JOURNAL OF BACTERIOLOGY, Jan. 1993, p. 474–478
0021-9193/93/020474-05$02.00/0

Vol. 175, No. 2

Nitrogen Fixation by the Thermophilic Green Sulfur Bacterium *Chlorobium tepidum*

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Received 11 August 1992/Accepted 10 November 1992

The thermophilic green sulfur bacterium *Chlorobium tepidum* grew with N_2, NH_4^+, or glutamine as the sole nitrogen source under phototrophic (anaerobic-light) conditions. Growth on N_2 required increased buffering capacity to stabilize uncharacterized pH changes that occurred during diazotrophic growth. Increased sulfide levels were stimulatory for growth on N_2. Levels of nitrogenase activity (acetylene reduction) in N_2-grown *C. tepidum* cells were very high, among the highest ever reported for anoxygenic phototrophic bacteria. Maximal acetylene reduction rates in *C. tepidum* cells were observed at 48 to 50°C, which is about 15°C higher than the optimum temperature for nitrogenase activity in mesophilic chlorobia, and nitrogenase activity in *C. tepidum* responded to addition of ammonia by a "switch-off/switch-on" mechanism like that in phototrophic purple bacteria. *C. tepidum* cells assimilated ammonia mainly via the glutamine synthetase-glutamate synthase pathway, elevated levels of both of these enzymes being present in cells grown on N_2. These results show that N_2 fixation can occur in green sulfur bacteria up to at least 60°C and that regulatory mechanisms important in control of nitrogenase activity in mesophilic anoxygenic phototrophs also appear to regulate thermally active forms of the enzyme.

Nitrogen fixation is a widespread property of anoxygenic phototrophic bacteria; most representatives of purple and green bacteria tested have been found to be diazotrophic (5–7, 9, 10, 14, 16, 17, 20). Exceptions include the nonsulfur purple bacterium *Rhodocyclus purpureus* (16, 17) and the thermophilic green nonsulfur bacterium *Chloroflexus aurantiacus* (8). Tests with another thermophilic phototroph, *Chromatium tepidum*, have also shown it to be incapable of N_2 fixation (15), although further experiments showed that *Chromatium tepidum* genomic DNA hybridized to an *nifHDK* probe prepared from the purple bacterium *Rhodospirillum rubrum* (13). The reason(s) why *Chromatium tepidum* remains phenotypically Nif^- (15) is unknown.

Postgate (19) has observed that diazotrophy and thermophily rarely coexist in a single organism, and relatively few examples of thermophilic nitrogen-fixing bacteria are known today. Perhaps the best examples are the methanogen *Methanococcus thermolithotrophicus* and the heterocystous cyanobacterium *Mastigocladus laminosus*; *M. thermolithotrophicus* fixes N_2 at 60°C (1), while *M. laminosus* fixes N_2 optimally at 45°C (22). In addition, *Clostridium thermosaccharolyticum* has been shown to fix N_2 up to 60°C (2). Among phototrophic bacteria, the mildly thermophilic nonsulfur purple bacteria *Rhodospirillum centenum* (5) and *Rhodopseudomonas* sp. strain GI (20) both fix N_2 up to 47°C.

We have recently isolated a thermophilic species of the genus *Chlorobium* (green sulfur bacteria) and have described it as a new species, *Chlorobium tepidum* (25). *C. tepidum* was isolated from a high-sulfide acidic hot spring and grows much faster than any known green sulfur bacterium (25). Although our initial attempts to grow *C. tepidum* on N_2 as the sole nitrogen source were equivocal, we have now developed culture conditions that support good diazotrophic growth of this thermophilic anoxygenic phototroph at temperatures of up to 51°C. Herein, we describe experiments on the basic properties of N_2-fixing *C. tepidum* cells, including regulatory aspects of nitrogenase and the route of fixed nitrogen incorporation.

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MATERIALS AND METHODS

Bacterial strains. *C. tepidum* TLS (ATCC 49652) was previously described (25) as was the mesophilic species *Chlorobium limicola* forma *thiosulfatophilum* 8327 (7). Stocks of both organisms were stored at −80°C in growth medium containing 10% (vol/vol) glycerol and fresh cultures were revived periodically.

