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Regulation by Molecular Oxygen and Organic Substrates of Hydrogenase Synthesis in *Alcaligenes eutrophus*

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Chemoautotrophic growth of *Alcaligenes eutrophus* 17707 is inhibited by 20% oxygen in the gas phase. Lowering the oxygen concentration to 4% results in chloramphenicol-sensitive derepression of soluble and membrane-bound hydrogenase activity (and of soluble hydrogenase antigen), showing that oxygen inhibition is due at least in part to repression of hydrogenase synthesis. Mutations resulting in derepression of hydrogenase activity (and antigen) under 25% oxygen (Ose^-) mobilized with a self-transmissable plasmid which is already known to carry genes necessary for hydrogenase expression. Plasmid-borne mutations resulting in loss of soluble hydrogenase activity have no effect on the Ose phenotype, but chromosomal mutations resulting in reduction or loss of both hydrogenase activities cannot be made Ose^-. The Ose^- mutation does not alter the thermostability of either hydrogenase, and soluble hydrogenase in the mutant reacts with complete identity with that of the wild type, indicating that the Ose^- phenotype does not result from structural alterations in either enzyme. Ose^- mutants are also relieved of normal hydrogenase repression by organic substrates, which aggravates hydrogenase-mediated inhibition of heterotrophic growth by hydrogen. Regulation of hydrogenase in Ose^- strains of *A. eutrophus* 17707 is nearly identical to that of wild-type *A. eutrophus* strains H1 and H16.

Alicaligenes eutrophus is a facultative chemoautotroph capable of growth on molecular hydrogen as the sole electron donor and carbon dioxide as the sole source of cell carbon (5). CO_2 is assimilated by a plant-like ribulose bisphosphate carboxylase in conjunction with the enzymes of the reductive pentosephosphate cycle (4). Hydrogen activation is accomplished by a soluble NAD^+-reducing hydrogenase (SH) and a membrane-bound (particulate) hydrogenase (PH) coupled to the electron transport chain (20, 24). Expression of both hydrogenase activities, but not of CO_2 assimilation or any required heterotrophic enzyme activities, is dependent upon the presence of a large, self-transmissable plasmid designated pHG1 (2, 8).

Regulation of H_2 uptake (Hup) is similar in the most extensively studied strains of *A. eutrophus*, H1 (ATCC 17698) and H16 (ATCC 17699). Both strains express Hup activity during chemoautotrophic growth under gas-phase O_2 concentrations exceeding 25% (26). Activity is repressed during rapid heterotrophic growth on such substrates as succinate, acetate, and malate, but highly derepressed on substrates that support only slow growth, e.g., glycerol and isoleucine (1, 10, 13). The inverse relationship between growth rate and Hup derepression is not linear; it has been suggested that the extent of derepression is related to the intracellular reductant supply (10). Molecular hydrogen is not required for Hup expression (13). A plasmid-coded, temperature-sensitive regulatory system in strain H16 has been reported recently (12).

This work describes Hup regulation in *A. eutrophus* strain 17707 (ATCC 17707), in which Hup is repressed by gas-phase oxygen concentrations exceeding 4% under autotrophic conditions and by organic substrates. This relative stringency (Ose, for oxygen sensitivity) appears to be determined by a single plasmid-borne genetic locus. A similar regulatory mechanism may operate in Hup^+ strains of *Rhizobium japonicum* (15) and may be cryptic in the oxygen-tolerant *A. eutrophus* strains H1 and H16.

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MATERIALS AND METHODS

Organisms and cultivation. Bacterial strains used in this study are listed in Table 1. Strains H1 and 17707 were obtained from the American Type Culture Collection. Bacteria were grown on a basal mineral medium (2) supplemented with trace elements (17). $CaCl_2$ was omitted. All supplements to the mineral medium are given as weight/volume percentages; gas mixtures are expressed as volume/volume percentages. The medium was supplemented with 0.05% $NaHCO_3$ for autotrophic growth or with 0.3% organic substrate for heterotrophic growth. The gas mixture for autotrophic growth was 10% CO_2, 3 to 30% O_2 (as indicated), and the balance was H_2. For liquid cultures, gas was supplied by continuous flow for the experiment in Fig. 1, otherwise by a gasometer system (3). Agar plates were incubated in gas-filled dessicators. Rich medium was L broth that contained 1% tryptone and 0.5% yeast extract. Formate minimal medium was 50% (vol/vol) basal mineral medium and 50% (vol/vol) 0.3 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH [pH 7.2] supplemented with 0.3% sodium formate, trace metals, and 0.001% sodium molybdate. Purified agar was used in solid autotrophic and formate media. All incubations were at 30°C. Cell numbers were determined as optical density at 420 nm.

Strain identity was routinely verified by drug resistance markers and by patterns of organic substrate utilization. Strain 17707 was discriminated from *A. eutrophus* strains H1 and H16 by its ability to grow rapidly on isoleucine (generation time of 3 h versus 7 h) and by its sensitivity to 50 μg of streptomycin per ml.

