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Identification of a Novel Gene, *aut*, Involved in Autotrophic Growth of *Alcaligenes eutrophus*

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The aerobic facultative chemoautotroph *Alcaligenes eutrophus* was found to possess a novel gene, designated *aut*, required for both lithoautotrophic (hydrogen plus carbon dioxide) and organoautotrophic (formate) growth (Aut^+ phenotype). Insertional mutagenesis by transposon Tn*5*-Mob localized the gene on a chromosomal 13-kbp *Eco*RI fragment. Physiological characterization of various Aut^- mutants revealed pleiotropic effects caused by the transposon insertion. Heterotrophic growth of the mutants on substrates catabolized via the glycolytic pathway was slower than that of the parent strains, and the colony morphology of the mutants was altered when grown on nutrient agar. The heterotrophic derepression of the *cbb* operons encoding Calvin cycle enzymes was abolished, although their expression was still inducible in the presence of formate. Apparently, the mutation did not affect the *cbb* genes directly but impaired the autotrophic growth in a more general manner. The conjugally transferred wild-type *Eco*RI fragment allowed phenotypic in *trans* complementation of the mutants. Further subcloning and sequencing identified a single open reading frame (*aut*) of 495 bp that was sufficient for complementation. The monocistronic *aut* gene was constitutively transcribed into a 0.65-kb mRNA. However, its expression appeared to be low. Heterologous expression of *aut* was achieved in *Escherichia coli*, resulting in overproduction of an 18-kDa protein. Database searches yielded weak partial sequence similarities of the deduced Aut protein sequence to some cytidylyltransferases, but no indication for the exact function of the *aut* gene was obtained. Hybridizing DNA sequences that might be similar to the *aut* gene were detected by Southern hybridization in the genome of two other autotrophic bacteria.

The aerobic, gram-negative bacterium *Alcaligenes eutrophus* is a facultative chemoautotroph able to utilize a wide variety of organic substrates for heterotrophic growth and hydrogen plus carbon dioxide or formate as energy and carbon sources for litho- or organoautotrophic growth, respectively (9). Autotrophic CO_2 fixation in this organism proceeds via the Calvin-Benson-Bassham carbon reduction cycle. The *cbb* genes coding for Calvin cycle enzymes are clustered within two highly homologous operons, one located on the chromosome and the other located on the megaplasmid pHG1 of *A. eutrophus* H16 (5, 30, 41). An activator gene, *cbbR*, encoded in divergent orientation immediately upstream of the chromosomal *cbb* operon was shown to control the expression of both *cbb* operons (54).

In *A. eutrophus* H16, the genetic information for the synthesis of the two hydrogenases is located within a large *hox* gene cluster upstream of the *cbb* operon on megaplasmid pHG1 (17). Thus, plasmid-cured strains lack the ability of lithoautotrophic growth but retain their organoautotrophic capacity, indicating that the chromosome contains the genes necessary for formate oxidation (6). Analogous to the hydrogenase system, *A. eutrophus* produces two formate dehydrogenases. The soluble enzyme reduces NAD^+, whereas the membrane-bound enzyme is coupled directly to the respiratory chain (15, 18).

In previous experiments performed to generate transposon Tn*5*-Mob insertional mutants of *A. eutrophus* with defective autotrophic CO_2 fixation, a class of mutants that did not carry the Tn*5*-Mob insertions within the *cbb* gene clusters was identified. The insertion sites of Tn*5*-Mob in these mutants were found to be on a chromosomal 13-kbp *Eco*RI fragment (7), resulting in the inability of the clones to grow autotrophically (Aut^- phenotype). The present investigation was undertaken to further characterize the phenotype and analyze the defect of the Aut^- mutants. It was shown that the mutants were also affected in heterotrophic growth on certain substrates as a consequence of the transposon insertion within a novel, monocistronic gene locus, designated *aut*. Apparently, the *aut* mutation impaired the carbon metabolism of *A. eutrophus* in a more general manner. Although the exact function of the newly identified gene was not elucidated, its involvement in autotrophic growth of the organism was clearly demonstrated. Southern hybridizations suggested that the gene may also be present in other autotrophic bacteria.

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MATERIALS AND METHODS

Strains, plasmids, phages, and culture conditions. Bacterial strains, phages, and plasmids used in this study are listed in Tables 1 and 2. Moreover, DNA isolated from *Paracoccus denitrificans* (ATCC 17741), *Pseudomonas* sp. ("*Pseudomonas oxalaticus*", ATCC 11883), *Rhizobium meliloti* (ATCC 9930), *Rhodobacter capsulatus* (DSM 152), *Rhodococcus* sp. (formerly *Nocardia opaca* 1b; DSM 427), *Thiobacillus intermedius* (ATCC 15466), or *Xanthobacter autotrophicus* (DSM 1393) was employed for Southern hybridizations. Unless stated otherwise, strains of *A. eutrophus* were grown aerobically at 30°C either in nutrient broth (NB) medium or in a mineral salts medium (42) supplemented with organic compounds at concentrations of 0.2 to 0.4% (wt/vol). Lithoautotrophic cultures were incubated under an atmosphere of H_2, CO_2, and O_2 (8:1:1, vol/vol/vol) in mineral medium containing additional 0.05% (wt/vol) $NaHCO_3$. For cultivation in the presence of fructose plus formate, cells were incubated in fructose medium until reaching an optical density at 436 nm of 1 to 2 (an optical

