase (step 4 in Fig. 1A). DAMC-dependent oxidation of NADH, but not of NADPH, could be detected; but again, the specific activity in crude extracts of strain H16 or N9A was low ($<0.04 \mu\text{mol/min} \cdot \text{mg}$ of protein). In contrast to Ao:DCPIP OR and FMP, this activity was formed constitutively on all growth substrates tested.

Cloning of Tn5-labeled fragments. In order to localize the genes that were essentially involved in the utilization of acetoin, total genomic DNAs of 17 randomly chosen class 2a mutants and six class 2b mutants were isolated, partially digested with *EcoRI*, and ligated with pHC79 DNA. The ligation mixtures were packaged with λ coat proteins and transfected into *E. coli* DH1. Recombinant clones which harbored Tn5::mob-labeled *EcoRI* restriction fragments were isolated from LB agar plates containing 140 μg of kanamycin per ml.

In order to identify the Tn5-labeled fragments in hybrid cosmids, the plasmid DNAs from eight recombinant clones obtained from each packaging reaction were isolated, digested with *EcoRI*, and separated by electrophoresis in an agarose gel. In addition to a few DNA fragments with various sizes, each recombinant plasmid contained a 6.4-kilobase-pair (kb) fragment (pHC79 DNA) and one fragment that was 25 to 30 kb if it was cloned from mutant strains N1230, N1236, N1239, N1240, N1244, N1246, N1247, and H1098; approximately 18.5 kb if it was cloned from all class 2b mutants; approximately 15 kb if it was cloned from mutant strains N1233, N1245, N1248, H1050, H1053, H1068, H1074, and H1094; or 10.5 kb if it was cloned from mutant strain N1237. The presence of Tn5::mob in these fragments was also demonstrated by hybridization with the biotinylated 4.1-kb *HindIII* restriction fragment of pSUP5011, which harbored the central region of Tn5::mob.

Analysis of Tn5-labeled fragments and mapping of Tn5 insertions. The Tn5::mob-harboring fragments isolated from

the 23 independent mutants investigated in this study were referred to as AT, BT, CT, DT, ET, and FT, with the mutant strains designations given as superscripts. These fragments and their subfragments were investigated in order to obtain a physical map of the native DNA fragments. They were isolated from agarose gels by electroelution and digested with *SaII*. For the class 2a mutants, the pattern of DNA restriction fragments obtained revealed that Tn5::mob was inserted into four different *EcoRI* fragments. Therefore, mutants belonging to class 2a were split into four different subclasses. The corresponding *EcoRI* restriction fragments in the wild type were referred to as A, C, D, and E. As discussed below, mutant strain N1237 did not represent a separate subclass. In class 2b mutants, Tn5::mob was inserted into only one wild-type fragment, which was referred to as fragment B (Fig. 2).

From the transposon-harboring *EcoRI* fragments, a bundle of *SaII* restriction fragments was derived; these fragments had identical sizes. The sizes of each two *SaII* restriction fragments varied, indicating a different location of Tn5::mob within the fragment; the presence of Tn5::mob DNA in these fragments was proved by hybridization. The *SaII* restriction pattern also revealed that in fragments A and C, two different *SaII* fragments were the target for Tn5::mob (0.35 and 1.75 kb or 1.6 and 2.5 kb, respectively), whereas in mutants belonging to subclasses B, D, and E, one distinct *SaII* fragment (2.8, 1.8, and 2.2 kb, respectively) was the target for Tn5::mob. As was apparent from the sizes of the Tn5::mob-harboring *SaII* fragments of mutant strain N1236, an irregular insertion of Tn5::mob DNA into fragment A occurred. However, this was not investigated in detail. Twenty-three insertions were cloned and analyzed. Data for 22 of these insertions are presented in this report; the insertion in mutant strain 1237 resulted from a cointegration event in fragment A (3; data not shown). Therefore, the insertion in strain 1237 does not define a unique locus that is required for acetoin utilization.

The locations of individual insertions of Tn5::mob in fragments A, B, C, D, and E were determined by restriction enzyme mapping to within about ± 50 base pairs, in consideration of the restriction map for Tn5 (23), which in pSUP5011 harbors the RP4 mob site (47). The results are shown in Fig. 5.