Culture media. Cultures of both *Chlorobium* species were grown routinely in medium Pf-7, a modification of Pfennig's sulfur phototroph medium (18), which contains both sulfide (2.5 mM) and thiosulfate (10 mM) as photosynthetic electron donors and acetate plus CO_2 as carbon sources (25). For growth on N_2, medium Pf-7 was modified as follows: sodium acetate (6.5 mM) was substituted for ammonium acetate, NH_4Cl was omitted, sulfide was increased to 5 mM, and 10 mM 3-*N*-morpholine propanesulfonic acid (MOPS) was added per liter (pH 7). This modified medium Pf-7 is referred to as medium Pf/N_2. It was found essential for reproducible diazotrophic growth of *C. tepidum* that medium Pf/N_2 have a final pH of 6.9 to 7.0.

Growth conditions. Cultures were grown photosynthetically on ammonia or glutamine as nitrogen sources in screw-capped tubes or bottles as previously described (25). For growth on N_2, cultures were grown in 250-ml Erlenmeyer sidearm flasks containing 120 ml of medium Pf/N_2, a small magnetic stirring bar, and a headspace of N_2-H_2 (99:1). Cultures were routinely inoculated (3 to 4% volume) in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) and then removed and placed in an illuminated (~5,400 lux, incandescent illumination) glass water bath held at 47 to 48°C. N_2-grown cells were agitated very gently (but not actively stirred) by action of the small stir bar driven by a magnetic stirring plate placed under the water bath.

Measurement of growth. Cell growth was routinely measured in a Klett-Summerson photometer fitted with a no. 66

(red) filter. Because young cultures of green sulfur bacteria typically produce copious amounts of elemental sulfur from the oxidation of sulfide (24, 25), Klett readings were not directly proportional to cell numbers during the first few hours of growth. However, after oxidation of sulfur by *C. tepidum*, linear functions relating photometer units to independent measures of cell growth were obtained (see Results).

Nitrogenase. The nitrogenase activity of intact cells was measured by a modification of the acetylene reduction method previously described (7). Late-log-phase cells were removed from growth vessels within the anaerobic chamber, and depending upon cell density, a 5- to 20-ml aliquot was transferred to a 70-ml serum vial and sealed with sleeve-type rubber stoppers under an N_2 atmosphere. Vials were removed from the chamber, incubated for 15 min in darkness at 48°C (or at the temperatures designated in Fig. 2), and returned to the light (5,400 lux), and acetylene was added to 10% to begin an assay. Ethylene was determined in a Varian model 2400 gas chromatograph fitted with a Poropak R column (70°C).

Enzymes of ammonia assimilation. Glutamine synthetase (GS; γ-glutamyltransferase activity) was assayed as described previously (17). Glutamate synthase (glutamine-oxoglutarate aminotransferase [GOGAT]) and glutamate dehydrogenase activities were measured by monitoring the 2-oxoglutarate-dependent oxidation of NADH, and the presence of alanine dehydrogenase was tested for by assaying pyruvate-plus-ammonia-dependent oxidation of NADH as previously described (17).

Protein. Protein contents of intact cells were determined after the cell pellets were boiled in 1 N NaOH for 20 min. Suitably diluted aliquots were assayed by using the Bio-Rad dye-binding system (Bio-Rad Laboratories, Richmond, Calif.); bovine serum albumin served as the protein standard.

Chemicals. All chemicals used were of reagent grade and most (including thiosulfate) were obtained from Fisher Scientific (St. Louis, Mo.). MOPS was obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Growth on N_2. Shortly after our isolation of the new thermophilic green sulfur bacterium *C. tepidum* (25), we tested several strains of this organism for the ability to grow on N_2. Initial experiments were not encouraging, and growth, when it did occur, was only scant. We then discovered that a rapid rise in pH was occurring in inoculated medium Pf-7 to which ammonium salts had been deleted. The chemical nature of this pH change was not identified, but from characterizational studies of *C. tepidum* we knew that the organism had a rather restricted pH range for growth (pH 6.8 to 7.2); media adjusted to a pH of 7.3 or higher failed to support growth (25). The pH in inoculated medium Pf-7 minus ammonia quickly rose to near 8, and this presumably halted further growth of *C. tepidum* on N_2. This problem was solved by employing the organic buffer MOPS in all media for growth of *C. tepidum* on N_2 (medium Pf/N_2); control experiments showed that MOPS did not serve as either a nitrogen or carbon source for *C. tepidum*. Increased sulfide levels for growth of *C. tepidum* on N_2 were also found to be stimulatory; optimal growth on N_2 occurred at 5 mM sulfide, where only about half this much supported optimal growth on ammonia (data not shown).

