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Ammonia Assimilation Enzymes in a Thermophilic *Bacillus* sp. of Marine Origin

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Abstract. The physiology of ammonia assimilation enzymes was examined in *Bacillus* sp. FE-1, a thermophilic marine bacterium. Glutamine synthetase (GS) and glutamate synthase (GOGAT) activities varied with the nitrogen source present in the medium, ranging as much as 10-fold for the former and 2.5-fold for the latter. Glutamate dehydrogenase (GDH) was detected but, under the growth conditions studied, levels were not affected by the nitrogen source. Anaerobic growth in the presence of nitrate yielded enzyme levels that were not significantly different from those measured under aerobic growth. Partially purified GS exhibited a temperature optimum between 65° and 75°C. The enzyme's Mn^{2+}-dependent reverse transferase activity was stimulated by K_2SO_4 and demonstrated some tolerance to NaCl. Hyperbolic kinetics were observed for ammonium, with an apparent K_M of 1.0 mM.

Members of the genus *Bacillus* exhibit an elaborate adaptive response to conditions of environmental stress. Nutrient deprivation, for example, will trigger a complex sequence of physiological and morphological changes resulting in endospore development. While research into the physiology and molecular biology of spore formation has focused on the mesophile *Bacillus subtilis*, comparatively little is known about the regulation of gene expression in the thermophilic and psychrophilic representatives of the genus. Furthermore, *Bacillus* strains isolated from the marine environment have not been examined in any detail. Although these species account for only a small percentage of the bacteria in the ocean, they may provide further insight into the strategies utilized by marine bacteria enabling them to adapt to their surroundings.

We have been studying strain FE-1, a thermophilic *Bacillus* sp. of marine origin, that was isolated from sediments of the West Florida Escarpment cold seep [10]. These seep communities resemble those associated with hydrothermal vents discovered along East Pacific Rise ridge crests. Escarpment sediment had characteristically high concentrations of ammonia and low amounts of dissolved sulfates [10]. The ammonia-rich environment from which FE-1 was isolated stimulated our interest in examining how this bacterium regulates the enzymes involved in assimilating ammonia. Gocke et al. [5] found evidence that FE-1 possessed a nitrogen metabolic pathway that has not been described for other members of the genus *Bacillus*. When grown anaerobically, FE-1 displayed a denitrification phenotype characteristic of *Pseudomonas aeruginosa*; the organism used nitrite, nitrous oxide in the presence of nitrite, and nitrous oxide alone after exhaustion of nitrite in a dissimilatory manner [5].

In this report we begin to examine regulation of GS (EC 6.3.1.2), GOGAT (EC 1.4.1.13), and GDH (EC 1.4.1.4.) in *Bacillus* sp. FE-1. Assimilation of ammonia in most prokaryotes occurs via the combined action of GS and GOGAT. GDH has also been shown to play a role in ammonia uptake under certain conditions. These enzymes are vital to metabolism since production of glutamine by GS and glutamate by GOGAT or GDH is used directly or indirectly in the formation of all macromolecules (for reviews, see [9, 12]). In most cases, levels of all three enzymes are regulated in some manner by the nitrogen source used for growth [9].

The regulation of ammonia assimilation has been studied in a number of bacteria, with the enteric

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organisms receiving most of the attention [9]. Nitrogen metabolism studies in members of the genus *Bacillus* have revealed regulatory mechanisms that appear to be unique to these bacteria (reviewed in [14]). While GS, GOGAT, and GDH from thermophilic *Bacillus* species have been identified, little is known about their regulation. We report the first study examining regulation of these enzymes in a thermophilic *Bacillus* of marine origin. In addition, we have partially purified GS from FE-1 and have examined some of its properties.