Localization of the wild-type genes. The fragments AT^{H1098}, CT^{N1245}, DT^{H1068}, and ET^{H1094}, each representing one gene locus that was essential for the catabolism of acetoin of each subclass, and fragment BT^{H1075}, representing class 2b, were isolated from a representative hybrid cosmid, biotinylated, and hybridized with wild-type genomic DNA that was completely digested with *EcoRI*. Signals appeared which represented 21-kb (with AT^{H1098}), 11.3-kb (BT^{H1075}), 7.7-kb (CT^{N1245}), 7.2-kb (DT^{H1068}), or 8.1-kb (ET^{H1094}) DNA fragments. This was in agreement with the size of the Tn5::mob-harboring *EcoRI* restriction fragments.

Cloning of the wild-type genes. To clone the *EcoRI* fragments B, C, D, and E, the Tn5-labeled fragments mentioned above were used to detect the corresponding native fragments in a λ L47 gene bank of *A. eutrophus* H16. *E. coli* WL7 was infected with recombinant λ L47 phages and plated onto LB medium. Plates with approximately 1,000 plaques were blotted onto nitrocellulose membranes. These membranes were hybridized with biotinylated BT^{H1075}, CT^{N1245}, DT^{H1068}, and ET^{H1094} DNAs. Signals appeared with all probes except CT^{N1245}. As was expected from the sizes of the Tn5::mob-harboring DNA fragments, the sizes of the cloned *EcoRI* restriction fragments B, D, and E in the

FIG. 5. Physical maps of *EcoRI* restriction fragments A, B, C, D, and E. Triangles indicate the insertion of *Tn5::mob* in the mutant strain. No cleavage sites were detected for *HindIII* in fragment A; for *HindIII* or *KpnI* in fragments B and D; for *KpnI* or *XhoI* in fragment C; or for *HindIII*, *XhoI*, *BamHI*, or *EcoRV* in fragment E.

recombinant phages were 11.3, 7.2, and 8.1 kb, respectively. As several attempts to clone fragments C via a λ L47 gene bank failed, we chose a different method. Total genomic DNA of strain H16 was partially digested with *EcoRI* and ligated to pHC79 DNA. Hybrid cosmids from recombinant strains of *E. coli* DH1 were prepared by alkaline lysis, digested with *EcoRI*, and separated in agarose gels. By hybridization with biotinylated CT^{N1245} DNA, it was shown

that 1 of 522 hybrid cosmids was positive for harboring the 7.7-kb *EcoRI* fragment C. The fragments were purified by electroelution and ligated to plasmid pVK101, pHP1016, pSUP202, and pUC9-1 DNAs.

With respect to fragment C, it should be mentioned that all hybrid cosmids and hybrid plasmids, which contained the fragments CT^{H1053}, CT^{N1233}, CT^{N1245}, or CT^{N1248} or the native fragment C, harbored a 8.6-kb *EcoRI* restriction

fragment, which was designated fragment G. It was not possible to clone fragment C independently from fragment G. In contrast, clones harboring only fragment G were easily obtained. The relationship between fragments C and G is unknown, and further detailed studies are needed to evaluate the function of fragment G.

The native fragment A was present in cosmid libraries that were prepared from the genomic DNAs of Tn5-induced mutants belonging to subclass C. As revealed by hybridization with biotinylated AT^{H1098} DNA and by the patterns of *Sal*I restriction fragments, the native fragment A was present in four of five pH79 hybrid cosmids containing CT^{N1233} and in one pH79 hybrid cosmid containing CT^{H1053} that was prepared from the total genomic DNA of mutant strains N1233 and H1053, respectively. Fragment A was not present in any hybrid cosmid which harbored fragments CT^{N1245} or CT^{N1248}. As genomic libraries could be constructed from incompletely digested genomic DNAs, fragments A and C are closely linked on the chromosome. This was also indicated by digestion of these hybrid cosmids with restriction endonucleases and by hybridization experiments (data not shown). Therefore, fragments A and C are probably linked, and the ends of fragments A and C, which are marked with Tn5 insertions, are probably the adjacent ends. However, it should be mentioned that fragment C was not detected in hybrid cosmids which were prepared from genomic DNAs of mutants belonging to subclass A. Fragment A was isolated from a hybrid cosmid harboring CT^{H1053} DNA by electroelution and was ligated to the plasmids mentioned above.

