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Characterization of Alcohol Dehydrogenase Genes of Derepressible Wild-Type *Alcaligenes eutrophus* H16 and Constitutive Mutants

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The nucleotide sequence of the gene that encodes the fermentative, derepressible alcohol dehydrogenase (ADH) in *Alcaligenes eutrophus* H16 and of adjacent regions was recently determined. Two potential −10 regions resembling the *Escherichia coli* σ^{70} consensus sequence were identified 77 and 93 nucleotides upstream of the structural gene. By determination of the 5′ mRNA terminus of the wild-type *adh* gene, the proximal −10 region was identified as responsible for *adh* expression under derepressive conditions. Transcription started seven nucleotides downstream of this region, at position 388. Sequence analysis of seven mutants expressing the *adh* gene under aerobic conditions revealed mutations in one or the other potential −10 region. In all seven strains, the mutations restored the invariant T of the *E. coli* promoter consensus sequence. Mutants altered in the proximal −10 region transcribed the *adh* gene under aerobic conditions with the same 5′ mRNA terminus as in the wild type; gene expression was impaired very little under aerobic conditions. Mutants altered in the distal −10 region also transcribed the *adh* gene aerobically but were still partially derepressible. The 5′ mRNA terminus was seven nucleotides downstream of the distal −10 region, at position 372. When these mutants were cultivated under conditions of restricted oxygen supply, the *adh* gene was transcribed from both −10 regions, resulting in the synthesis of two mRNA species with different 5′ termini.

Although strictly dependent on oxygen or nitrate as electron acceptors for growth, *Alcaligenes eutrophus* is able to derepress typical pyridine nucleotide-dependent fermentation enzymes such as a multifunctional alcohol dehydrogenase (ADH) and a lactate dehydrogenase (LDH). Both enzymes are synthesized only if cells are cultivated under conditions of restricted oxygen supply (27, 33, 34). In addition to the ability to produce molecular hydrogen (15, 34) and to synthesize poly(β-hydroxybutyric acid) (34), both enzymes appear to provide a safety valve for the release of excess reducing power in the absence of exogenous hydrogen acceptors such as oxygen or nitrate, as indicated by the excretion of ethanol, 2,3-butanediol, and lactate into the medium (33). The fermentative ADH consists of four identical subunits with a molecular weight of 38,549, as calculated from the nucleotide sequence (13). The ADH catalyzes the NAD(P)-dependent oxidation of ethanol, 2,3-butanediol, and acetaldehyde and the reduction of acetaldehyde, acetoin, and diacetyl (31).

Our interest focuses on the regulation of expression of fermentation enzymes in the strict aerobe *A. eutrophus*. Recently, we described the cloning and sequencing of the gene coding for the fermentative ADH of *A. eutrophus* H16 (13, 14). Sequence analysis of the 5′ upstream *adh* region revealed two adjoining potential −10 regions that exhibited homologies to the *Escherichia coli* σ^{70} consensus sequence (9, 22). In both putative promoters, the invariant T of the *E. coli* σ^{70} consensus sequence was replaced by a G. Since the consensus sequence of *A. eutrophus* promoters is still unknown, the significance of these two potential −10 regions is unclear. Identification of the *adh* transcription start should indicate the location of the promoter.

In previous studies, we described the isolation of mutants expressing the *adh* gene also under aerobic conditions (14, 29). In this study, we continued the physiological characterization of these mutants. In addition, we describe the cloning and sequencing of the regions located 5′ upstream of the structural gene and determined the *adh* transcription start in the wild type and in the mutants mentioned above. Taking into consideration all available data, we are now able to identify the *adh* promoter of *A. eutrophus* H16.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The strains of *A. eutrophus* and *E. coli* as well as plasmids and phages used in this study are listed in Table 1.

Media and growth conditions. *A. eutrophus* was routinely grown with mineral salts medium (26) in sidearm flasks on a rotary shaker. Growth was monitored with a Klett-Summerson colorimeter equipped with a 520- to 580-nm filter. For derepression of fermentation enzymes, cells were grown aerobically in glass fermentors containing 4 liters of mineral medium supplemented with 1.5% (wt/vol) sodium gluconate. After exponential growth with an unrestricted supply of oxygen (approximately 500 ml of air per min) to an optical density at 436 nm of 10, 1.5% (wt/vol) sodium gluconate were added and the aeration rate was decreased to 50 ml of air per min (30).

E. coli was grown in Luria-Bertani (LB) medium or in M9 mineral salts medium at 37°C (18). If necessary, antibiotics or other supplements were added in the following concentrations (micrograms per milliliter): (50), thiamine (1), proline (50), and nalidixic acid (10 to 100), and coumermycin (10 to 100).

Isolation and analysis of DNA. Total genomic DNA of *A. eutrophus* was isolated from cells grown in fructose (0.2%, wt/vol) mineral salts medium at 30°C by the procedure described by Marmur (19).