TABLE 1. Bacterial and phage strains used in this work

Strain	Relevant characteristics[a]	Source or reference
A. eutrophus		
HF 39	Aut$^+$ Hox$^+$; pHG1	48
HF 210[b]	Aut$^+$ Hox$^-$	28
HB 3[c]	Aut$^-$ Hox$^+$ Km^r; pHG1	55
HB 4[c]	Aut$^-$ Hox$^+$ Km^r; pHG1	55
HB 23[c]	Aut$^-$ Hox$^+$ Km^r; pHG1	55
HB 24[c]	Aut$^-$ Hox$^+$ Km^r; pHG1	55
HB 28[c]	Aut$^-$ Hox$^+$ Km^r; pHG1	55
HB 38[d]	Aut$^-$ Hox$^-$ Km^r	55
E. coli		
S17-1	Sm^r Tp^r *mod*$^+$ *res thi pro recA*; integrated plasmid RP4-Tc::Mu-Km::Tn*7*	46
K38	HfrC (λ)	38
Phage λAEC3[e]	13-kbp *Eco*RI chromosomal fragment inserted into λL47 and carrying the *aut* gene from *A. eutrophus* H16	This study

[a] All *A. eutrophus* strains were Cfx$^+$ Fox$^+$ Sm^r. Aut$^{+/-}$, ability or inability of autotrophic growth; Cfx$^+$, ability to fix CO_2; Hox$^{+/-}$, ability or inability to oxidize H_2; Fox$^+$, ability to oxidize formate; pHG1, megaplasmid; Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistent; Tc^r, tetracycline resistant; Tp^r, trimethoprim resistant.
[b] Plasmid-cured mutant derived from *A. eutrophus* HF39.
[c] Tn*5*-Mob-induced mutant derived from strain HF39.
[d] Tn*5*-Mob-induced mutant derived from strain HF210.
[e] Isolated from a λ gene library of *A. eutrophus* H16 (27).

density at 436 nm of 1 corresponds to 0.2 mg of cells [dry weight] per ml), before formate was added, and the incubation was continued for another 4 h. Cultures of *P. denitrificans*, *Pseudomonas* sp., *Rhodococcus* sp., *T. intermedius*, or *X. autotrophicus* were grown in NB medium, and those of *Escherichia coli*, *R. meliloti*, or *R. capsulatus* were grown in LB medium (39) at 30°C or 37°C (*E. coli*).

Enzyme assays. Activities of ribulose-1,5-bisphosphate carboxylase (RubisCO) and phosphoribulokinase (PRK) were determined in whole-cell assays (32).

Transposon mutagenesis, conjugation, and transduction by λ phages. Insertional mutagenesis of *A. eutrophus* with transposon Tn*5*-Mob was performed as described previously (53) by using suicide plasmid pSUP5011, which was conjugally delivered from the mobilizing strain *E. coli* S17-1 by the spot-agar mating technique (48). Hybrid plasmids, constructed from broad-host-range vectors (pLAFR1 or pMP92) and *aut*-containing DNA fragments, were transferred from *E. coli* S17-1 to *A. eutrophus* strains. Transconjugants were selected on NB plates supplemented with 20 μg of tetracycline per ml and 120 μg of kanamycin per ml. DNA of hybrid cosmids was transduced into *E. coli* S17-1 after packaging into phage λ particles by using a commercial packaging kit (Boehringer, Mannheim, Germany).

Gene expression. Heterologous gene expression in *E. coli* was carried out by using the T7 RNA polymerase expression system developed by Tabor and Richardson (50) as described previously (54). An expression plasmid carrying the *aut* gene downstream of a canonical ribosome-binding site was constructed by insertion of *aut* into pT7-7 (see "Construction of plasmids"). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (31). Cells from 1-ml culture samples were collected and subsequently resuspended in 100 μl of cracking buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithioerythritol, 2% [wt/vol] SDS, 0.1% [wt/vol] bromophenol blue, 10% [wt/vol] glycerol). The suspensions were boiled for 3 to 5 min, and 10-μl samples of such lysates were applied to the gel. Visualization of the proteins was achieved by staining with Coomassie blue.

Preparation and manipulation of nucleic acids. Isolation of total bacterial DNA was performed by the method of Ausubel et al. (3) and of phage DNA by using a method described by Sambrook et al. (39). Large-scale plasmid preparations were done by the alkaline-SDS lysis procedure (4). A miniscale version of this method was used to check *A. eutrophus* transconjugants for plasmid content, whereas transformants of *E. coli* were analyzed by a rapid boiling method (21). DNA manipulations and cloning of DNA fragments were carried out by using standard procedures (3, 39). Total RNA of *A. eutrophus* was prepared by the method of Oelmüller et al. (34).

Hybridization techniques. For Southern hybridizations, DNA was digested with restriction endonucleases and separated by agarose gel electrophoresis. In Northern (RNA) hybridization experiments, total RNA was separated in formaldehyde-containing agarose gels (39). Transfer of DNA or RNA onto nylon membranes (Biodyne B; Pall, Dreieich, Germany) was achieved by vacuum blotting; transfer of phage DNA was done by a plaque-lifting method (39). DNA probes were labelled with [α-^{32}P]dCTP by nick translation or random priming (39). Nonradioactive labelling of probes with digoxigenin and detection of hybrids were done in some experiments by using the DIG Kit (Boehringer). Southern and Northern hybridizations were performed as described by Sambrook et al. (39) or Oelmüller et al. (34), respectively.

DNA sequence determination. Both strands of DNA fragments subcloned into pUC vectors were completely sequenced by the dideoxy chain termination method (40). The sequencing reactions were primed by either the universal or the reverse primer of the pUC system or by sequence-specific oligodeoxynucleotides (17-mers) synthesized on the Gene Assembler Plus DNA Synthesizer (Pharmacia, Freiburg, Germany). Nucleic acid and deduced amino acid sequences were analyzed with the aid of the latest available version of the programs from the University of Wisconsin Genetics Computer Group (14). Searches in various databases used the programs BLAST (1) and TFastA (36).