Complementation of class 2a mutants. Broad-host-range vectors pVK101 or pHP1016, which harbored fragment A, B, C, D, or E, were mobilized from *E. coli* S17-1 into two strains of each subclass by conjugation. The ability to utilize acetoin as a carbon source for growth was restored in *trans* in mutants belonging to subclasses A, D, or E only upon transfer of that fragment, which was insertionally inactivated in the genome of the respective mutant; e.g., mutant strain H1098 was complemented by plasmid pVK101::A. Mutants belonging to subclass D or E were not affected by any fragment except fragment D or E, respectively, which fully restored the ability of the bacteria to grow on acetoin without a prolonged lag phase. Interestingly, plasmid pHP1016::C did not complement any mutant belonging to subclass C or any other subclass. As was shown with transconjugants of selected mutants harboring pHP1016::G, the puzzling fragment G did not complement any of the class 2a or class 2b mutants. Plasmid pVK101::B complemented none of the class 2a mutants.

Complementation of class 2b and *hno* mutants. In mutant strains belonging to class 2b, the ability to use acetoin as a sole carbon source for growth was restored only upon the transfer of fragment B. Together with the ability to grow on acetoin, the ability for chemolithoautotrophic growth was restored as well. The highly pleiotropic *hno* mutant strains HF149 and HF09, which are impaired in chemolithoautotrophic growth and are deficient in several other diverse metabolic activities such as nitrate and urea assimilation, denitrification, and transport systems for dicarboxylic acids (38), were also impaired for growth on acetoin. Growth on acetoin and chemolithoautotrophic growth could be restored upon the transfer of plasmid pVK101::B in these mutants as well. The other defects were not the subject of this study. In contrast, fragments A, C, D, and E were not able to complement class 2b or *hno* mutants.

Further evidence that the locus on fragment B was iden-

FIG. 6. Growth of *A. eutrophus* on succinate-acetoin. *A. eutrophus* wild-type strain H16 (□) and acetoin-negative mutant N1248 (○) were grown aerobically at 30°C in a mineral salts medium containing 0.18% (wt/vol) succinate and 0.2% (wt/vol) acetoin. Cultures were inoculated with cells that were grown on succinate. Growth was monitored with a Klett-Summerson photometer. S1 and S2 indicate withdrawal of samples.

tical to the *hno* locus came from a comparison of the physical maps for the 11.3-kb fragment B isolated in this study and the 12.3-kb *Eco*RI restriction fragment present in plasmid pCH148, which harbors the *hno* locus (39). Both fragments were similar with respect to the positions of restriction sites for *Bgl*II, *Bam*HI, *Sal*I, and *Xho*I, with only a few deviations. In addition, Tn5::mob mapped in the same area in class 2b mutants as it did in *hno* mutants (Fig. 5 [39]).

Heterologous expression of cloned genes. As revealed by physiological experiments, the genes for Ao:DCPIP OR (50), AcDH-II (21, 50), and FMP (50) were the most likely candidates to be carried by fragments A, C, D, and E. In addition, other as yet unknown structural components of the acetoin catabolic pathway and regulatory genes may be encoded by these fragments as well. However, all attempts to identify one of the hypothetical proteins in *E. coli* DH1, HB101, or S17-1 harboring one of these fragments in pVK101, pHP1016, or pSUP202 by enzymatic (Ao:DCPIP OR, AcDH-II), gel electrophoretic (FMP), or immunological methods (AcDH-II) failed. In addition, clones of *E. coli* JM83 or JM101 harboring recombinant pUC9-1 plasmids did not express any of the suspect proteins if the cells were grown in LB or M9 medium, even if 0.2 mM isopropyl-β-D-thiogalactopyranoside was added.