Figure 1 shows growth of *C. tepidum* on ammonia or on N_2 as the sole nitrogen source. Cultures of *C. tepidum* grew very rapidly on ammonia at 48°C; minimum generation times of ca. 2 h were typically observed. By contrast, growth of *C. tepidum* on N_2 was slower, minimum generation times averaging ca. 10 h (Fig. 1). Control flasks containing argon in place of N_2 (Fig. 1) showed that growth of *C. tepidum* in the absence of fixed nitrogen was indeed N_2 dependent but also showed an unavoidable problem encountered with green sulfur bacteria grown at high light intensities such as those used herein. Turbidimetric growth measurements of green sulfur bacteria are initially influenced by the light-scattering effects of elemental sulfur produced from the oxidation of sulfide (Fig. 1). In cultures of *C. tepidum* grown on either ammonia or N_2 as the nitrogen source, sulfur production was maximal during the first 4 to 6 h of growth, and then sulfur was consumed during the production of sulfate (25). Thus turbidimetric measurements of growth of *C. tepidum* (Fig. 1) were initially overestimates of true cell growth. This can be seen as an inflection in growth curves followed by true exponential growth (Fig. 1). However, measurements taken of any parameter of bacterial growth (cell numbers, protein, or dry weight) from about 7 h into the growth cycle or thereafter (Fig. 1) were found to be strictly proportional to turbidimetric measurements and thus were indicative of true exponential growth. In the argon control culture (Fig. 1), growth due to residual N_2 combined with sulfur production yielded some turbidity, but a plateau was reached within 12 h, beyond which turbidity in the culture remained constant; during this same period N_2-supplemented cultures grew to high cell densities (Fig. 1).

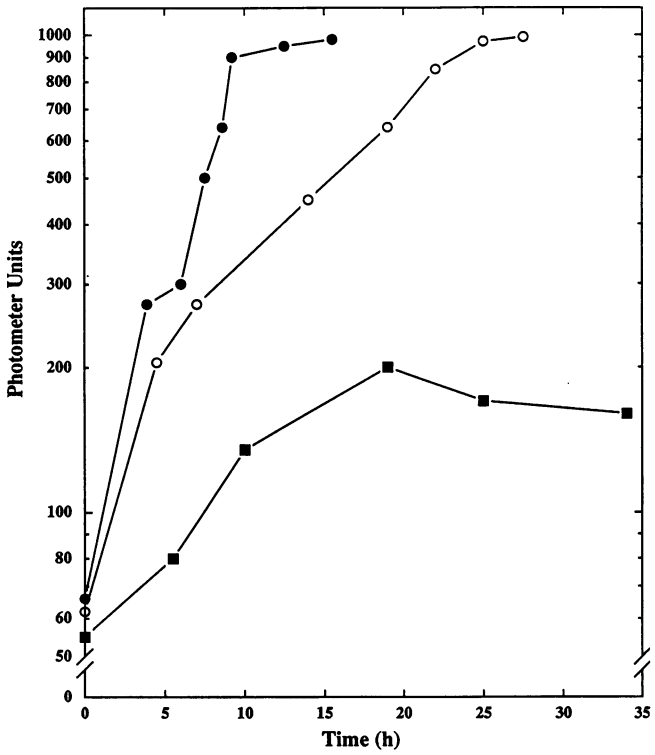

FIG. 1. Growth of *C. tepidum* in medium Pf-7 on ammonia (●), medium Pf/N_2 on N_2 (○), or medium Pf/N_2 sparged with argon and containing an argon headspace (■). Each culture was incubated at 47 to 48°C and 5,400 lux. Minimum generation times on ammonia and N_2 were ca. 2 and 10 h, respectively. Nine hundred photometer units is equivalent to $\sim 6 \times 10^9$ cells · ml^{-1}.