Mutant isolation. Plasmid curing was carried out either by treatment with 2 μg of mitomycin C per ml (2) or by repeated transfers on rich medium at 40°C. Cured strains were identified as autotrophic-negative clones that grew normally on formate and on organic substrates and were verified by agarose gel electrophoresis (2).

Ethyl methanesulfonate (EMS) mutagenesis was carried out by the method of Friedrich and Schlegel (9). Mutants with defects in hydrogenase activity were identified as slow-

TABLE 1. *A. eutrophus* strains

Strain	Relevent phenotype[a]	Source
H1	Wild type	ATCC 17698[b]
17707	Wild type	ATCC 17707
7-1	Rp^r	Spontaneous derivative of 17707
7-300	Ose^-	Spontaneous derivative of 17707[b]
7-301	Sm^r Ose^-	Spontaneous derivative of 7-300[b]
7-355	Rp^r Ose^-	Spontaneous derivative of 7-1
7-361	Rp^r Ose^-	Spontaneous derivative of 7-1
7-301C1	Sm^r SH^- PH^-	7-301 cured by mitomycin C[b]
7-1C1	Rp^r SH^- PH^-	7-1 cured by growth at 40°C
7-68	His^-	EMS mutagenesis of 17707[b]
7-113	Rp^r SH^-	EMS mutagenesis of 7-1
7-114	Rp^r SH^- Ose^-	Spontaneous derivative of 7-113
7-207	Rp^r SH^-	EMS mutagenesis of 7-1
7-421	Rp^r SH^- PH^-	EMS mutagenesis of 7-1
7-438	Rp^r SH^- PH^-	EMS mutagenesis of 7-1

[a] Abbreviations: Rp, rifampin (50 μg/ml); Sm, streptomycin (50 μg/ml); SH, soluble hydrogenase; PH, particulate hydrogenase; His, histidine; Ose, oxygen sensitivity.
[b] Gift of Kjell Anderson.

growing autotrophic clones that grew normally on formate and on organic substrates. In some cases, Hup-defective mutants were enriched by growth on glycerol mineral medium under 80% H_2 and 20% O_2. Spontaneous drug-resistant mutants were isolated by plating on media containing 50 μg of the appropriate antibiotic per ml. Spontaneous Ose^- mutants were isolated by incubation on mineral bicarbonate plates under an atmosphere of 10% CO_2, 60% H_2, and 30% O_2.

Conjugation experiments. Inocula for matings were grown on rich medium. Spot matings (8) were carried out by mixing donor and recipient cells at a ratio of 2:1 and spotting 0.3 ml on L agar plates. After 24 h of incubation, cells were streaked directly onto selective media. From 10 to 20 isolated exconjugant colonies were streak purified on rich medium, and unselected phenotypes were determined. All selections were carried out under 4% O_2 in the presence of 50 μg of antibiotic per ml. Exconjugants occurred at a frequency of about 10^{-5} per recipient cell. No colonies appeared when 10^8 donor or recipient cells alone were plated on selective media.

Enzyme assays. Total Hup activity (SH plus PH) in whole cells was determined at 30°C by suspending 5×10^8 cells in 4 ml of basal mineral medium with 500 μM methylene blue in 25-ml flasks. Flasks were sealed and flushed thoroughly with N_2, and 10 μmol of H_2 was injected. Loss of H_2 from the gas phase was determined by gas chromatography. Assays were performed in triplicate.

For determination of individual hydrogenase activities, exponential-phase cells were washed, frozen and thawed, treated with 800 U of lysozyme per ml in 50 mM potassium phosphate buffer (pH 7.0) for 30 min, and broken in a French press. Cytoplasmic and membrane fractions were separated by centrifugation at 120,000 × *g* for 1 h at 4°C. SH activity was determined spectrophotometrically as hydrogen-dependent NAD^+ reduction by cytoplasmic fractions at 25°C (24). PH activity in washed membranes (11) was determined spectrophotometrically as H_2-dependent methylene blue reduction at 25°C (19). No NAD^+ reduction activity was observed in membrane preparations. Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as a standard.

Dissolved oxygen was measured with a Clark-type oxygen electrode.

Immunochemical experiments. SH from isoleucine-grown *A. eutrophus* H1 was purified by the large-scale procedure of Schneider et al. (23) to a final specific activity of 1,380 μmol h^{-1} (mg of protein)$^{-1}$. Ninety-eight percent of the protein measured in a scan of a sodium dodecyl sulfate-polyacrylamide gel corresponded to the subunits of SH (22). The single diffuse band corresponding to a 29,000 M_r subunit reported for strain H16 (22) resolved into two closely spaced bands on most gels.