Plasmid isolation from *E. coli* was performed by the alkaline lysis method (18) or by a modified Birnboim-Doly procedure (5). Plasmid DNA or genomic DNA was digested

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant characteristics[a]	Source or reference
Strains		
A. eutrophus		
H16	Wild type; ethanol$^-$ 2,3-butanediol$^-$ *adh*(Drp)	36; DSM 428, ATCC 17699
HC1409, HC1421	Km^r *adh*(Con) ethanol$^-$ 2,3-butanediol$^+$	14
CF101, CF17, CF303	*adh*(Con) ethanol$^+$ 2,3-butanediol$^+$	29
CF100, CF106, CF108	*adh*(Con) ethanol$^+$ 2,3-butanediol$^+$	C. Fründ, diploma thesis, 1985
DJ21	*adh*(Con) ethanol$^+$ 2,3-butanediol$^+$	D. Jendrossek, diploma thesis, 1985
HF09	*hno rpoN*	25
HF149	Km^r *hno rpoN*	10, 28
E. coli		
JM83	*ara* Δ(*lac-proAB*) *thi* φ80 *lacZ*ΔM15	32
JM101	*supE* Δ(*lac-proAB*) *thi*(F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}ZΔM15)	39
WL87	*recBC*	Amersham Buchler
Plasmid		
pUC91	Ap^r *lacPOZ′*	8
Phage		
Lambda L-47		17

[a] Abbreviations: Drp, derepressible; Con, constitutive.

with restriction endonucleases, using buffers supplied by the manufacturer. DNA fragments were routinely analyzed in agarose gels in TBE buffer (50 mM Tris, 50 mM boric acid, 2 mM disodium EDTA, pH 8.5).

DNA sequence analysis. DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. (23) with double-stranded plasmid DNA isolated from strain JM83 and with [α-^{35}S]dATP, using a T7 polymerase sequencing kit as described by the manufacturer (Pharmacia-LKB, Uppsala, Sweden). An oligonucleotide (5′-GGTACA TGCGTTGCTCC-3′) complementary to +6 to −10 of the wild-type *adh* gene (13) was synthesized with a Gene Assembler apparatus (Pharmacia-LKB) and served as a primer; alternatively, commercial pUC primers were used. Because of the high G+C content of *A. eutrophus*, DNA sequencing reactions were routinely performed at 45°C. Products of the sequencing reaction were separated in an S2 sequencing apparatus (GIBCO/BRL Bethesda Research Laboratories GmbH, Eggenstein, Federal Republic of Germany) or in a Macrophor sequencing apparatus at 65°C (Pharmarcia-LKB) in 7% (wt/vol) polyacrylamide gels in a buffer (pH 8.3) containing 100 mM Tris hydrochloride, 83 mM boric acid, 1 mM disodium EDTA, and 42 to 45% (wt/vol) urea.

Construction of lambda L-47 libraries and detection of clones harboring DNA fragments homologous to *adh*. Genomic DNA was digested with *Eco*RI. Fragments were purified by phenol-chloroform extraction and ligated to lambda L-47 *Eco*RI arms. The products were packaged with lambda coat proteins by using an in vitro packaging kit prepared as described by Hohn (11). The resulting phage preparation was used to infect *E. coli* WL87 cells. DNA of recombinant lambda L-47 phages were transferred from plaques to nitrocellulose or nylon filters (pore size, 0.45 μm) by capillary blotting. The DNA was then alkali denatured, neutralized, washed with 2× SSC (0.3 M sodium chloride, 0.03 M trisodium citrate, pH 7), and fixed to the filter by baking at 80°C or by UV irradiation. The filters were hybridized with biotinylated DNA of the 11.5-kilobase-pair fragment HS10 as a DNA probe as described recently (14). Hybridization products were visualized by applying the biotin detection kit purchased from GIBCO/BRL. Positive plaques were purified by two additional infection cycles. *Eco*RI-digested phage DNA was separated in agarose gels, and the fragment of interest was isolated by electroelution.

Preparation of RNA. RNA was isolated from *A. eutrophus* according to a procedure described recently (20).

Determination of the transcription start site. The transcription start site was determined in nuclease S1 protection assays as described by Berk and Sharp (3). To provide a reference, an oligonucleotide complementary to positions 503 to 520 of the *adh* gene (13) (5′-CTTGTCTGCCAGTTCG AT-3′) was used both as a primer in a standard dideoxy-sequencing reaction and for the ^{35}S-labeling reaction.

Determination of enzyme activities. The activities of ADH, LDH, and acetaldehyde dehydrogenase II were determined photometrically in soluble protein fractions obtained after centrifugation at 100,000 × *g* of ultrasonically broken cells as described recently (12, 30). Specific activities were calculated as units per gram of protein. Protein was measured according to Beisenherz et al. (1).

Chemicals. Restriction endonucleases, biotin-11-dUTP, the nick translation kit, and DNA detection kit, T4 DNA ligase, top agar, and all complex media were obtained from GIBCO/BRL. Substrates used in enzyme assays were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany, except 2,3-butanediol, which was from Fluka, Neu-Ulm, Federal Republic of Germany. Agarose NA, T7 DNA polymerase sequencing kit, and DNase (RNase free) were purchased from Pharmacia-LKB. Antibiotics and ethidium bromide were from Sigma Chemical Co., St. Louis, Mo.; 5-bromo-4-choro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Biomol, Ilvesheim, Federal Republic of Germany. Most other chemicals were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany.