Repression of Ao:DCPIP OR, FMP, and AcDH-II formation by succinate or fructose. Recently, it has been shown that Ao:DCPIP OR, FMP, and AcDH-II are only synthesized if acetoin or acetoinogenic substrates are used (50). The formation of AcDH-II is also induced during growth on ethanol (21, 50). When *A. eutrophus* H16 was incubated aerobically in a mineral salts medium containing fructose-acetoin or succinate-acetoin as carbon sources, growth was diauxic (Fig. 6). Ao:DCPIP OR, FMP, and AcDH-II were only present in samples (S2) which were withdrawn at the end of the second growth phase, whereas these proteins were absent from samples (S1) which were withdrawn at the end of the first growth phase.

Evaluation of hypothetical functions of gene loci. In order to assign structural genes to specific loci, we cultivated numerous Tn5-induced, acetoin-negative mutants in the presence of succinate-acetoin. With all mutants belonging to sub-

TABLE 2. Compilation of genetic results

Class	Growth on acetoin	Locus	Mutant strain	Hypothetical function
2a	None	A	e.g., H1098, N1239	Genes for Ao:DCPIP OR or FMP
2a	None	C	e.g., H1053, N1233	Genes for Ao:DCPIP OR or FMP
2a	Slow ($t_d^a = 28$ h)	D	H1068, H1074	Unknown (regulator ?, AcDH-II ?)
2a	Slow ($t_d = 6$ h)	E	H1050, H1094	Unknown (regulator ?, AcDH-II ?)
2b	None	B	e.g., H1070, N1242	<i>rpoN</i> gene

^a t_d , doubling time.

classes A, B, and C, growth terminated at approximately 200 Klett units (Fig. 6), whereas the growth continued very slowly after a prolonged lag phase with mutants belonging to subclasses D and E.

With one exception, all mutant strains belonging to subclasses A, B, or C did not form Ao:DCPIP OR or FMP when the cells were grown in a medium containing succinate-acetoin and when the cells were harvested in the acetoin-utilizing growth phase (S2 in Fig. 6). Only mutant strain N1240 (subclass A) synthesized Ao:DCPIP OR; however, FMP was also absent from this mutant. The rationale for this is unknown and remains to be elucidated. In contrast, mutant strains belonging to subclasses D or E synthesized Ao:DCPIP OR as well as FMP. The same results were obtained when the cells were grown in a medium containing fructose plus acetoin. Acetaldehyde dehydrogenase activity was detected in all mutant strains. In mutants belonging to subclass D or E, this activity was only approximately 50% of that in the wild type or in the other mutants. As *A. eutrophus* possesses three different proteins that exhibit acetaldehyde dehydrogenase activity (these proteins were also present in the crude extracts), it was not possible to assign AcDH-II to one of the fragments; further detailed biochemical studies are necessary.

DISCUSSION

In this study we mapped the gene loci which are essential for the utilization of acetoin on five different chromosomal *EcoRI* restriction fragments. This and previous physiological studies demonstrate that the catabolism of acetoin is complex. Although many bacteria can grow on 2,3-butanediol or acetoin as the carbon source, little is known about the catabolic pathway of these substrates. Juni and Heym (25, 26) have provided evidence that in *Acinetobacter calcoaceticus* 2 mol of 2,3-butanediol is oxidized in a cyclic pathway (Fig. 1A), which releases acetate, via acetoin, diacetyl, DAMC, and acetylbutanediol. Characteristic intermediates or enzyme activities of this cycle have also been detected in *Corynebacterium* sp., *Klebsiella oxytoca*, and *Pseudomonas putida* (25); *Bacillus cereus* (28); and *Pseudomonas fluorescens* (19). Other investigators have provided evidence that the direct cleavage of acetoin (Fig. 1B and C) occurs in *Bacillus subtilis* (32), *Lactobacillus brevis* (12), *Candida utilis* (C. J. Breedveld et al., Proc. 4th European Congress on Biotechnol. vol. III, p. 374, 1987), and *Pelobacter carbinolicus* (36, 37). In *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Streptococcus faecalis* cleavage of diacetyl (Fig. 1F) may occur (7, 45).