To obtain antiserum, 1.2 mg of purified SH was emulsified in Freund complete adjuvant and injected subcutaneously in the neck and lower back of a female rabbit. Serum taken before injection of antigen showed no reaction with purified SH or with strain H1 extracts on immunodiffusion plates. After the appearance of low-titer anti-SH (about 2 weeks), a booster consisting of 0.6 mg of SH emulsified in Freund incomplete adjuvant was injected subcutaneously in the neck. Serum was taken at intervals for the next 6 weeks.

The procedure of Ouchterlony (16) was used in all immunodiffusion experiments.

Materials. Inorganic salts for media and assay buffers were obtained from Mallinckrodt, Inc., Paris, Ky. Agar, purified agar, and L broth components were obtained from Difco Laboratories, Detroit Mich. All other chemicals, including organic substrates, mutagens, antibiotics, NAD^+, and methylene blue, came from Sigma Chemical Co., St. Louis, Mo. A Beckman KVIII spectrophotometer was used for photometric determinations.

RESULTS

Regulation of Hup by oxygen in *A. eutrophus* 17707. Strain 7-1, a spontaneous rifampin-resistant derivative of wild-type *A. eutrophus* 17707, grew autotrophically with a generation time of about 3 h under an atmosphere of 3% O_2, 10% CO_2, and 87% H_2. An atmosphere of 25% O_2, 10% CO_2, and 65% H_2 allowed only very slow growth (Table 2). Heterotrophic growth rates on fructose under 3% O_2 and 97% N_2 and under 25% O_2 and 75% N_2 were nearly identical, as were those on formate, which requires the enzymes of CO_2 fixation but not

TABLE 2. Effect of oxygen on growth and Hup activity in wild-type and oxygen-tolerant strains

Strain	O_2 concn (%)	Generation time (h) on:			Sp act[a]	
		H_2-CO_2	Fructose	Formate	SH	PH
7-1 (Ose^+)	3	2.7	2.4	10.2	22.4	154.7
7-1 (Ose^+)	25	43.8	2.5	10.7	1.1	4.1
7-301 (Ose^-)	3	2.8	2.5	10.5	27.5	181.8
7-301 (Ose^-)	25	3.5	2.5	10.2	23.8	122.4

[a] Determined for cells growing on H_2-CO_2. Units are micromoles of substrate reduced $hour^{-1}$ milligram of $protein^{-1}$.

of H_2 uptake (11). The oxygen sensitivity of strain 17707, therefore, is specific to hydrogen-dependent growth.

Figure 1 shows that oxygen inhibition is due at least in part to repression of Hup activity at the level of protein synthesis. Two identical cultures of strain 7-1 were grown autotrophically on minimal medium bubbled with a gas mixture consisting of 4% O_2, 10% CO_2, and 86% H_2. At 0 h in the figure, the gas mixture was shifted to 20% O_2, 10% CO_2, and 70% H_2. In the next 16 h, only slight growth occurred, and total hydrogenase (SH plus PH) activity dropped from 3.0 μmol of H_2 h^{-1} 10^9 $cells^{-1}$ to barely detectable levels. At 16 h, 250 μg of chloramphenicol per ml was added to one of the cultures, and after evacuation of the flasks, the gas mixture was shifted back to 4% O_2 (see figure legend for dissolved oxygen concentrations). Hup activity in the chloramphenicol-free culture was derepressed almost immediately, followed by resumption of growth, whereas neither growth nor derepression of Hup activity was observed in the culture to which the protein synthesis inhibitor had been added. Growth was not inhibited by a gas mixture consisting of 4% O_2, 10% CO_2, 70% H_2, and 16% Ar (not shown), indicating that it is increased oxygen and not decreased hydrogen which is inhibitory.

Total hydrogen uptake by whole cells was determined in the above experiment by using methylene blue as electron acceptor. Both hydrogenases of *A. eutrophus* are capable of this reaction (20, 24); thus, neither was active in whole cells of strain 7-1 incubated on autotrophic medium under high oxygen. This conclusion was confirmed by determination of individual hydrogenase activities in crude extracts (Table 2). That the repression of Hup by oxygen occurs at the level of protein synthesis was supported by comparison of SH antigen levels in strain 7-1 incubated on autotrophic medium under high O_2 (Fig. 2A, well f) and low O_2 (Fig. 2A, wells b through e).

Plasmid-borne oxygen-tolerant mutations. Oxygen sensitivity-negative (Ose^-) mutants of strain 17707 appeared spontaneously on autotrophic plates incubated under 30% O_2 at a frequency of about 5×10^{-7} per plated cell, compared with 1×10^{-8} and 3×10^{-9} for rifampin and streptomycin resistance, respectively. In contrast to the wild-type, autotrophic growth and Hup expression in the Ose^- mutant 7-301 were only slightly inhibited by 25% oxygen (Table 2). This strain did not differ from the wild-type in its response to oxygen during growth on organic substrates or on formate (Table 2), which indicates that the mutation affects hydrogen metabolism only.