RESULTS AND DISCUSSION

Determination of N-terminal amino acid sequence of purified ADH. Since three of the first five codons of the *adh* gene encode for methionine (13), we were not sure about the correct N terminus of the protein (Met-1–Thr-2–Ala-3–Met-4–Met-5–). A functional Shine-Dalgarno sequence was located six nucleotides upstream of the first methionine codon, which led us to the conclusion that this codon represents the start of the gene (13). To determine the N-terminal amino acid sequence of the ADH, the enzyme was purified to homogeneity as described previously (31). The denatured (with sodium dodecyl sulfate and mercaptoethanol) protein

was electroblotted onto a polyvinylidene fluoride membrane (Immobilon; 0.2-μm pore size; Millipore Corp., Bedford, Mass.) and then subjected to Edman degradation. A total of 32 amino acids were identified, and the sequence of these amino acids was identical to amino acids 2 to 33 derived from the nucleotide sequence. Both methionine codons (Met-4 and Met-5) were found to be within the sequence; however, the N terminus was not a methionine but a threonine.

The lack of the first methionine is in agreement with the observation that the amino acid Ala, Ser, Gly, Pro, Thy, or Val, in contrast to Arg, Asn, Asp, Gln, Glu, Ile, Leu, Lys, or Met, permits the posttranslational removal of the N-terminal formylmethionine from proteins in *E. coli* and in other organisms (24, 35). This rule seems to be also valid for *A. eutrophus*: whereas the N termini of the α and δ subunits of the soluble hydrogenase (Met-Asp and Met-Arg [40]) and of the fermentative LDH (Met-Lys [unpublished data]) were not modified, the N termini of the α and τ subunits of the soluble hydrogenase (Ser [40]), and of the fast-migrating protein (Ala [K. Zeh, personal communication]) were modified.

Formation of fermentative enzymes in mutants with semiconstitutive *adh* expression. In cells of *A. eutrophus* H16 grown under restricted oxygen supply, the multifunctional ADH and LDH are derepressed. Mutants that synthesize the ADH also under aerobic conditions are able to utilize 2,3-butanediol (t_d = 2.2 to 6.4 h) or ethanol (t_d = 15 to 50 h) as a carbon source for growth (29). To study the regulation of ADH, its expression in nine mutants was investigated in detail. Strains CF100, CF101, CF106, and CF108 are spontaneous mutants, strains CF17 and DJ21 are nitrite-induced mutants, and CF303 is an ethyl methanesulfonate-induced mutant (29; C. Fründ, diploma thesis, University of Göttingen, Göttingen, Federal Republic of Germany, 1985; D. Jendrossek, diploma thesis, University of Göttingen, 1985). Strains HC1409 and HC1421 were isolated after Tn*5* mutagenesis (14).

To analyze the ability of the mutants to form ADH and LDH in oxygen-limited cultures, the cells were grown in 4-liter fermentors as described previously (30). The wild-type strain H16 synthesized ADH and LDH immediately after the oxygen supply had been restricted (Fig. 1A). As shown in Fig. 1E for mutant HC1421, both Tn*5*-induced mutants formed ADH at very low specific activities during the aerobic growth phase (50 to 65 U/g). Restriction of oxygen supply did not result in a significant increase of specific activity (65 to 80 U/g). The formation of LDH was not affected.

Mutant strains CF108 (Fig. 1B), CF101, and CF106 (Fig. 1C) synthesized ADH at an intermediate level during aerobic growth (300 to 350 U/g). Since restriction of the oxygen supply resulted in an increase of ADH activity to a level comparable to the level of wild-type expression of ADH, these mutants will be referred to as semiconstitutive. In these three mutants, the formation of LDH was not affected.

Mutant strains DJ21, CF100, CF303 (Fig. 1D), and CF17 synthesized ADH at a high level during the aerobic growth phase (800 to 1,200 U/g). Oxygen limitation resulted in only a slight increase of the specific ADH activity. Obviously, the *adh* gene was derepressed under aerobic conditions. Again, the regulation of LDH was not affected in these three strains.

In *E. coli*, the formation of several anaerobic proteins is dependent on the *ntrA* (*rpoN*) gene, which encodes a novel subunit of RNA polymerase, the sigma factor σ^{54} (4). In *A. eutrophus*, several metabolic functions are also dependent on the presence of an *rpoN*-like gene (21). For example, growth on acetoin, which is the product of ADH-catalyzed oxidation of 2,3-butanediol, is dependent on such a gene (7). If expression of ADH or LDH depends on the presence of the σ^{54} subunit of RNA polymerase, mutants impaired in the synthesis of this sigma factor should be unable to derepress ADH or LDH. However, in HF09 and HF149 (both *rpoN*), as in the wild type, both fermentative enzymes were formed and subjected to derepression (Fig. 1F). Therefore, repression of ADH and LDH is independent of σ^{54} in *A. eutrophus*.