Materials and Methods

Strain and media. *Bacillus* sp. FE-1 was a gift from Dennis Bazylinski (Virginia Polytechnic Institute and State University). The organism was maintained aerobically on tryptone-base blood agar plates at 65°C; storage was at 4°C. Minimal media for growth contained 81.8% Tris-Salts medium [3], 9.1% (v/v) M1 salts, and 0.045% (w/v) casein amino acids (CAA). M1 salts consisted of (all w/v) 1.95% NaCl, 1.26% $MgCl_2$, 0.32% K_2SO_4, 0.18% $CaCl_2$, 0.055% KCl, 0.016% $NaHCO_3$, 0.012% $FeCl_3$, 0.01% sodium citrate, 0.008% KBr, 0.0024% NaF, and 0.0022% boric acid. M1 salts were sterilized by filtration and stored in the dark. Nitrogen source supplements were NH_4Cl, L-glutamate, L-glutamine, and KNO_3, all at a final concentration of 0.36% (w/v).

Conditions of Growth. Cultures were grown in 22 ml of minimal medium in a sidearm flask at 65°C in a hot-air rotatory shaker set at 200 rpm. Overnight growth in the same medium as the experimental growth condition was used to inoculate cultures. Growth was measured turbidimetrically with a Klett-Summerson colorimeter equipped with a No. 54 filter. Cells were harvested at one generation time prior to stationary phase. For anaerobic growth, cultures were grown in minimal medium supplemented with KNO_3 and treated as described by Gocke et al. [5], with the exception that inoculated tubes (10 ml) were transferred to an anaerobic jar, which was placed into a 65°C incubator.

Preparation of crude extracts. Cells used for the measurement of enzyme activities were harvested by centrifugation at 18,000 *g* for 5 min at 4°C, washed twice with cold 50 mM Tris, pH 7.5, 150 mM KCl, and then frozen at −20°C. Extracts were prepared by suspending cells in either IMED buffer [4] for GS assays, or 50 mM Tris · HCl, pH 7.5, mM β-mercaptoethanol, and 1 mM Na_2EDTA, for the assay of GOGAT and GDH, and disrupted by two passages through an Aminco french pressure cell (20,000 lb/in^2). Debris was removed by centrifugation for 10 min at 14,000 *g*, 4°C.

Enzyme assays. Unless otherwise noted, GS activity was measured with the Mn^{2+}-dependent reverse transferase assay described previously [4], with the omission of hexadecyltrimethylammonium bromide. The Mn^{2+}-dependent biosynthetic activity was measured according to Wedler et al. [19], monitoring phosphate release. All assays were done for 5 min at 65°C, and controls were included to account for nonspecific ATPase and hydroxamate synthesis. GOGAT [16] and GDH [11] activities were measured spectrophotometrically at 65°C with NADPH as cofactor.

Enzyme activities were measured shortly after preparing cell extracts. Protein was determined spectrophotometrically [7].

Preparation of GS from strain FE-1. Partial purification of GS was done by a modification of the procedure described for the enzyme from *B. subtilis* [2]. Eight liters of cells, grown to mid-exponential in Luria broth at 65°C, were harvested and washed three times with buffer A (50 mM Imidazole, pH 7.0, 0.2 mM Na_2EDTA, and 1 mM β-mercaptoethanol) supplemented with 250 mM KCl. The cells were suspended in buffer A containing 2 mM phenylmethylsulfonyl fluoride (2 g cells per ml buffer) and were passed twice through a french pressure cell at 20,000 lb/in^2. Cell debris was removed by centrifugation at 18,000 *g* for 10 min, and streptomycin sulfate was added to the supernatant fraction to a final concentration of 1%. After being stirred for 30 min, the preparation was cleared by centrifugation, resulting in an extract with a protein concentration greater than 40 mg/ml. Following the addition of ATP, sodium glutamate, and $MnCl_2$ to a final concentration of 10, 30, and 10 mM respectively, the preparation was incubated at 80°C for 5 min. Dropwise addition of 1 M acetic acid (40 mM final concentration) to the prepration resulted in the appearance of a cloudy white precipitate, which was removed by centrifugation at 14,000 *g* for 10 min. The supernatant fraction was then applied to a 2.5 × 100 cm Sephacryl S-300 (Pharmacia) gel filtration column previously equilibrated with buffer A. Fractions containing GS activity were pooled and applied to a 1.5 × 15 cm DEAE-sephacel (Pharmacia) column equilibrated with buffer A. Proteins were eluted with a 120-ml linear NaCl gradient (0 to 1 M) prepared in buffer A. GS-containing fractions were pooled, polyethyleneglycol-concentrated, and dialyzed (twice) against 1000 volumes of buffer A. All procedures were carried out at 4°C.