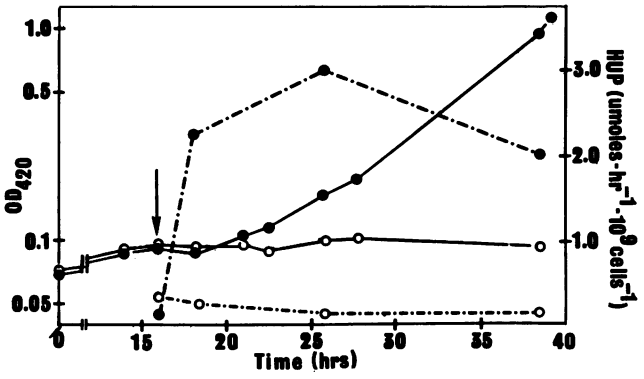

FIG. 1. Derepression of Hup activity by reduced oxygen tension. Two cultures of strain 7-1 were grown autotrophically under 4% O_2 and then 20% O_2, as described in text. At 16 h (arrow), the atmosphere supplied to both cultures was shifted from 20% O_2 to 4% O_2, and 250 μg of chloramphenicol per ml was added to one culture (○), whereas the other was left untreated (●). Turbidity (solid lines) and Hup activity (broken lines) were followed. Dissolved oxygen concentrations in the flask without chloramphenicol were 0.4 μmol/ml at 16 h (before shift), 0.1 μmol/ml at 19 h, and 0.01 μmol/ml at 38 h.

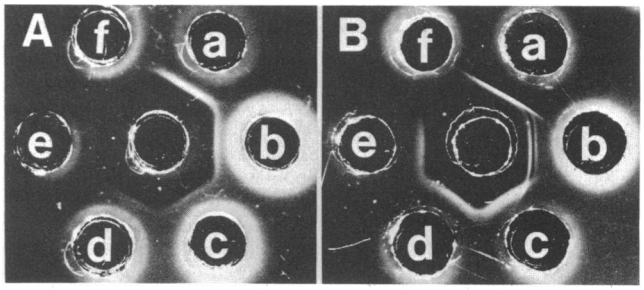

FIG. 2. SH antigen in autotrophically grown cells. Stationary-phase cells grown on fructose minimal medium were washed and incubated in autotrophic medium under the indicated oxygen concentrations for 18 h. Center wells contained 20 μl of anti-*A. eutrophus* H1 SH, and outer wells contained 20 μl each of cytoplasmic extract of the indicated strains (protein concentrations [mg/ml] are indicated in parentheses). (A) a, purified *A. eutrophus* H1 SH (0.09); b, 7-1, 4% 0_2 (16.8); c, 7-1, 4% O_2 (8.4); d, 7-1, 4% O_2 (4.2); e, 7-1, 4% O_2 (2.1); f, 7-1, 25% O_2 (9.75). (B) a, purified H1 SH (0.13); b, 7-113, 4% O_2 (15.3); c, 7-1, 4% O_2 (6.8); d, 7-301, 4% O_2 (6.2); e, 7-301, 25% O_2 (6.0); f, 7-207, 4% O_2 (8.6).

The Hup plasmid of strain 17707 is self-transmissable, like that of other *A. eutrophus* strains (8). Strains that are cured of the plasmid lose the ability to oxidize hydrogen and grow autotrophically, but they grow normally on organic substrates and on formate (2, 8). The Ose^- marker was found to be plasmid-borne in mating experiments with Ose^+ and Ose^- donors and cured recipients (Table 3). Autotrophic growth of recipient strains under 4% O_2 was selected. The recipient strains 7-1C1 and 7-301C1 were cured derivatives of strains 7-1 (Ose^+) and 7-301 (Ose^-), respectively. In both matings, the response to 25% O_2 characteristic of the plasmid donor was observed in all exconjugants. Of eight independently isolated Ose^- mutants, all donated the mutant phenotype with the plasmid.

Involvement of hydrogenase proteins in oxygen sensitivity. To determine whether either of the hydrogenase enzymes are involved in oxygen regulation, the effects of Hup^- mutations on the Ose phenotype were determined, and the properties of hydrogenases in Ose^+ and Ose^- strains were compared.

The mutants listed in Table 4 were isolated after EMS mutagenesis and are defective in one or both hydrogenase activities. Unlike the completely Hup^- phenotype of cured strains, strains 7-113, 7-207, 7-421, and 7-438 had partial Hup activity (Hup^p), allowing them to grow autotrophically at a reduced rate. Strains 7-113 and 7-207 lacked SH activity only and donated their slow-growth phenotype with the Hup plasmid in crosses with the cured strain 7-301C1 (Table 3). Strain 7-113 produced an altered SH antigen, whereas strain 7-207 did not produce detectable (on immunodiffusion plates) amounts of SH antigen (Fig. 2B, wells b and f). Strains 7-421 and 7-438 were pleiotropically defective in both Hup activities, did not donate their slow-growth phenotype with the Hup plasmid in crosses with 7-301C1 (exconjugants are Hup^+; Table 3), and did not produce detectable SH