Growth of semiconstitutive mutants on 2,3-butanediol and ethanol. The growth of all mutants on 2,3-butanediol and on ethanol was studied. In the late exponential growth phase, cells were harvested and the activity of the fermentative ADH was determined. The doubling times of all spontaneous and of all chemically induced mutants on 2,3-butanediol were nearly identical (2.2 to 2.7 h; Table 2); only the two Tn*5*-induced mutants grew more slowly (t_d = 4 h).

Measurements in 2,3-butanediol-grown cells revealed three groups of mutants exhibiting different levels of specific ADH activity. Highest activities (868 to 1,190 U/g) were measured in strains CF17, CF100, CF303, and DJ21 (group A), strains CF101, CF106, and CF108 (group B) exhibited intermediate levels (230 to 290 U/g), and both Tn*5*-induced mutants (group C) had very low levels (88 to 110 U/g). Therefore, the oxidation of 2,3-butanediol seems to be the growth-limitating step only in the Tn*5*-induced mutants; otherwise, mutants belonging to group A should grow faster than group B mutants.

Growth on ethanol (Table 2) was much slower than on 2,3-butanediol (16.5 to 40 h). The doubling times of group A and B mutants were 17 ± 2 and 36 ± 4 h, respectively. The Tn*5*-induced mutants (group C) did not grow on ethanol. As shown previously (14), the mutants were not affected in growth on acetoin or acetate. Therefore, it seemed likely that the differences in the doubling times on ethanol reflect different amounts of fermentative ADH present in the cells: in ethanol-grown cells, the specific activity of ADH was much higher than in 2,3-butanediol-grown cells (1,700 to 3,240 U/g). Again, all group A mutants had the highest activity (2,460 to 3,240 U/g; Table 2), whereas group B mutants exhibited significantly lower activity (1,700 to 1,870 U/g). Since the specific activities correlate with the doubling times on ethanol, the oxidation of ethanol is probably the growth-limiting step in these strains. It seems unlikely that the oxidation of acetaldehyde to acetate is the limiting step, because the specific activities of acetaldehyde dehydrogenase II, which is involved in the catabolism of 2,3-butanediol and acetoin in *A. eutrophus* (7, 12), were nearly identical in all mutants tested (110 to 165 U/g). Because the Tn*5*-induced mutants do not grow on ethanol, the corresponding values could not be measured. However, since the promoter provided by Tn*5* is very weak (see below), expression of ADH is probably too low to allow growth of group C mutants on ethanol.

Cloning and sequencing of the *adh* gene from mutants with constitutive *adh* expression. The physiological data presented above indicated three distinct groups mutants expressing the *adh* gene under aerobic conditions. In contrast to the wild type, oxygen starvation in mutant strains CF17, CF100, CF303, and DJ21 (group A) resulted in a less than twofold increase of ADH specific activity. Mutants CF101, CF108, and CF106 (group B) were characterized by semiconstitutive expression of ADH; oxygen starvation resulted in a two- to fivefold increase of ADH activity. The Tn*5*-induced mutants (group C) synthesized ADH at a low level; expression was affected very little by the aeration rate.