Construction of plasmids. DNA fragments containing the entire *aut* gene or part of it were ligated into the *Xba*I site of pMP92 after filling in the cohesive ends by means of the Klenow fragment of DNA polymerase I. This resulted in the mobilizable plasmids pAF1111, pAF1112, pAF1113, and pAF1115. The 557-bp *Ava*I-*Dde*I fragment was excised from pAF1112 by double digestion with *Eco*RI and *Pst*I and cloned into the corresponding restriction sites of pT7-5 and pT7-6. The resulting expression plasmids were designated pAEC5014 and pAEC5015, respectively.

Furthermore, the *aut* gene was fused in frame to the start codon downstream of the canonical ribosome-binding site in expression vector pT7-7. The excision of the 579-bp *Bam*HI-*Dde*I fragment from pAF1111 by *Eco*RI-*Pst*I double digestion and then insertion of the fragment into the *Eco*RI-*Pst*I sites of pT7-7 were the first of several construction steps. This initial construct (pAEC5016) was completely digested by *Nde*I and then incompletely cleaved with *Rsa*I. The resulting gap between the *Nde*I (start codon of the *aut* gene) and *Rsa*I sites (8 bp downstream of the start codon) was closed by inserting a corresponding resynthesized sequence portion of the *aut* gene. This manipulation reconstructed the *aut* gene and positioned the start codon at the appropriate distance from the ribosome-

TABLE 2. Plasmids used in this work

Plasmid	Relevant characteristics[a]	Source or reference
pLAFR1	Tc^r Mob^+ Tra^-; λ-*cos*	16
pMP92	Tc^r Mob^+ Tra^-	47
pMP921	Derivative of pMP92 lacking the *Hin*dIII site adjacent to the *Sst*I site	10
pMP922	Derivative of pMP92 lacking the *Hin*dIII site adjacent to the *Sph*I site	10
pSUP5011	Km^r Ap^r Cm^r mob^+; integrated transposon Tn*5*-Mob	45
pT7-5	Ap^r; promoter P_{T7}	49
pT7-6	Ap^r; promoter P_{T7}	49
pT7-7	Ap^r; promoter P_{T7}	49
pGP1-2	Km^r, T7 polymerase; *cI857*	50
pUC18	Ap^r; *lacPOZ'*	56
pAEC5009	pUC18::0.7-kbp *Bam*HI-*Xho*I fragment	This study
pAEC5010	pUC18::0.6-kbp *Bam*HI-*Dde*I fragment	This study
pAEC5013	pUC18::0.5-kbp *Rsa*I-*Dde*I fragment	This study
pAEC5014	pT7-5::0.6-kbp *Ava*I-*Dde*I fragment	This study
pAEC5015	Inserted fragment inversed relative to pAEC5014	This study
pAEC5016	pT7-7::0.6-kbp *Bam*HI-*Dde*I fragment	This study
pAEC5018	*aut* gene contained in pAEC5016 fused to start codon in pT7-7	This study
pAEC5019	Filled-in *Sal*I site within the *aut* gene contained in pAEC5009	This study
pAF1	pLAFR1::13-kbp *Eco*RI fragment	This study
pAF2	Inserted fragment inversed relative to pAF1	This study
pAFTn5	pLAFR1::20-kbp *Eco*RI plus 1.8-kbp *Eco*RI plus 1.1-kbp *Eco*RI plus 0.9-kbp *Eco*RI fragments, carrying a Tn*5*-Mob insertion within the 20-kbp subfragment	This study
pAF1110	pMP92::0.9-kbp *Eco*RI-*Xho*I fragment from pAF1	This study
pAF1111	pMP92::579-bp *Bam*HI-*Dde*I fragment from pAEC5010	This study
pAF1112	pMP92::557-bp *Ava*I-*Dde*I fragment from pAEC5011	This study
pAF1113	pMP92::491-bp *Bam*HI-*Pvu*II fragment from pAEC5012	This study
pAF1115	pMP92::487-bp *Rsa*I-*Dde*I fragment from pAEC5013	This study
pAF1116	pMP921::0.7-kbp *Xba*I-*Hin*dIII fragment from pAEC5018	This study
pAF1117	pMP922::0.7-kbp *Xba*I-*Hin*dIII fragment from pAEC5018 (inversed relative to pAF1116)	This study
pAF1118	pMP921::0.7-kbp *Bam*HI-*Hin*dIII fragment from pAEC5009	This study
pAF1119	pMP922::0.7-kbp *Bam*HI-*Hin*dIII fragment from pAEC5009 (inversed relative to pAF1118)	This study
pAF1120	pMP921::0.7-kbp *Bam*HI-*Hin*dIII fragment from pAEC5019	This study
pAF1121	pMP922::0.7-kbp *Bam*HI-*Hin*dIII fragment from pAEC5019 (inversed relative to pAF1120)	This study

[a] Tra, conjugal transfer functions; Mob, mobilization functions.

binding site. The final construct (pAEC5018) was verified by sequencing.

The reconstructed *aut* gene was removed from pAEC5018 by digestion with *Xba*I and *Hin*dIII and subsequently ligated into the corresponding restriction sites of pMP921 and pMP922, generating the mobilizable plasmids pAF1116 and pAF1117, respectively. A frameshift mutation was introduced into the *aut* gene after linearization of pAEC5009 by *Sal*I digestion, followed by filling in of the cohesive ends and religation. The *Bam*HI-*Hin*dIII fragment containing the modified *aut* gene was excised from the resulting plasmid pAEC5019 and inserted into the *Bam*HI-*Hin*dIII sites of pMP921 and pMP922 to yield pAF1120 and pAF1121, respectively. The unmodified *Bam*HI-*Hin*dIII fragment from pAEC5009 was also cloned into pMP921 and pMP922, producing pAF1118 and pAF1119, respectively.