TABLE 3. Determination of plasmid or chromosomal location of Hup and Ose markers[a]

Donor	Recipient	Selection	Exconjugant phenotype[b]
7-301 (Sm^r Ose^-)	7-1C1 (Rp^r Hup^-)	Rp^r Hup^+	Sm^s Ose^-
7-68 (His^- Ose^+)	7-301C1 (Sm^r Hup^-)	Sm^r His^+ Hup^+	Ose^+
7-113 (Rp^r Hup^p)	7-301C1 (Sm^r Hup^-)	Sm^r Hup^+ or Hup^p	Rp^s Hup^p
7-207 (Rp^r Hup^p)	7-301C1 (Sm^r Hup^-)	Sm^r Hup^+ or Hup^p	Rp^s Hup^p
7-421 (Rp^r Hup^p)	7-301C1 (Sm^r Hup^-)	Sm^r Hup^+ or Hup^p	Rp^s Hup^+
7-438 (Rp^r Hup^p)	7-301C1 (Sm^r Hup^-)	Sm^r Hup^+ or Hup^p	Rp^s Hup^+

[a] Abbreviations: Hup^+, wild-type hydrogenase activity (fast autotrophic growth); Hup^p, partial Hup activity (slow autotrophic growth); Hup^-, no detectable hydrogenase activity due to absence of plasmid (no autotrophic growth).
[b] Hup^p exconjugants are distinguished from Hup^+ by small colony size.

antigen (not shown). None of the mutants listed in Table 4 showed detectable alterations of growth rates on heterotrophic substrates or on formate.

Since Hup^p mutants are capable of slow autotrophic growth, their Ose phenotype can be determined. All are sensitive to oxygen (Ose^+). Ose^- derivatives of strains 7-113 and 7-207 which retained the slow-growth phenotype of their Ose^+ SH^- PH^+ parents occurred spontaneously at the normal frequency. Ose^- derivatives of strains 7-421 and 7-438 could not be obtained.

That the Ose^- phenotype is not due to an alteration in the soluble hydrogenase structural genes is indicated by two additional lines of evidence. First, structural alterations in enzymes frequently result in altered thermodenaturation rates, but SH activity in cytoplasmic extracts decayed with a half-life of about 20 min at 38°C whether it came from strain 7-1 or Ose^- strain 7-301 (Fig. 3). Second, a reaction of complete identity was observed between cytoplasmic extracts of strains 7-1 and 7-301 on immunodiffusion plates (Fig. 2B, wells c, d, and e). Thus SH antigen appears to be unaltered by the Ose^- mutation. Its level of expression under 25% O_2 was, however, increased (compare Fig. 2A, well f, with Fig. 2B, well e).

Thermodenaturation of PH activity in membrane fragments was nonlinear at 67°C, indicating the involvement of more than one component in this reaction (Fig. 3). No significant differences in pattern or rate of decay were observed between strains 7-1 and 7-301, however. Antiserum against *A. eutrophus* PH was unavailable.

Effect of the Ose^- mutation on other types of Hup regulation. Two previously reported types of Hup regulation in *A. eutrophus* are the plasmid-coded, temperature-sensitive regulation reported by Friedrich et al. (12) and growth rate-related regulation under heterotrophic conditions (10, 13). None of the Ose^- mutants examined were altered in temperature-sensitive regulation (all are Hup^+ at 30°C and Hup^- at 37°C), but all were derepressed for whole-cell Hup activity on glycerol. Figure 4 shows Hup activities in Ose^+ strain 7-1, Ose^- strains 7-301 and 7-355, and wild-type *A. eutrophus* strain H1 on three different organic substrates. Hup expression in strain 7-1 was consistently greater on glycerol (g = 8 h) than on succinate (g = 1.3 h), but still much less than that observed in strain H1. Heterotrophic Hup expression in Ose^- mutants of strain 17707 resembled the pattern and magnitude observed in the wild-type strain H1. Hup activity in glycerol- and fructose-grown strain 7-1 could not

TABLE 4. Hup mutants of strain 17707

Strain	Generation time (h) for autotrophic growth	Sp act[a]	
		SH	PH
7-1	3.5	46.34	152.12
7-301C1		<0.02	<0.02
7-113	12.5	0.07	196.87
7-207	14.0	0.13	186.60
7-421	20.0	1.05	3.21
7-438	27.0	3.31	<0.02

[a] Units are micromoles substrate reduced $hour^{-1}$ milligram of $protein^{-1}$. All strains were grown autotrophically except 7-301C1 which does not grow autotrophically; it was grown on glycerol.