In *A. eutrophus* two different proteins which are only synthesized if the cells are grown on acetoin have been identified recently and are most likely involved in the catabolism of acetoin (50). (i) Ao:DCPIP OR catalyzes the oxidative-hydrolytic cleavage of acetoin into acetate and acetaldehyde (Fig. 1B). The natural electron acceptor of this

enzyme is still unknown. Furthermore, it remains to be shown whether this reaction, as measured in vitro, also occurs in vivo. An oxidative-thiolytic cleavage of acetoin, which results in the formation of acetyl coenzyme A instead of acetate (Fig. 1C), is not as advantageous for an aerobic bacterium as it is for an anaerobic bacterium like *Pelobacter carbinolicus* (36). (ii) The function of the second protein, FMP, is completely unknown. However, the abundance of this protein in crude extracts of acetoin-grown cells indicates that it is a catalytic rather than a regulatory protein. Enzymatic and molecular analyses are under way which will result in the molecular characterization of this protein and in an understanding of its function.

The cocloning of fragment A and Tn5-labeled fragments C and the occurrence of DNA fragments in these hybrid cosmids, which contained fragment A and C DNAs, provided further evidence that both fragments are closely linked in the genome. Furthermore, these experiments revealed that the ends of fragments A and C, which are marked with Tn5 insertions, are the adjacent ends. Because of the locations of Tn5 insertions, the cluster of genes might cover a region of approximately 5 kb. On the basis of the experiments performed in this study, the following hypothesis is made. (i) The genes for Ao:DCPIP OR, FMP, and at least one additional unknown protein are located in this region (Table 2). The claim for the unknown protein is unnecessary if Ao:DCPIP OR is composed of two different subunits. (ii) The genes are organized in one operon. (iii) Transcription is initiated on fragment A. (iv) The gene for FMP is located distally from the promoter. On the basis of these assumptions it is possible to explain why fragment C in *trans* did not complement mutants belonging to subclass C and why mutants belonging to subclasses A and C did not express either Ao:DCPIP OR or FMP. The assumption would also explain why it is difficult to purify active Ao:DCPIP OR. The unknown protein may contribute to the oxidative-hydrolytic cleavage of acetoin and may be closely associated with Ao:DCPIP OR. Our hypothesis must be proven by cloning a common DNA fragment that covers the relevant regions of fragment A and C and by molecular analysis of this fragment.

AcDH-II is induced not only during growth on acetoin but also during growth on ethanol (21). Therefore, the regulation of expression of AcDH-II on the one hand and of Ao:DCPIP OR or FMP on the other hand relies on different mechanisms. As two other proteins also exhibit AcDH activity in *A. eutrophus* (AcDH-I [21] and fermentative alcohol dehydrogenase [51]) which might compete with AcDH-II activity, insertional inactivation of the gene for AcDH-II would probably not completely prevent growth on acetoin. As mutants belonging to groups D and E were still able to grow slowly on acetoin, the gene for AcDH-II may be located on fragment D or E (Table 2).

Complementation of the *hno* mutation by fragment B and great similarities of the physical maps revealed that the gene locus, which is located on fragment B, is identical to the *hno* gene identified by Römermann et al. (39) and encodes for a *rpoN*-like protein. This sigma factor seems to be necessary for the expression of genes involved in the catabolism of acetoin and in chemolithoautotrophic metabolism, as well as in the formation of many other proteins (38). Experiments that showed that Ao:DCPIP OR and FMP are not expressed in class 2b mutants indicated that the expression of these two proteins is *rpoN* dependent. Recently, it has been shown that in *E. coli* the expression of the anaerobically inducible formate dehydrogenase and hydrogenase isoenzyme 3 is *rpoN* dependent (4). Because class 2b mutants of *A. eutrophus* synthesize fermentative alcohol and lactate dehydrogenase (29), this locus does not seem to be essential for the expression of these fermentation enzymes which are formed under conditions of restricted oxygen supply (44). However, expression of proteins involved in anaerobic respiration seems to be *rpoN* dependent (38).

The induction of AcDH-II, FMP, and Ao:DCPIP OR indicates the involvement of pathway-specific regulator proteins in *A. eutrophus*. The regulatory genes may be essential for the expression of the three structural genes. This may explain why heterologous expression of the genes did not occur in *E. coli*. For expression, the genes must be cloned directly downstream from an *E. coli* promoter or they must be cotransferred with the regulator gene, which is probably located on fragment D or E (Table 2).

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