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were obtained from GIBCO-BRL (Eggenstein, Germany), Boehringer, or Pharmacia, which was also the supplier of Klenow fragment of DNA polymerase I, T7 DNA polymerase, nucleotides, RNase-free DNase, and chemicals used for oligodeoxynucleotide synthesis. Serva (Heidelberg, Germany) provided several antibiotics. Ribulose-1,5-bisphosphate and ribulose-5-phosphate were purchased from Sigma (Deisenhofen, Germany). RubisCO was purified from *A. eutrophus* as reported previously (8).

Nucleotide sequence accession number. The nucleotide sequence data shown in Fig. 1 have been deposited in GenBank under accession no. U07639.

RESULTS

Phenotypic characterization of Aut^- mutants. The Aut^- mutants HB3, HB4, HB23, HB24, HB28, and HB38, isolated after Tn*5*-Mob transposon insertions into the genome of *A. eutrophus* HF39 or the pHG1-cured strain *A. eutrophus* HF210, had been selected for their inability to grow litho- and/or organoautotrophically. They were shown to carry Tn*5*-Mob insertions in a chromosomal 13-kbp *Eco*RI fragment, not linked to the *cbb* operon, and be able to oxidize H_2 and formate (55). However, the mutants derived from strain HF39 no longer exhibited the partial heterotrophic derepression of the *cbb* genes, measured as RubisCO and PRK activities (exemplified by HB3 in Table 3), that is typical of pHG1-containing strains of *A. eutrophus* when grown with fructose or gluconate (6). It was, nevertheless, possible to induce the expression of these genes by addition of formate to cultures growing on fructose (Table 3), although formate supplied as a single substrate did not support growth of the mutants. Incubation of such cultures under an atmosphere of H_2-CO_2-O_2 also induced the *cbb* genes in the mutant strains but to a much lower extent (Table 3). These data show that the mutants were not impaired in the expression of the *cbb* genes and consequently in the CO_2 assimilation by the Calvin cycle reactions.

Growth experiments revealed a pleiotropic effect of the Tn*5*-Mob insertions. Heterotrophic growth of the mutants in mineral medium with fructose, gluconate, glycerol, or glycolate was significantly slower than that of the parent strains, whereas pyruvate, acetate, or succinate supported the same growth rate

TABLE 3. Activities of RubisCO and PRK in parent strain *A. eutrophus* HF39 and the Aut⁻ mutant HB3 grown under various conditions

Substrate	Sp act (U/mg of cell protein)			
	A. eutrophus HF39		Mutant HB3	
	RubisCO	PRK	RubisCO	PRK
Gluconate	0.035	0.066	<0.001	<0.001
Fructose	0.014	0.022	<0.001	<0.001
Fructose + formate[a]	0.046	0.090	0.056	0.084
Fructose + H_2-CO_2[b]	0.027	0.042	0.005	0.007

[a] Addition of formate to the culture at an optical density at 436 nm of 1 to 2.
[b] Incubation of the culture under an atmosphere of H_2-CO_2-O_2 after reaching an optical density at 436 nm of 1 to 2.

in all strains (Table 4). A possible deficiency in the glycolytic carbon metabolism of the mutants was ruled out by activity analyses of the corresponding enzymes (data not shown). In addition to reduced heterotrophic growth rates, the mutants showed an altered colony morphology when grown on NB plates, although they grew at the same rate as the parent strains in NB medium. Mutant colonies were whitish-opaque in contrast to the transparent colonies of the parent strains. Determination of poly(hydroxybutyric) acid, the main storage component of *A. eutrophus*, indicated no increased accumulation of the polyester in the mutant cells.

Isolation and nucleotide sequence of *aut*. To isolate the genomic region containing the inserted Tn*5*-Mob, total DNA from mutant strain HB3 was completely digested with *Eco*RI, ligated with the mobilizable, broad-host-range cosmid pLAFR1, and packaged into λ phage particles. Transduction of *E. coli* S17-1 and selection for tetracycline and kanamycin resistances yielded hybrid plasmid clones carrying the 13-kbp *Eco*RI fragment with an integrated Tn*5*-Mob. An *Eco*RI-*Hin*dIII subfragment of pAFTn5 was used as a hybridization probe to select from a genomic library of *A. eutrophus* H16 a λ phage clone (λAEC3) containing the wild-type 13-kbp *Eco*RI fragment. After cloning of this fragment into pLAFR1, the resulting plasmids pAF1 and pAF2 were conjugally transferred into mutant HB3. Transconjugants showed recovery of the Aut⁺ phenotype. Their autotrophic and heterotrophic growth rates as well as their colony morphologies on NB agar were similar to those of the parent strains (data not shown). Further subcloning delimited the region required for phenotypic complementation in *trans* to a 0.9-kbp *Eco*RI-*Xho*I fragment (pAF1110) which was subsequently sequenced.

An open reading frame of 495 bp (designated *aut*) was identified by analysis of the determined sequence (Fig. 1). Its deduced potential protein product consisted of 164 amino acid residues and had a calculated M_r of 17,923. The DNA sequence immediately upstream of the translation start codon of *aut* (ATG in nucleotide positions 327 to 329) had only weak similarity to the consensus sequence of a ribosome-binding site (44), and no obvious σ^{70}- or σ^{54}-dependent promoter structures were found. The G+C content of *aut* (67.1 mol%) was in good agreement with the overall G+C content of *A. eutrophus* genomic DNA (66.3 to 66.8 mol%) (13), and the codon usage was very similar to that of other *A. eutrophus* genes (2, 29, 30, 41). Sequence comparisons revealed partial similarities between the deduced amino acid sequence of the *aut* product and a few known sequences in databases (see Discussion). A hydropathy analysis indicated no extended hydrophobic regions within the Aut sequence, suggesting that it might be a soluble protein.