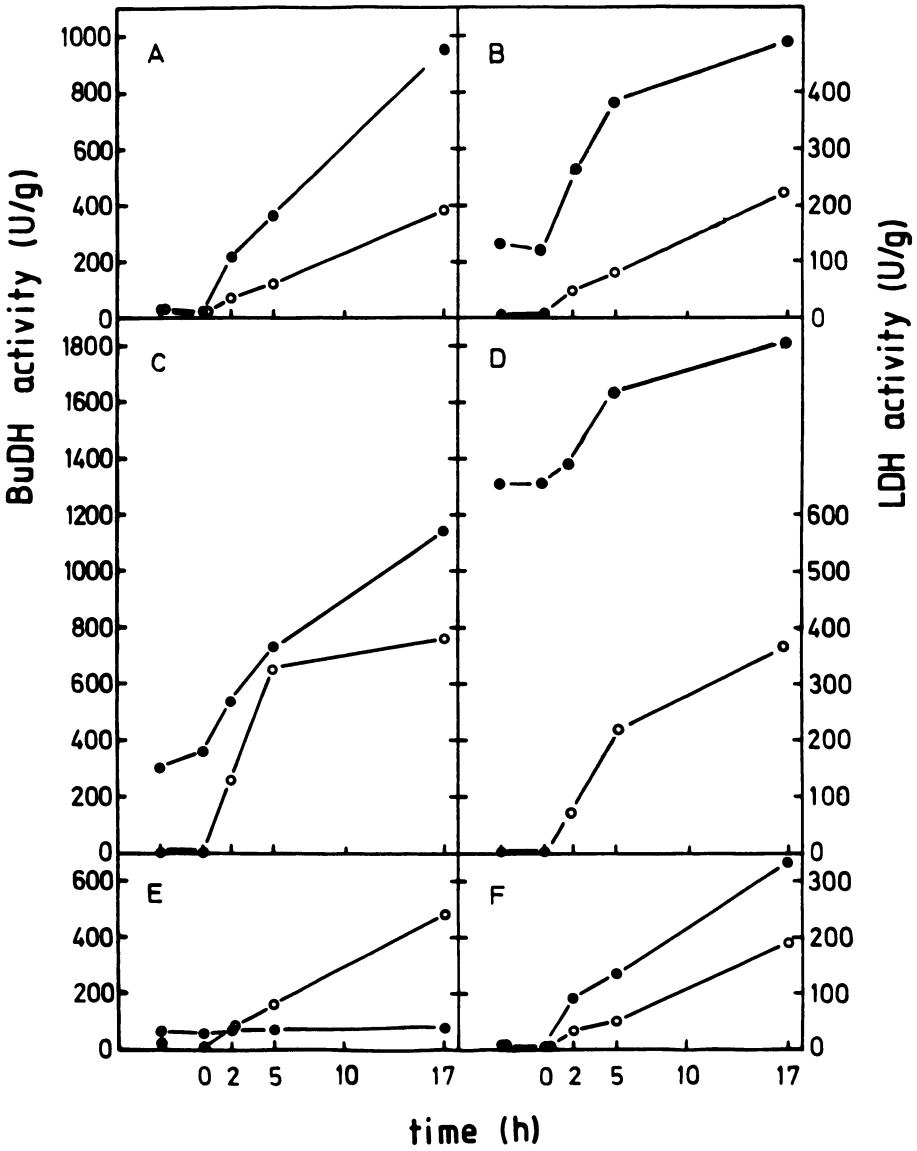

FIG. 1. Specific activities of fermentation enzymes in *A. eutrophus* H16 (A), CF108 (B), CF106 (C), CF303 (D), HC1421 (E), and HF09 (*rpoN*) (F) during cultivation on gluconate. When the cell density had reached an optical density at 436 nm of 10, the aeration rate was decreased from 500 to 50 ml of air per min (zero time). Symbols: ○, LDH; ●, ADH (measured as 2,3-butanediol dehydrogenase [BuDH]).

The insertions of Tn*5* in HC1409 and HC1421 are located 56 or 66 bases upstream of the *adh* gene (13). Since regions of Tn*5* can serve as a weak but constitutive promoter for genes located downstream of the insertion (2), the underrepressible low-level ADH constitutive phenotype of the Tn*5*-induced mutants may be due to the function of this promoter. Sequence analysis of the 5′ upstream region of the wild-type *adh* gene had revealed two putative −10 regions with homologies to the *E. coli* σ^{70} consensus sequence (Fig. 2). Neither putative promoter sequence contained the invariant T (9, 22) but instead contained a G. Consequently, the *adh* wild-type gene was not expressed by its own promoter in *E. coli* (13). If one assumes that one or both putative promoter sequences are responsible for *adh* regulation in *A. eutrophus*, the integration of Tn*5* between these sequences and the structural *adh* gene should abolish the wild-type regulation, which would explain the observed phenotype of HC1421 and HC1409.

It seemed probable that the altered *adh* regulation in the mutants belonging to groups A and B was due to mutations in the putative promoter regions or at other locations in the 5′ upstream *adh* region. Therefore, we cloned the *adh* genes of strains CF100, CF101, CF106, CF108, CF17, CF303, and DJ21) from lambda L-47 libraries by using the wild-type *adh* gene as a hybridization probe. All phage clones harbored a 11.5-kilobase-pair *Eco*RI fragment that was homologous to the wild-type fragment HS10, as indicated by identical *Pst*I restriction patterns. Suitable subfragments were sequenced to determine the 5′ upstream region of the mutant *adh* gene (Fig. 2). All seven mutants analyzed revealed mutations in the 5′ upstream *adh* region. Mutants belonging to group A were altered in the gene-proximal −10 region; the G at position 381 in the wild type (13) had been replaced by a T in mutants CF17, CF100, and CF303; and in strain DJ21, a deletion of the G at position 381 was identified, thus integrating the T at position 382 within the putative −10 region.

TABLE 2. Doubling times and ADH specific activities in mutants of *A. eutrophus*

Strain	Determination with given substrate			
	2,3-Butanediol		Ethanol	
	t_d (h)	ADH sp act[a] (U/g)	t_d (h)	ADH sp act (U/g)
HC1421	4	88	NG[b]	ND[c]
HC1409	4	110	NG	ND
CF101	2.3	230	36	1,820
CF108	2.2	260	36	1,870
CF106	2.3	290	36	1,700
CF100	2.4	870	19	2,460
CF303	2.6	930	19	2,440
CF17	2.4	940	19	2,600
DJ21	2.7	1,200	16.5	3,240

[a] Measured with 2,3-butanediol as the substrate (31).
[b] NG, No growth.
[c] ND, Not determined.

Group B mutants were altered in the −10 region distal from the gene. The G at position 365 in the wild type had been replaced by a T in mutants CF101 and CF108. In mutant CF106, five nucleotides of the proximal −10 region were duplicated, resulting in two overlapping putative −10 regions (TATCA-T-ATCAG) (Fig. 2).