TABLE 1. Nitrogenase activities in intact *C. tepidum* cells[a]

Growth condition(s)[b]	Nitrogenase activity[c]
N_2	6,300
Glutamine (1 mM)	300
Glutamine (10 mM)	<0.1
Ammonia (7.5 mM)	<0.1
Ammonia (1 mM)[d]	4,240
N_2, limiting $S_2O_3^{2-}$[e]	2,450
N_2, limiting $S_2O_3^{2-}$ with $S_2O_3^{2-}$ supplement[f]	5,000
N_2, assayed in darkness	<0.1

[a] All assays were conducted in the light (N_2 headspace) unless otherwise specified. Results are averages of three or more experiments. Cells were assayed from late-log-phase cultures.

[b] Glutamine and ammonia cells were grown in completely filled screw-capped tubes; N_2 cells were grown in flasks with a large N_2 headspace. The following amino acids did not support growth of *C. tepidum* at 1, 5, or 10 mM concentrations: alanine, arginine, asparagine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

[c] Nanomoles of ethylene produced per hour per milligram of cell dry weight.

[d] Cells assayed after reaching stationary phase.

[e] Cells grown in medium Pf/N_2 containing 6 mM $S_2O_3^{2-}$.

[f] Cells grown as described in footnote *e* but supplemented with 10 mM $S_2O_3^{2-}$ at time of assay.

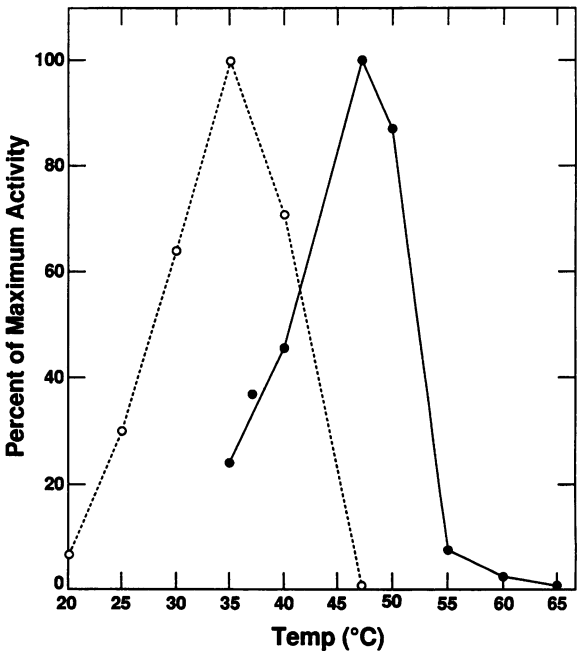

FIG. 2. Nitrogenase activity as a function of temperature in *C. tepidum* (●) and *C. limicola* forma *thiosulfatophilum* (○) cells. Cells from a single batch culture of each organism grown at 48°C (*C. tepidum*) or 35°C (*C. limicola*) were assayed for nitrogenase activity by acetylene reduction at different temperatures. Values represent micromoles of ethylene produced in 1 hour at a given temperature and are plotted as the percentage of maximum activity observed for each species. One hundred percent values were 18.8 μmol of ethylene per 5 ml of culture for *C. tepidum* and 16.4 μmol of ethylene per 20 ml of culture for *C. limicola*.

Nitrogenase in *C. tepidum*. Once culture conditions supporting growth of *C. tepidum* on N_2 were established, it was found that such cells readily reduced acetylene to ethylene (Table 1). Levels of nitrogenase activity in intact N_2-grown *C. tepidum* cells were very high (Table 1), higher in fact than that of any N_2-grown anoxygenic phototrophic bacterium previously reported (7, 9, 10, 16, 17, 20). As expected, cells grown on excess ammonia did not fix N_2 (Table 1). But cells grown on low levels (<1 mM) of glutamine, the only nitrogen source other than ammonia or N_2 tested that was used by *C. tepidum* cells, showed some nitrogenase activity; cells grown on excess glutamine (10 mM), however, were completely inactive (Table 1). Light was also required for demonstrable nitrogenase activity in *C. tepidum* (Table 1). In addition, thiosulfate supplementation of *C. tepidum* cultures grown on limiting levels of thiosulfate greatly stimulated nitrogenase activity (Table 1). This suggests that this relatively high-potential ($E_0' = +0.025$ V) reduced sulfur source, which is an excellent photosynthetic electron donor for *C. tepidum* (25), can feed electrons to nitrogenase, probably after photosynthetic conversion to a low-potential electron donor.