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      EcoRI
1     GAATTCGTGGTGTAATGCCTAGCAATTCCGATAATATCGGAATTAGCGCCAGCCGCAGCT   60

61    GGCAGCAACCGCAGGCCGCGCATATCGACGCCGTGCGGCAGGCGCTGCAGGGCGCCTTGC  120

121   GCGGCATTGCCACCGCGTCGGTGCAGCAGGCCCGGCCGGTCTCCGATCCGGCAGCCAGGG  180

181   ATGTCGCCAACGACGCGGCCATGAGGCTGTGCGTGACGGGGTAACCAGGACCCCGCCGCC  240

          BamHI                    AvaI
241   GGCGGGGATCCCAAGAAGGGGTACCATCTCGGGCAAGCACGCGGGCTCCGACCAGGGGCC  300

                    RBS          RsaI
301   AGCCTCCACTTTCCGATGCCGACCCCATGTCCGTACCCGCTTTCGAATCCAAGCTGACCC  360
1                              M  S  V  P  A  F  E  S  K  L  T  L   12

361   TGCCTGACACCCCCGCCGCGCTGGCCGCGCGCATCGCCGCCTTGCCGCGCCCGCTGGTGT  420
13      P  D  T  P  A  A  L  A  A  R  I  A  A  L  P  R  P  L  V  F   32

421   TCACCAATGGCGTCTTCGACATCCTGCACCGCGGCCATGCCACCTACCTGGCACAGGCGC  480
33      T  N  G  V  F  D  I  L  H  R  G  H  A  T  Y  L  A  Q  A  R   52

481   GCGCACTGGGCGCAAGCCTGGTGGTTGGCGTCAACAGCGATGCTTCCGTAAAGATGCTGG  540
53      A  L  G  A  S  L  V  V  G  V  N  S  D  A  S  V  K  M  L  G   72

541   GCAAGGGCGACGACCGTCCGCTGAACCATGAATCGGACCGCATGGCGCTGCTGGCCGCGC  600
73      K  G  D  D  R  P  L  N  H  E  S  D  R  M  A  L  L  A  A  L   92

          SalI                                          ♀
601   TGGAGTCGGTCGACCTGGTGGCGATGTTCCGCGAACAGACCCCGGTGGAACTGATCCGCC  660
93      E  S  V  D  L  V  A  M  F  R  E  Q  T  P  V  E  L  I  R  L  112

661   TGGTGCGCCCCGACATCTACGTCAAGGGCGGCGACTACGACATCGACACGCTGGAAGAAA  720
113     V  R  P  D  I  Y  V  K  G  G  D  Y  D  I  D  T  L  E  E  T  132

          PvuII
721   CCCGGCTGGTGCGCAGCTGGGGCGGGCAGGCCTACGCCATCCCCTTCCTGCACGACCGCT  780
133     R  L  V  R  S  W  G  G  Q  A  Y  A  I  P  F  L  H  D  R  S  152

                                        DdeI
781   CGACCACCAAGCTGCTGACCAGGGTGCGCCAGGGCAGCTAAGGCAGCCCAGGGCGCTCAG  840
153     T  T  K  L  L  T  R  V  R  Q  G  S  *                      164

                                              XhoI
841   GAGCAGGCCAGCACGTCCTCGGCGCCGTAGCGCTGGCGCATGGCCTCGAG  890
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FIG. 1. Nucleotide sequence of the 0.9-kbp *Eco*RI-*Xho*I fragment from *A. eutrophus* containing the *aut* gene (nucleotide positions 327 to 821). The deduced amino acid sequence of Aut is given in the one-letter code. A possible ribosome-binding site (RBS) is printed in bold. The insertion site of transposon Tn*5*-Mob after position 650 in the mutant HB3 was determined by sequencing and is marked ♀. Relevant restriction sites are indicated.

TABLE 4. Doubling times of parent strain *A. eutrophus* HF39 and the Aut⁻ mutant HB3 during growth on various organic substrates

Substrate	Doubling time (h)	
	A. eutrophus HF39	Mutant HB3
Gluconate	1.8	2.3
Fructose	2.2	3.3
Glycerol	31	112
Glycolate	2.6	3.3
Pyruvate	1.6	1.7
Acetate	1.5	1.6
Succinate	1.4	1.4

Verification of *aut* by phenotypic complementation of Aut⁻ mutants. Functional confirmation of the presumed *aut* gene was obtained by subcloning the entire gene, or parts of it, into the mobilizable broad-host-range vector pMP92 and subsequently using the resulting plasmids in complementation experiments with the Aut⁻ mutants. Restoration of the Aut⁺ phenotype was observed only with plasmids containing the

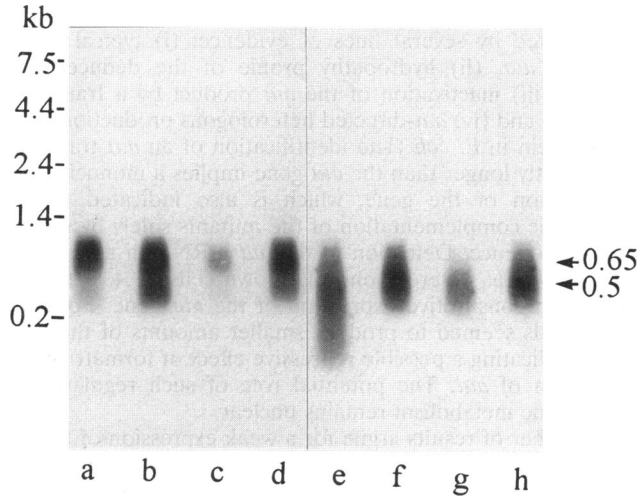

FIG. 2. Autoradiograph of Northern hybridizations of total RNA from *A. eutrophus* HF39 or mutant HB3 with an *aut* gene probe. Lanes: a to d, RNA from HF39 grown with pyruvate (a), fructose (b), formate (c), or H_2-CO_2 (d); e to h, RNA from HB3 grown with pyruvate (e), fructose (f), fructose plus formate (g), or fructose plus H_2-CO_2 (h). The lengths of the transcripts (arrows) and RNA size markers are given in kilobases. Between 10 and 20 μg of RNA was applied to each lane.