As a result of these mutations, the invariant T of the *E. coli* σ^{70} consensus sequence was restored in all group A and group B mutants (TATAAT or TATCAT; Fig. 2). Consequently, in contrast to the wild-type *adh* gene, all mutant genes were expressed aerobically in *E. coli* from their own promoters independently of the orientation of the *adh* gene in pUC9-1.

Determination of the *adh* transcription start in wild-type and mutant strains. The studies on ADH formation had shown the inability of group A mutants to repress ADH synthesis under aerobic conditions. Since all strains were mutated in the proximal −10 region, the involvement of this DNA region in *adh* regulation was confirmed. Independent of the type of mutation, all four strains had lost the G in position 381. This observation does not mean that *adh* regulation depends on only one nucleotide in this specific

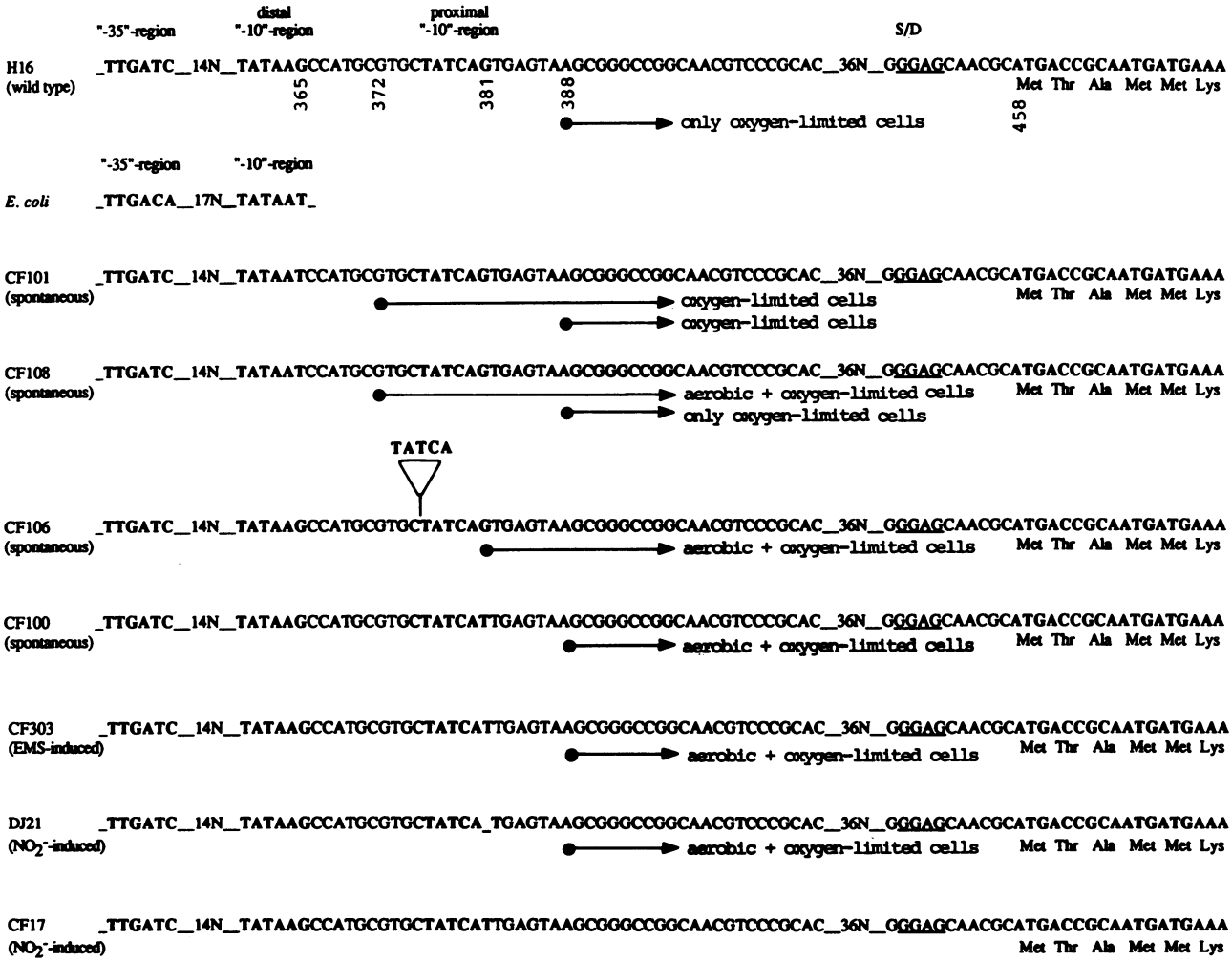

FIG. 2. 5′ upstream nucleotide sequences of the *adh* gene of *A. eutrophus* H16 and of mutants. The putative Shine-Dalgarno sequence (S/D) is underlined, and the beginning of the translated gene is indicated. Numbering of important nucleotides is according to reference 13. The origins of *adh* transcription in the wild type and in mutants are indicated by arrows.