Temperature optimum for *C. tepidum* nitrogenase activity. Because of its thermophilic phenotype and the fact that N_2 fixation is rarely associated with thermophilic bacteria (8, 19), we tested the ability of cultures of *C. tepidum* to reduce acetylene as a function of temperature. *C. limicola* forma *thiosulfatophilum*, a mesophilic green sulfur bacterium phylogenetically related to *C. tepidum* (25), was used as a nonthermophilic control. As shown in Fig. 2, the optimum temperature for nitrogenase activity in the mesophilic and thermophilic *Chlorobium* species differed by 12 to 15°C. More significantly, however, at the optimum temperature for *C. tepidum* nitrogenase activity, nitrogenase from *C. limicola* was completely inactive (Fig. 2), presumably because of heat denaturation of critical cell components needed for nitrogenase activity (or perhaps even of nitrogenase itself). In *C. tepidum* cells, nitrogenase activity was near maximal even at 50°C but fell off sharply above this temperature; low but detectable levels of nitrogenase activity were still measurable at 60°C (Fig. 2). The maximal growth temperature for *C. tepidum* on ammonia, 51 to 52°C (25), was also confirmed as the maximal temperature for growth of the organism on N_2 as the sole nitrogen source (data not shown).

Control of nitrogenase activity in *C. tepidum* cells. Anoxygenic phototrophic bacteria employ a mechanism for regulation of nitrogenase activity called the nitrogenase "switch-off/switch-on" effect (14, 21), and our previous studies with *C. limicola* have shown that mesophilic green bacteria are subject to nitrogenase switch-off by ammonia (7). In the thermophilic species *C. tepidum*, ammonia switch-off of nitrogenase activity in intact cells was also observed and, depending on the amount of ammonia added, could be shown to be a reversible phenomenon (Fig. 3). Low levels of ammonia (100 μM) switched-off *C. tepidum* nitrogenase with the same kinetics as did higher levels of ammonia (1 mM), but within 1 h, the lower level of ammonia was presumably consumed, leading to reactivation of nitrogenase (Fig. 3).

Ammonia assimilation. Assays of extracts of *C. tepidum* cells indicated that fixed N_2 or exogenous ammonia was incorporated into an organic form primarily by the GS-GOGAT route (Table 2). Low but detectable levels of glutamate dehydrogenase were present in *C. tepidum* cells (Table 2), but assays for alanine dehydrogenase showed no activity. Both glutamate dehydrogenase and GS activities in *C. tepidum* were linked to NADH as a coenzyme. Levels of both glutamine synthetase and glutamate synthase in N_2-grown *C. tepidum* cells were elevated some fivefold over those of excess-ammonia-grown cells (Table 2). All enzymes

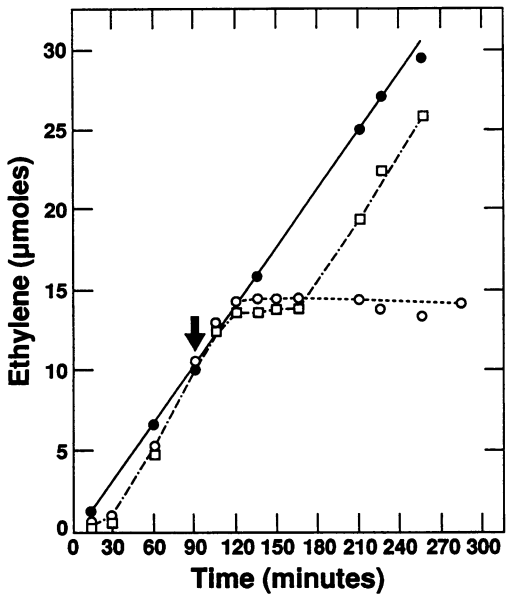

FIG. 3. Ammonia switch-off of nitrogenase activity in intact N_2-grown *C. tepidum* cells. Ammonia (□, 100 µM; ○, 1 mM) was added at the point indicated by the arrow, and acetylene reduction assays were performed at 15-min intervals as described in Materials and Methods. N_2-grown *C. tepidum* cells to which no ammonia was added (●) served as the control.

were assayed at the growth temperature optimum (48°C) of *C. tepidum* (25).