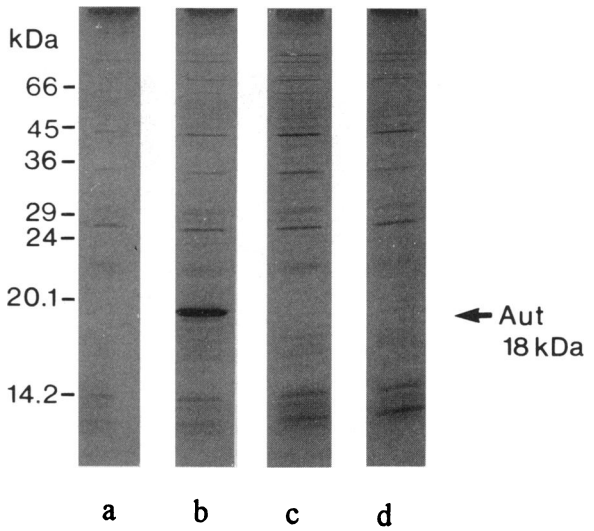

FIG. 3. Heterologous expression of the *aut* gene in *E. coli*. SDS-PAGE was performed with the crude extracts from several transformants of *E. coli* K38(pGP1-2) containing pT7-7 (lane a), pAEC5018 (lane b), pAEC5014 (lane c), or pAEC5015 (lane d). The position of the overproduced Aut protein is marked by the arrow. Numbers indicate the sizes of molecular mass markers in kilodaltons. About 10 μg of protein was applied to each lane.

complete *aut* coding sequence (pAF1111 or pAF1112), whereas plasmids with a truncated *aut* gene (pAF1113 or pAF1115) were unable to complement the mutants. These results provided evidence that (i) the *aut* gene was both necessary and sufficient to establish the Aut^+ phenotype and (ii) all Aut^- mutants were deficient in *aut*.

The introduction of a frameshift mutation into *aut* by filling in its unique *Sal*I restriction site also served to corroborate the functionality of the gene. The mutated gene cloned in vectors pMP921 or pMP922 (resulting plasmids pAF1120 or pAF1121) no longer complemented the Aut^- mutants. The gene was apparently inactivated by the frameshift mutation, providing additional evidence that *aut* constitutes a protein coding gene. Only transconjugant mutants harboring pAF1118 or pAF1119 with the unmodified *aut* grew autotrophically at the rates of the parent strains. Again, all Aut^+ transconjugants showed complete restoration of normal heterotrophic growth rates and colony morphology on NB agar. Moreover, the RubisCO activities of these transconjugants reached the levels of the wild-type strains under all conditions tested (data not shown).

Transcript analysis. The activity of the *aut* gene was studied by analyzing its transcription products in parent strain HF39 and in *aut* mutant HB3. For Northern blot analysis, total RNA was isolated from cells of HF39 and HB3 grown under various conditions. A 0.6-kbp *Eco*RI-*Pst*I fragment from pAEC5010 that encodes the entire *aut* (*Bam*HI-*Dde*I fragment in Fig. 1) served as the hybridization probe. Surprisingly, transcripts of *aut* were detected in the RNA of cells from both strains regardless of the growth conditions, although the abundance of *aut* mRNA seemed to be significantly lower in cells grown in the presence of formate (Fig. 2). Nevertheless, the transcription of *aut* is constitutive rather than induced under autotrophic conditions. The sizes of hybridizing mRNA from HF39 were determined to be about 0.65 kb, which was only slightly larger than that of the *aut* gene, indicating that it was a monocistronic transcript. The *aut* mRNAs from HB3 were significantly smaller (about 0.5 kb) than the transcripts from the parent strain. This observation suggests that the defect in the mutant is due to a premature transcription termination within *aut*, caused by the inserted Tn*5*-Mob.

Heterologous expression of the *aut* gene. Two-dimensional gel electrophoretic comparisons of protein patterns from parent strain HF39 and *aut* mutant HB3 failed to identify a potential *aut* product (data not shown). Thus, heterologous expression of the gene in *E. coli*, by using the T7 RNA polymerase promoter system (50), was performed to examine whether it would direct protein synthesis in the foreign host. The gene cloned in the expression vectors pT7-5 or pT7-6 (plasmids pAEC5014 and pAEC5015, respectively) yielded no detectable overproduced proteins in crude extracts of *E. coli* K38 as judged by SDS-PAGE (Fig. 3). Since the apparently very low expression was probably due to the weak ribosome-binding site of *aut*, the gene was fused in frame to the translational start codon downstream of the strong ribosome-binding site in vector pT7-7 (plasmid pAEC5018) to overcome this difficulty. Transformant *E. coli* K38(pAEC5018) did in fact overproduce a protein of about 18 kDa (Fig. 3), corresponding in size to the expected *aut* gene product. The functionality of the *aut* gene as present in pAEC5018 was verified by cloning in vectors pMP921 and pMP922. Conjugal transfer of the resulting plasmids pAF1116 and pAF1117, respectively, into HB3 restored the Aut^+ phenotype in the mutant, although *aut* lacked its own promoter. Presumably, promoter activities residing on the vectors sufficed to produce the required amount of Aut protein.