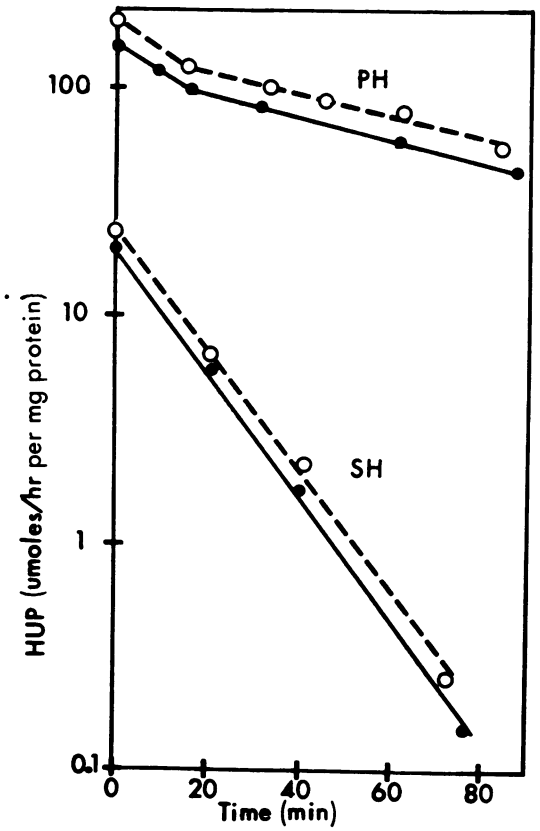

FIG. 3. Thermodenaturation of hydrogenase activities in Ose^+ and Ose^- strains. Strains 7-1 (closed circles) and 7-301 (open circles) were grown autotrophically under 4% O_2, and soluble and particulate fractions were prepared. Thermodenaturation of PH and SH activity was carried out at 67 and 38°C, respectively. Samples were assayed at 25°C.

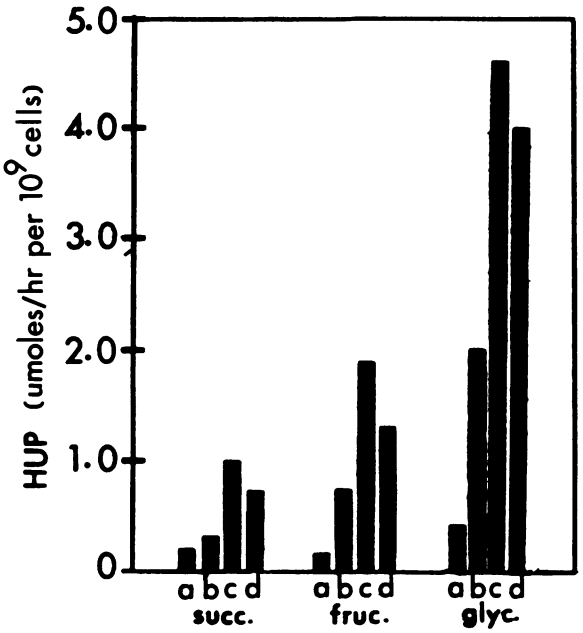

FIG. 4. Hup activities in heterotrophically grown cells. Strains were grown under air to mid-exponential phase on minimal medium with 0.3% organic substrate, and whole-cell Hup activity was determined. Care was taken to compare activities in cultures that were at nearly identical turbidities. Doubling times were 1.3 h for strains 7-1 and H1 on succinate, 2.5 h for strain 7-1 and 2.3 h for strain H1 on fructose, and 8.0 h for strain 7-1 and 10.0 h for strain H1 on glycerol. Strains were (a) 7-1, (b) 7-301, (c) 7-355, and (d) H1.

be derepressed by lowering the oxygen supply, even to the point of growth limitation (data not shown).

Regulation of SH during heterotrophic growth was reflected in the levels of SH antigen in Ose^+ and Ose^- strains. Substantial amounts were detected in crude extracts of glycerol-grown Ose^- strains 7-361 (Fig. 5A, wells b through e) and 7-301 (data not shown), whereas glycerol-grown Ose^+ strain 7-1 showed no SH antigen (Fig. 5A, well f). Even the inactive SH antigen of strain 7-113 was repressed on glycerol in the Ose^+ background (Fig. 5B, well f) and derepressed in its Ose^- derivative strain 7-114 (Fig. 5B, wells b through e).

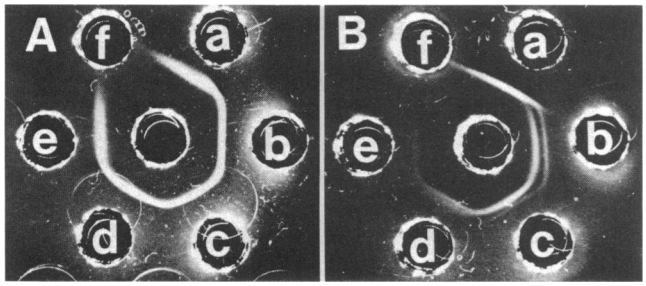

FIG. 5. SH antigen in heterotrophic cells. Cells were grown on glycerol under air and harvested in early stationary phase. Center wells contained 20 μl of anti-*A. eutrophus* H1 SH, and outer wells contained 20 μl each of the following cytoplasmic fractions (protein concentrations [mg/ml] are indicated in parentheses). (A) a, Purified H1 SH (0.26); b, 7-361 (13.6); c, 7-361 (6.8); d, 7-361 (3.4); e, 7-361 (1.7); f, 7-1 (26.2). (B) a, Purified H1 SH (0.26); b, 7-114 (17.0); c, 7-114 (8.5); d, 7-114 (4.2); e, 7-114 (2.1); f, 7-113 (25.2).