DISCUSSION

We have established through growth and acetylene reduction experiments that the thermophilic green sulfur bacterium *C. tepidum* can fix molecular nitrogen. However, although *C. tepidum* grows well on N_2, it does so only in media sufficiently buffered to prevent the dramatic pH rise that occurs when ammonia is omitted from the relatively unbuffered medium used for growth on ammonia (25). A growth-inhibitory pH shift does not occur in media used for diazotrophic growth of nonsulfur purple bacteria (16) or in cultures of the mesophilic relative of *C. tepidum*, *C. limicola* forma *thiosulfatophilum*. In the latter organism, good growth on N_2 was achieved without the need for additional buffering capacity or higher sulfide levels (7). The nature of the pH shift catalyzed by N_2-grown cells of *C. tepidum* remains unidentified.

Nitrogen fixation by *C. tepidum* up to 60°C represents the highest temperature reported for N_2 fixation in anoxygenic phototrophic bacteria. Among cyanobacteria, *M. laminosus* is the only well-documented thermophilic diazotroph. *M. laminosus* will grow on N_2 at 55°C or lower (23), but shows a temperature optimum for N_2 fixation at 45°C (22); the latter is below that observed herein with *C. tepidum*. *C. thermosaccharolyticum* was the first thermophilic member of the *Bacteria* (eubacteria) reported to fix N_2 and shows acetylene reduction activity up to 60°C (2). The thermophilic methanogen *M. thermolithotrophicus* is able to fix N_2 at 65°C (1) and is currently the most thermophilic of all known diazotrophic bacteria. Growth of the thermophilic methanogen *Methanobacterium thermoautotrophicum* on N_2 has also been reported (4), but this finding has recently been questioned (12). The moderately thermophilic nonsulfur purple bacteria *Rhodopseudomonas* sp. strain GI (20) and *R. centenum* (5) also fix dinitrogen up to about 47°C, but both of these organisms have growth temperature optima well below that of *C. tepidum* (5, 20).

The temperature optimum for *C. tepidum* nitrogenase activity of about 48°C agrees nicely with the temperature profile for growth of this organism (25). By contrast, in the chemotrophic diazotroph *Klebsiella pneumoniae*, temperature regulates expression of nitrogenase (26). *K. pneumoniae* will grow on N_2 at 30°C but not at 37°C, although the latter temperature is near the temperature optimum for growth of the organism on ammonia (26). In *C. tepidum*, however, the maximal temperature for growth of the organism on N_2 was the same as that for growth on ammonia. These results suggest that expression of *C. tepidum* nitrogenase is not regulated by temperature and that the enzyme itself is a thermally stable protein, although in vitro experiments would be necessary to establish the latter with certainty (11).

Nitrogenase activities in N_2-grown *C. tepidum* cells were much higher than those reported from purple bacteria (16) or from mesophilic chlorobia (7) and were subject to switch-off by ammonia. For example, the specific activities of nitrogenase in cells of the mesophilic *Chlorobium* species *C. limicola* are about one-third those of *C. tepidum* (7). This difference in enzyme activity between the two species is also reflected in growth rates; the doubling time for *C. limicola* cells grown on N_2 is about twice that measured herein for *C. tepidum* (7). Switch-off of *C. tepidum* nitrogenase activity by ammonia, which was shown to be a reversible phenomenon, further solidifies the importance of this regulatory mechanism for diazotrophic growth of all anoxygenic phototrophic bacteria. Ammonia switch-off is universally observed in anoxygenic phototrophs (14, 21) and has even been observed in phototrophic heliobacteria, phylogenetic relatives of the clostridia (10). The reversibility of the switch-off effect observed in intact *C. tepidum* cells is consistent with the operation of a DRAT-DRAG-based nitrogenase regulatory system (14), and indeed, preliminary experiments employing Western blotting (immunoblotting) have shown that these enzymes do exist in *C. tepidum* cells (13). It is possible that *C. tepidum* could be a source of thermostable DRAT and DRAG proteins.