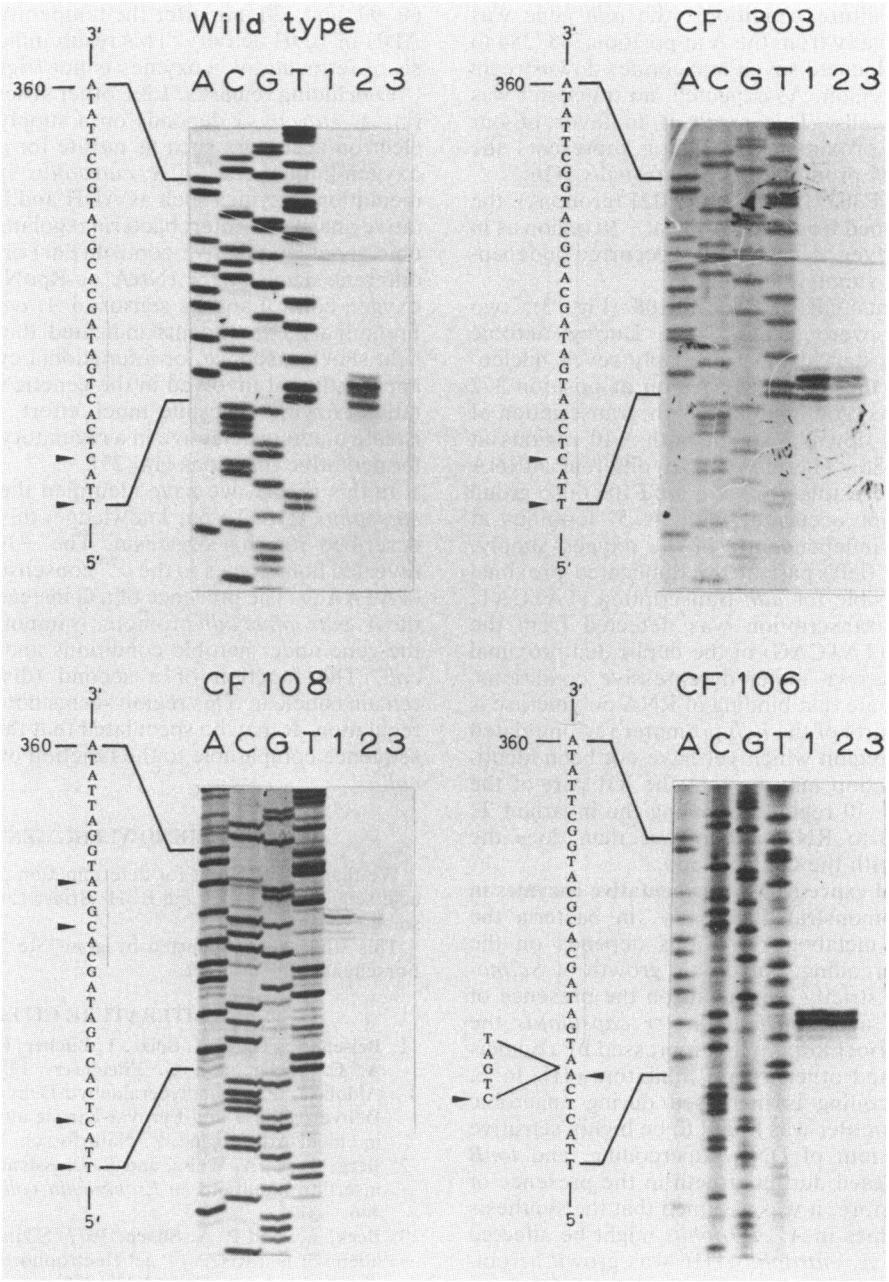

FIG. 3. Identification of the origin of *adh* transcription in *A. eutrophus* H16 and in mutants by nuclease protection assay. Cells were grown in 4-liter glass fermentors aerobically (500 ml of air per min) (lanes 1) or with a restricted supply of oxygen (50 ml of air per min) (lanes 2); lanes 3 contain control without RNA. The regions of the origin of *adh* transcription are indicated in the sequence by arrowheads.

position but that a change in this position is sufficient to seriously affect the regulation mechanism.

The distal −10 region was obviously less important for *adh* regulation, because group B mutants (strains CF101, CF108, and CF106) were still partially derepressible. It was likely that CF101 and CF108 used the mutated distal −10 region during aerobic growth, resulting in expression of ADH at an intermediate level, whereas the intact proximal −10 region was responsible for expression of ADH at a higher level under oxygen starvation. To confirm this assumption, the start site of *adh* transcription in the wild type and in mutants was determined. Group A mutants, which used the proposed proximal −10 region as a promoter, should initiate mRNA synthesis in this region regardless of the oxygen supply, whereas mRNA synthesis from group B mutants should be oxygen dependent. During aerobic growth, mRNA synthesis should initiate at the mutated distal −10 region; under oxygen-limited conditions, both promoters should be active, resulting in the synthesis of two mRNA species differing in size by 16 nucleotides, i.e., the distance between both putative −10 regions.