These data suggest that Ose regulates Hup expression at the level of protein synthesis during heterotrophic growth, as it does during autotrophic growth.

Effect of the Ose^- mutation on inhibition of heterotrophic growth by hydrogen. The hydrogen effect, in which H_2 inhibits the growth of wild-type *A. eutrophus* on certain organic substrates by inhibiting catabolic enzyme activity, is not observed in Hup^- mutants (19, 21). It was of interest to determine what effect the Ose^- mutation, which results in overexpression of Hup activity on glycerol, would have on inhibition of growth by hydrogen.

Figure 6 shows that although strain 7-1 and Ose^- strain 7-361 grew at the same rate on glycerol under air, an atmosphere of 80% H_2 and 20% O_2 resulted in greater inhibition of growth of the mutant than of the wild type. Strain 7-1C1, a cured derivative of strain 7-1, did not respond at all to the hydrogen atmosphere, as expected from its Hup^- phenotype. The difference between Ose^+ and Ose^- strains was also seen with all other Ose^+ (wild-type strain 17707) and Ose^- (strains 7-301, 7-355, and 7-361) strains tested. Nearly identical results were obtained with an atmosphere of air with 4% H_2, but the effect was transitory due to consumption of the hydrogen.

DISCUSSION

Reduced oxygen tension in autotrophic cultures of *A. eutrophus* 17707 brings about chloramphenicol-sensitive

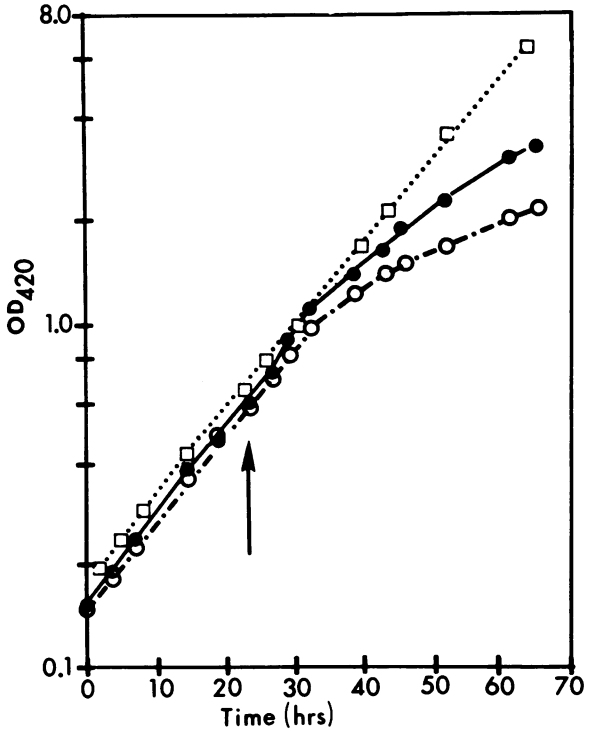

FIG. 6. Effect of Ose^- mutation on hydrogen inhibition of growth on glycerol. Strains 7-1 (●), 7-361 (○), and 7-1C1 (□) were cultured on glycerol minimal medium under air. At the time indicated by the arrow, culture flasks were evacuated and filled with an atmosphere consisting of 80% H_2 and 20% O_2. Parallel cultures which were gassed with 80% N_2 and 20% O_2 continued to grow at the rate characteristic of growth under air.

induction of SH and PH activity and of SH antigen, showing that oxygen represses Hup at the level of protein synthesis. Mutants which are derepressed for Hup activity and SH antigen under elevated (>20%) oxygen concentrations occur spontaneously at relatively high frequency. These Ose^- mutants are also relieved of the normal repression of Hup activity by organic substrates. Comparison of SH antigen levels in mutant and wild-type strains grown on glycerol indicates that Ose-dependent repression of Hup during heterotrophic growth also occurs at the level of protein synthesis.