The ATP-dependent GS-GOGAT system is the major route for assimilation of exogenous ammonia and fixed N_2 in *C. tepidum*. GS activities in N_2-grown *C. tepidum* cells were significantly higher than those reported previously for mesophilic chlorobia (3). Elevated levels of GS under diazotrophic growth conditions are commonly observed in nitrogen-fixing bacteria and presumably serve as a mechanism to scavenge ammonia produced by nitrogenase (3). Glutamate synthase (GOGAT) activities in cells of *C. tepidum* also responded to N_2-fixing growth conditions, showing the same fivefold increase in activity as seen with GS under the same

TABLE 2. Enzymes of ammonia assimilation in *C. tepidum*[a]

Growth condition	GS[b]	GOGAT[c]	Glutamate dehydrogenase[c]
N_2	5,078	149	8
Ammonia	946	33	12

[a] Cells were grown photosynthetically to the late logarithmic phase in medium Pf/N_2 or Pf-7 (14 mM NH_4^+).
[b] Nanomoles of γ-glutamylhydroxymate formed per minute per milligram of protein.
[c] Nanomoles of NADH oxidized per minute per milligram of protein.

conditions. However, such an increase in GOGAT activity has not been observed in mesophilic chlorobia, in which the activities of this enzyme have been found to vary little with ammonia concentration (3).

In conclusion, the thermophilic phototroph *C. tepidum* is clearly a nitrogen-fixing bacterium and shows many of the features common to other nitrogen-fixing phototrophs, with a major exception being its thermally active properties. Because of its likely thermal stability, it will be interesting to determine the sequence of the structural genes for the *C. tepidum* nitrogenase complex (*nifHDK*) in order to compare amino acid sequences of *C. tepidum* nitrogenase and nitrogenase reductase with those from other diazotrophs. In this connection, we have recently cloned a region of the *C. tepidum* chromosome that hybridizes strongly to *nifHDK* probes from *K. pneumoniae* and *R. rubrum* (data not shown), and work is now in progress towards sequencing this region to obtain structural information on *C. tepidum* nitrogenase proteins.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the National Research Initiative Competitive Grants Program of the U.S. Department of Agriculture (grant 91-37305-6600).