RNA was isolated from the wild type and from mutants CF101, CF108, CF106, CF100, CF303, and CF17 during growth in 4-liter fermentors from the aerobic and from the oxygen-limited growth phase. The 5′ mRNA termini were determined by S1 mapping. In *A. eutrophus* H16 grown

under derepressive culture conditions, the *adh* gene was transcribed most probably from the A at position 388 (384 to 388; Fig. 3), which is located seven nucleotides downstream of the proximal −10 region. As expected, no transcript was detected in aerobic cells. This result is in favor of our assumption that the proximal −10 region represents the oxygen-dependent *adh* promoter of *A. eutrophus* H16.

In strains CF100, CF303 (Fig. 3), and DJ21 (group A), the *adh* gene was transcribed from the proximal −10 region as in the wild type. However, transcription occurred independently on the oxygen supply.

In group B mutants CF101 and CF108 (Fig. 3), two different transcripts were synthesized. During aerobic growth, transcription started most probably seven nucleotides downstream of the distal −10 region at position 372 (368 to 372). During oxygen-limited growth, transcription of the *adh* gene started downstream of both −10 regions at positions 372 and 388. Therefore, two different mRNA species appeared. Interestingly, in strain CF106 (also group B) only one transcript occurred, with its 5′ terminus at position 382 or 381, independently of the oxygen supply. Obviously, the distal (left) part of the duplicated proximal −10 region is responsible for *adh* transcription (TATCAT; Fig. 2 and 3). No transcription was detected from the proximal (right) part (TATCAG) of the duplicated proximal −10 region in cells grown under derepressive conditions. This finding may indicate that binding of RNA polymerase is dependent on other parts of the *adh* promoter region related to the proximal −10 region which yet have not been identified. Another explanation may be that the left part of the duplicated proximal −10 region, including the invariant T, has a higher affinity to RNA polymerase than does the wild-type sequence with the G at the end.

Oxygen-independent expression of fermentative enzymes in *A. eutrophus*. As demonstrated recently, in bacteria the expression of some metabolic functions depends on the degree of DNA supercoiling. Anaerobic growth of *Salmonella typhimurium* is strictly dependent on the presence of gyrase activity (37), and in *Rhodobacter capsulatus* the biosynthesis of bacteriochlorophyll is repressed by coumermycin, novobiocin, and other gyrase inhibitors (41). In *E. coli*, negative supercoiling is increased during anaerobic growth. The *tonB* promoter was found to be highly sensitive to changes in the extent of DNA supercoiling, and *tonB* expression was increased during growth in the presence of novobiocin (6). Therefore, it was assumed that the synthesis of fermentative enzymes in *A. eutrophus* might be affected by gyrase inhibitors. *A. eutrophus* H16 was grown aerobically at 30°C in mineral salts medium with sodium gluconate as a carbon source. In the mid-logarithmic growth phase, coumermycin A or nalidixic acid was added at a concentration of 0, 10, 25, 60, or 100 μg/ml. When after 2 h the cells were harvested and analyzed for ADH and LDH, no activity of these enzymes was detected. This result indicated that the formation of fermentative enzymes in *A. eutrophus* is not affected by gyrase inhibitors.

In another series of experiments, potassium cyanide was added to the cells at a concentration of 0, 200, 300, 400, or 600 μg/ml in order to mimic oxygen deficiency. Depending on the concentration of cyanide, growth ceased transiently for a period of 3 to 7 h. However, ADH or LDH activity was not detected in any sample. Thus, the synthesis of fermentative enzymes was unaffected by cyanide inhibition.

Since in *E. coli* synthesis of some proteins is induced by exposing cells to a heat shock (16, 38), growing cells of *A. eutrophus* were shifted from 27 to 39°C. Samples taken 0, 40, 60, 90, and 120 min after the temperature shift exhibited no ADH or LDH activity. This result indicates that the synthesis of fermentative enzymes is not triggered by heat shock.

Concluding remarks. Like other strictly respiratory bacteria, *A. eutrophus* depends on a supply of oxygen or other electron acceptors such as nitrate for growth. Surprisingly, oxygen-limited cells of *A. eutrophus* synthesize typical fermentation enzymes such as ADH and LDH. Whereas facultative anaerobic enterobacteria regulate expression of anaerobic genes by positive control (Fnr) or by the function of a different sigma factor (NtrA or RpoN), the mechanism of oxygen control and its sensor in *A. eutrophus* is unknown. Preliminary experiments indicated that DNA supercoiling, heat shock response, or a functional cytochrome oxidase is apparently not involved in the genetic regulation of fermentative enzymes. Despite much effort, we were not able to isolate mutants defective in a regulatory gene responsible for fermentative functions (14, 29).

In this report, we have identified the *adh* promoter of *A. eutrophus* H16. To our knowledge, this is the first promoter described for this organism. The −10 region (TATCAG) revealed homologies to the σ^{70} consensus sequence of *E. coli* (TATAAT). The presence of a G instead of the invariant T in the *A. eutrophus adh* promoter is important for repression of the gene under aerobic conditions and for expression in *E. coli*. The function of a second (distal) −10-like region remains unclear. This region seems not to be involved in *adh* regulation. It may be speculated that this region represents a sequence comparable to the function of the −35 region in *E. coli*.

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