Detection of possibly *aut*-homologous DNA regions in other organisms. Considering the essential role of *aut* in autotrophic growth of *A. eutrophus*, it seemed reasonable to search for the presence of similar sequences in the genome of other bacteria, including autotrophic and heterotrophic species. For this purpose, total DNA of various organisms was hybridized with an *aut*-specific probe (487-bp *Rsa*I-*Dde*I fragment from pAEC 5013) (Fig. 1) in a Southern blot experiment. Only two of the tested DNAs, those from the autotrophs *Pseudomonas* sp. and

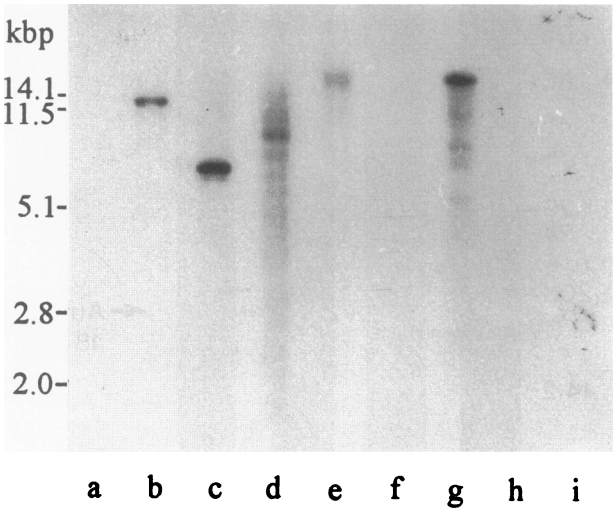

FIG. 4. Autoradiograph of Southern hybridizations of *Eco*RI-digested total DNA of *E. coli* WL87 (lane a), *A. eutrophus* HF39 (lane b), *Pseudomonas* sp. (lane c), *P. denitrificans* (lane d), *R. capsulatus* (lane e), *X. autotrophicus* (lane f), *T. intermedius* (lane g), *R. meliloti* (lane h), or *Rhodococcus* sp. (lane i) with an *aut* gene probe. About 2 μg of DNA from each species was applied to each lane, with the exception of *A. eutrophus* (1 μg) and *P. denitrificans* (5 μg). Numbers indicate the lengths of DNA size markers in kilobase pairs.

T. intermedius, showed distinct, rather strong hybridization signals (Fig. 4). No or weak, presumably unspecific, signals were obtained with the other isolates. These results suggest a possible occurrence of *aut*-homologous sequences in *Pseudomonas* sp. and *T. intermedius*.

DISCUSSION

A novel gene locus, *aut*, which is essential for litho- as well as organoautotrophic growth of *A. eutrophus* was identified. The characterization of Tn*5*-Mob-induced Aut$^-$ mutants revealed pleiotropic effects caused by a defect in *aut* in addition to the loss of the autotrophic capability. Whereas the heterotrophic derepression of the *cbb* operons was abolished in the mutants, induction or derepression was still possible in the presence of formate or, although to a remarkably smaller extent, under an atmosphere of H_2-CO_2. An *aut* defect possibly causes a metabolic imbalance in the central carbon metabolism that prevents an efficient expression of the *cbb* operons. The inability of the mutants to grow autotrophically as well as their retarded growth on substrates catabolized to pyruvate via glycolytic reactions may be due to a bottleneck limiting the carbon flow in this section of metabolism between 3-phosphoglycerate and pyruvate. However, no apparent enzyme deficiency was detected in the mutants, but a lack or malfunction of an isoenzyme specifically required during autotrophic growth cannot be completely ruled out as the primary cause of the mutant phenotype. The phenotype of phosphoglycerate mutase mutants, another class of known Aut$^-$ mutants of *A. eutrophus*, is different (37). Moreover, the altered colony morphology of the newly isolated *aut* mutants, compared with that of the parent strains when growing on NB agar, remains a puzzling observation.

Since it was not possible to identify the defective function in the Aut$^-$ mutants by phenotypic characterization, a genetic analysis that resulted in the identification of the *aut* gene was required. The gene obviously codes for a *trans*-acting function, i.e., a diffusible gene product, most probably a soluble protein as indicated by several lines of evidence: (i) typical codon usage in *aut*, (ii) hydropathy profile of the deduced Aut protein, (iii) inactivation of the *aut* product by a frameshift mutation, and (iv) *aut*-directed heterologous production of an Aut protein in *E. coli*. The identification of an *aut* transcript only slightly longer than the *aut* gene implies a monocistronic organization of the gene, which is also indicated by the phenotypic complementation of the mutants solely by the *aut* coding sequence. Detection of the *aut* mRNA in autotrophically as well as heterotrophically grown cells of *A. eutrophus* suggests a constitutive expression of the *aut* gene. Formate-grown cells seemed to produce smaller amounts of the transcript, indicating a possible repressive effect of formate on the expression of *aut*. The potential role of such regulation in autotrophic metabolism remains unclear.

A number of results argue for a weak expression of the *aut* gene in *A. eutrophus*, i.e., that the Aut protein is a minor component of the cells. Aut could not be identified in the pattern of soluble or membrane-associated proteins of *A. eutrophus* analyzed by two-dimensional PAGE. The DNA sequence upstream of *aut* showed no apparent structures reminiscent of either σ^{70}- or σ^{54}-dependent promoters. Furthermore, the putative ribosome-binding site of the *aut* gene has a low similarity to the consensus sequence. These findings were corroborated by the attempts to overproduce the Aut protein in *E. coli*, which were successful only after placing the *aut* gene behind a canonical ribosome-binding site in the expression vector pT7-7. Presumably, translation of the *aut* mRNA was not favored by the authentic weak ribosome-binding site poorly recognized in *E. coli*. The fact that the *aut* gene construct as used for heterologous expression was able to complement the *aut* mutants when cloned in the broad-host-range vectors pMP921 or pMP922 is remarkable. Despite lacking its own promoter, the gene is apparently expressed at a rate sufficient to furnish the required Aut protein. Promoter activities originating on the vectors have to be responsible for this expression. Given a low expression of *aut* in *A. eutrophus*, it is tempting to speculate that its product has a regulatory rather than a direct metabolic (catabolic or anabolic) function.