REFERENCES

1. **Belay, N., R. Sparling, and L. Daniels.** 1984. Dinitrogen fixation by a thermophilic methanogenic bacterium. Nature (London) **312:**286–288.
2. **Bogdahm, M., and D. Kleiner.** 1986. N_2 fixation and NH_4^+ assimilation in the thermophilic anaerobes *Clostridium thermosaccharolyticum* and *Clostridium thermoautotrophicum*. Arch. Microbiol. **144:**102–104.
3. **Brown, C. M., and R. A. Herbert.** 1977. Ammonia assimilation in purple and green sulphur bacteria. FEMS Lett. **1:**39–42.
4. **Fardeau, M.-L., J.-P. Peillex, and J.-P. Belaïch.** 1987. Energetics of growth of *Methanobacterium thermoautotrophicum* and *Methanococcus thermolithotrophicus* on ammonium chloride and dinitrogen. Arch. Microbiol. **148:**128–131.
5. **Favinger, J., R. Stadtwald, and H. Gest.** 1989. *Rhodospirillum centenum* sp. nov., a thermotolerant cyst-forming anoxygenic photosynthetic bacterium. Antonie van Leeuwenhoek J. Microbiol. Serol. **55:**291–296.
6. **Gest, H., and J. L. Favinger.** 1983. *Heliobacterium chlorum*, an anoxygenic brownish-green photosynthetic bacterium containing a "new" form of bacteriochlorophyll. Arch. Microbiol. **136:**11–16.
7. **Heda, G. D., and M. T. Madigan.** 1986. Aspects of nitrogen fixation in *Chlorobium*. Arch. Microbiol. **143:**330–336.
8. **Heda, G. D., and M. T. Madigan.** 1986. Utilization of amino acids and lack of diazotrophy in the thermophilic anoxygenic phototroph *Chloroflexus aurantiacus*. J. Gen. Microbiol. **132:** 2469–2473.
9. **Heda, G. D., and M. T. Madigan.** 1988. Nitrogen metabolism and N_2 fixation in phototrophic green bacteria, p. 175–187. *In* J. M. Olson, J. G. Ormerod, J. Amez, E. Stackebrandt, and H. G. Trüper (ed.), Green photosynthetic bacteria. Plenum Press, New York.
10. **Kimble, L. K., and M. T. Madigan.** 1992. Nitrogen fixation and nitrogen metabolism in heliobacteria. Arch. Microbiol. **158:**155–161.
11. **Ljungdahl, L. G., and D. Sherod.** 1976. Proteins from thermophilic microorganisms, p. 147–186. *In* M. R. Heinrich (ed.), Extreme environments. Academic Press, New York.
12. **Lobo, A. L., and S. H. Zinder.** 1992. Nitrogen fixation by methanogenic bacteria, p. 191–211. *In* G. Stacey, R. H. Burris, and J. E. Evans (ed.), Biological nitrogen fixation. Routledge, Chapman and Hall, Inc., New York.
13. **Ludden, P. W., G. P. Roberts, and M. T. Madigan.** Unpublished data.
14. **Ludden, P. W., and G. P. Roberts.** 1989. Regulation of nitrogenase activity by reversible ADP ribosylation. Curr. Top. Cell Regul. **30:**23–56.
15. **Madigan, M. T.** 1986. *Chromatium tepidum* sp. nov., a thermophilic photosynthetic bacterium of the family *Chromatiaceae*. Int. J. Syst. Bacteriol. **36:**222–227.
16. **Madigan, M. T., S. S. Cox, and R. A. Stegeman.** 1984. Nitrogen fixation and nitrogenase activities in members of the family *Rhodospirillaceae*. J. Bacteriol. **157:**73–78.
17. **Masters, R. A., and M. T. Madigan.** 1983. Nitrogen metabolism in the phototrophic bacteria *Rhodocyclus purpureus* and *Rhodospirillum tenue*. J. Bacteriol. **155:**222–227.
18. **Pfennig, N., and H. G. Trüper.** 1989. Anoxygenic phototrophic bacteria, p. 1635–1709. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. Williams & Wilkins, Baltimore.
19. **Postgate, J. R.** 1974. Prerequisites for biological nitrogen fixation in free-living heterotrophic bacteria, p. 663–686. *In* A. Quispel (ed.), The biology of nitrogen fixation. Elsevier North-Holland Publishing Co., Amsterdam.
20. **Resnick, S. M., and M. T. Madigan.** 1989. Isolation and characterization of a mildly thermophilic purple bacterium containing bacteriochlorophyll *b*. FEMS Microbiol. Lett. **65:**165–170.
21. **Roberts, G. P., and P. W. Ludden.** 1992. Nitrogen fixation by photosynthetic bacteria, p. 135–165. *In* G. Stacey, R. H. Burris, and J. E. Evans (ed.), Biological nitrogen fixation. Routledge, Chapman and Hall, Inc., New York.
22. **Stevens, E. S., V. B. Mehta, and L. S. Lane.** 1985. Heterocyst development and nitrogen fixation by *Mastigocladus laminosus*, p. 235–243. *In* P. W. Ludden and J. E. Burris (ed.), Nitrogen fixation and CO_2 metabolism. Elsevier Science Publishing Co., New York.
23. **Stewart, W. D. P.** 1970. Nitrogen fixation by blue-green algae in Yellowstone thermal areas. Phycologia **9:**261–268.
24. **Trüper, H. G.** 1978. Sulfur metabolism, p. 677–687. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Press, New York.
25. **Wahlund, T. M., C. R. Woese, R. W. Castenholz, and M. T. Madigan.** 1991. A thermophilic green sulfur bacterium from New Zealand hot springs, *Chlorobium tepidum* sp. nov. Arch. Microbiol. **159:**81–90.
26. **Zhu, J., and W. J. Brill.** 1981. Temperature sensitivity of the regulation of nitrogenase synthesis by *Klebsiella pneumoniae*. J. Bacteriol. **145:**1116–1118.