The Ose^- mutation is transmitted on a plasmid that is already known to carry genes necessary for the expression of both Hup activities in *A. eutrophus* (2, 8). So far, no plasmid genes have been conclusively identified. Mutants of strain H1 which lack soluble but not membrane-bound hydrogenase activity ($SH^- PH^+$) were isolated after mitomycin treatment and were found by agarose gel electrophoresis to have large deletions of plasmid DNA (1, 2). Mutants of strain 17707 lacking SH activity only were isolated during this work by EMS mutagenesis and were found by conjugation experiments to have plasmid lesions. One of them (7-113) produces an altered SH antigen, making it likely that this plasmid-borne mutation is in an SH structural gene. Neither of the plasmid-borne $SH^- PH^+$ mutations examined in this work had any effect on the Ose phenotype. Both mutants were Ose^+ as isolated and yielded Ose^- derivatives at the same frequency as $SH^+ PH^+$ strains. This is physiological evidence that the Ose^- mutation is in a different plasmid gene from those which are altered in the $SH^- PH^+$ mutants, and that its phenotypic expression is independent of SH activity.

So far, no $SH^+ PH^-$ mutants have been isolated, perhaps because the membrane-bound hydrogenase is not required for normal-looking colony formation under laboratory conditions (19). Chromosomal mutations resulting in reduced SH activity and reduced to undetectable PH activity were isolated during this study; these strains were found to be Ose^+, but they did not yield Ose^- derivatives. The altered genes are clearly unlinked to the plasmid-borne Ose marker, so their incompatability with the Ose^- phenotype remains unexplained. Membrane-bound hydrogenase activity might be required for growth under excess oxygen. It has been suggested that hydrogenase stimulates oxygen-sensitive nitrogen fixation in legume nodules not only by providing reductant, but also by reducing the oxygen tension (7). The possibility that the membrane-bound hydrogenase plays a role in Ose-dependent regulation cannot now be ruled out.

The biochemical relationship of the hydrogenases of *A. eutrophus* to oxygen is complex. Both assume extremely stable, inactive forms in the presence of oxygen in vitro and are activated by the removal of O_2 (18, 24). SH has been found to catalyze the production of toxic superoxide anions in the presence of oxygen and reductant (25). Since all of the Hup^p mutants are Ose^+, it is not likely that oxygen sensitivity results from Hup-dependent toxicity. The observation that thermodenaturation of both hydrogenases and antigenic identity of the SH are unaltered in the Ose^- mutant 7-301 supports the conclusion that oxygen tolerance does not result from a structural alteration in either enzyme.

The high frequency of spontaneous occurrence of the Ose^- mutation indicates a large target size, suggesting that oxygen tolerance results from inactivation of a gene product. Since both hydrogenases are active and apparently unaltered in Ose^- mutants, we conclude that the Ose^- mutation probably inactivates an as-yet-unidentified plasmid-coded product which regulates Hup expression at the level of protein synthesis.

The physiological significance of oxygen-sensitive regulation of Hup during autotrophic growth of strain 17707 is unclear. Oxygen cannot be the sole effector, since it has no effect on Hup expression during heterotrophic growth. Autotrophic growth under 70% H_2, 10% CO_2, and 20% O_2 is probably extremely rare in nature, but mixotrophic growth (in which small quantities of hydrogen provide supplemental energy during growth on organic substrates) may not be. The Ose system may hence be important to mixotrophically (rather than autotrophically) growing cells. The growth of *A. eutrophus* on certain organic compounds is known to be inhibited by hydrogen if hydrogenase is synthesized, a phenomenon termed the hydrogen effect (19, 21). Since the magnitude of the hydrogen effect is proportional to the specific activity of hydrogenase and since the Ose system acts to reduce the activity of hydrogenase in heterotrophically growing cells (see Fig. 4), we expect the growth rates of *A. eutrophus* under conditions allowing mixotrophy to be higher in Ose^+ than in Ose^- cells. The experiment shown in Fig. 6 confirms this expectation. We tentatively conclude that the selective advantage of the Ose system of regulation is to minimize the hydrogen effect and hence to facilitate mixotrophic growth.

Although autotrophic growth under excess hydrogen and 10 to 20% oxygen must be rare in nature, such an atmosphere is routinely used in the enrichment and isolation from nature of hydrogen-oxidizing bacteria (3). It is possible, therefore, that oxygen-tolerant *A. eutrophus* strains such as H1, which regulates Hup in a manner similar to the Ose^- mutants of strain 17707 under both autotrophic (26) and heterotrophic (Fig. 4) conditions, are themselves laboratory-selected mutants. Davis et al. (6), in their survey of aerobic hydrogen bacteria, observed the selection of autotrophic oxygen tolerance in *Alcaligenes paradoxus* and cautioned against the assignment of taxonomic significance to oxygen sensitivity. The *A. eutrophus* strains used in that study were all oxygen tolerant, including strain 17707 (which was received second hand, rather than directly from the American Type Culture Collection as in this study). Recently, mutants of *Rhizobium japonicum* were isolated which are derepressed for hydrogen uptake in the presence of elevated oxygen concentrations, and these were also found to be partially relieved of the normal repression of Hup by organic substrates (15). Such observations suggest that the Ose system of regulation may be a general feature of hydrogen bacteria.

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