There were no indications for the presence of known, defined functional domains in the deduced sequence of the Aut protein. However, the sequence comparisons disclosed partial similarities of Aut to some proteins, especially cytidylyltransferases (Fig. 5). TagD coding for glycerol-3-phosphate cytidylyltransferase (GlyCT) of *Bacillus subtilis* (33) showed the highest overall similarity (30% residue identity and 50% similarity), followed by cholinephosphate cytidylyltransferase (CCT) of the yeast *Saccharomyces cerevisiae* (25% identity and 45% similarity) (51). Considering the possible functions of Aut, both GlyCT and CCT activities are not expected to occur in *A. eutrophus* because of the physiological roles of the two enzymes in teichoic acid synthesis in gram-positive bacteria and phospholipid synthesis in eucaryotes, respectively. This assumption was confirmed by the results of enzyme assays with cell extracts of the parent strain HF39 and *aut* mutant HB3. Neither GlyCT nor CCT activities, which were also absent in *E. coli* even after overproduction of the Aut protein (data not shown), were detected. Comparable partial resemblances to Aut (22% overall identity and 45% similarity) were found for the X proteins (of unknown function) from *E. coli* (24) (Fig. 5) and *Pseudomonas fluorescens* (23). The *x* gene is part of the *x-ileS-lsp* operon. Known sequences of various other cytidylyltransferases (12, 20, 22, 26) showed no significant similarities to Aut.

A function of Aut as one of the following cytidylyltrans-

```
Aut     1 MSVPAFESKLTLPDTPAALAARIAALPRPLVFTNGVFDILHRGHATYLAQ  50
TagD    1                              MKKVITYGTFDLLHWGHIKLLER  23
CCT    76 RYTNELPKELRKYRPGKGFRFNLPPTDRPIIYADGVFDLFHLGHMKQLEQ 126
X       1                MKLIRGIHNLSQAPQEGCVLTIGNFDGVHRGHRALLQG  38

Aut    51 ARAL--GASL--VVGVNSDASVKMLGKGDDRP-LNHESDRMALLAALESV  95
TagD   24 AKQL--GDYL--VVAISTDEFNLQK---QKKA-YHSYEHRKLILETIRYV  65
CCT   127 CKKAFPNVTL--IVGVPSDKITHKL---KGLT-VLTDKQRCETLTHCRWV 170
X      39 LQEEGRKRNLPVMVMLFEPQPLELFATDKAPARLTRLREKLRYLAECG-V  87

Aut    96 DLVA--MFREQTPVELIRLVRPDIYVKGGDYDIDTLEETRLVRSWGGQAY 143
TagD   66 DEVIPEKNWEQKKQDIIDHNIDVFVMGD------DWEGKFDFLKDQCEVV 109
CCT   171 DEVVPNAPWCVTPEFLLEHKIDHVAHDDIPYVSADSDDIYKPIKEMGKFL 220
X      88 DYVLCVRFDRRFAALTAQNFVSDLLVKHLRVKFLAVGDDFPLALVVKAIS 137

Aut   138 AIPFLHDRSTTKLLTRVRQGS 164
TagD  110 YLPRTEGISTTKIKEEIAGL  129
CCT   221 TTQRTNGVSTSDIITKIIRDY 240
X     138 CYYRKLAWNTA-SISPVRKLF 157
```

FIG. 5. Alignment of the deduced amino acid sequence (one-letter code) of the Aut protein from *A. eutrophus* with several sequences contained in databases. Residues identical to those in Aut are printed in bold. Dashes indicate gaps introduced to optimize the alignment. TagD, GlyCT of *B. subtilis* (33); CCT, CCT of *S. cerevisiae* (51); X, gene product of unknown function from the *x-ileS-lsp* operon of *E. coli* (24).

ferases could be excluded on a metabolic basis. Like GlyCT, ribitol-5-phosphate cytidylyltransferase catalyzes a reaction in teichoic acid synthesis of gram-positive organisms (43). Ethanolamine cytidylyltransferase is involved in phospholipid synthesis in eucaryotes (25), and *N*-acylneuraminate cytidylyltransferase (glycolipid synthesis) is also principally a eucaryotic enzyme, its possible presence in some procaryotes not confirmed (19). On the other hand, a regulatory function of Aut as a nucleotidylating enzyme that modifies another protein is conceivable. Few sequences of such protein-modifying nucleotidylyltransferases are known. These enzymes, like the uridylyltransferase-uridylyl-removing enzyme of *E. coli* (35) and *Azotobacter vinelandii* (11) or adenylyltransferase of *E. coli* (52), are involved in the regulation of glutamine synthetase. However, no significant sequence similarities were observed between these proteins and Aut.

It is an intriguing question whether the *aut* gene encodes a special function only required in *A. eutrophus* for autotrophic growth or if homologous genes occur in other autotrophic bacteria as well. In the latter case, a more general significance of the gene for bacterial autotrophic metabolism may be postulated. Our probing of DNA from six different autotrophic bacteria by Southern hybridization detected sequences presumably homologous to *aut* present in the genomes of the chemoautotrophs *Pseudomonas* sp. and *T. intermedius* but not in the other strains. The weak signals observed with the DNA from *P. denitrificans* and *R. capsulatus* might indicate the occurrence of less-similar *aut* sequences within these organisms, although unspecific hybridization cannot be excluded in these two cases. Our findings suggest that *aut*-homologous genes do not belong to the common genetic inventory of autotrophs and thus do not play a general, essential role in autotrophic carbon metabolism. The function the Aut protein serves in *A. eutrophus* might, however, be accomplished in other organisms by alternative, isofunctional gene products. More studies with *A. eutrophus* and possibly additional autotrophic bacteria will be necessary to elucidate the exact function of the novel *aut* gene.

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