Materials. Electrophoretically pure glutamine was from Calbiochem (La Jolla, California). All reagents used were of the highest purity available.

Results

Growth of FE-1 in minimal medium. Strain FE-1 was grown aerobically in minimal medium supplemented with glutamine, glutamate, ammonia, or nitrate as described in Methods. We found that FE-1, like most thermophilic *Bacillus* spp., required some medium component that could be satisfied by the addition of low concentrations of CAA; we did not characterize its requirements further. Growth occurred in the absence of added nitrogen sources, with a generation time of 60 min. The addition of individual nitrogen sources to the CAA-containing minimal medium did not significantly affect generation time. However, a two- to three-fold higher cell density was reached with nitrogen source addition, indicating that the unsupplemented medium was limited for nitrogen. Under strict anaerobic conditions growth occurred with a generation time of 100 min, and, as described previously, FE-1 would not grow anaerobically unless nitrate was present [5].

Regulation of ammonia assimilation enzymes. We assayed crude extracts of FE-1 cultures grown in the presence of various nitrogen sources (Table 1).

Table 1. Levels of ammonia assimilation enzymes in *Bacillus* sp. FE-1 grown in the presence of various nitrogen sources[a]

Nitrogen source	Specific activity (nmoles min^{-1} mg $protein^{-1}$)		
	GS	GOGAT	GDH
Glutamine	1.5	53	26
NH_4Cl	1.6	78	29
Glutamate	11	39	23
KNO_3 (aerobic)	10	82	18
KNO_3(anaerobic)	8.0	62	24
No addition	15	96	25

[a] Enzyme levels were measured in FE-1 grown to mid-exponential phase in minimal medium containing the indicated nitrogen source. Results are the averages of duplicate analyses of extracts prepared from at least three separate sets of cultures. The variation between the result of an individual assay and the final average was less than ±25%.

GS levels were found to range 10-fold, being lowest when glutamine or ammonia was added to the CAA-minimal medium; the other nitrogen source supplements yielded high GS levels. This pattern of enzyme regulation is similar to that observed in *Bacillus licheniformis* [17] and differs from that observed in *B. subtilis*, which yields partially derepressed GS levels when ammonia is the sole nitrogen source [15].

FOR GOGAT, levels were lowest when glutamate was present, being 2.5-fold lower than the levels found in the absence of added nitrogen compounds. This small variation differs from other *Bacillus* spp., e.g., *B. licheniformis*, which was found to have GOGAT levels that were regulated 30-fold [17]. However, glutamate or an amino acid whose catabolism produces glutamate has been shown to affect GOGAT in most *Bacillus* spp. examined [14], and the presence of CAA in the medium may have played a role in enzyme expression.

GDH activity in crude extracts of FE-1 was found to require NADPH as cofactor, and NADH could not be used for enzymatic activity. It has been shown that the NADH-dependent GDH from several *Bacillus* spp. is cold-labile [6], requiring the preparation of extracts at room temperature for detection of activity. Crude extracts from FE-1 prepared at room temperature did not contain detectable NADH-specific activity, when assayed in either direction. Production of the NADPH-dependent GDH did not vary significantly with the nitrogen source used for growth (Table 1). This lack of regulation was also reported for the *Bacillus stearothermophilus* GDH [13]. As was noted above for GOGAT, the presence of CAA in the medium may also influence GDH, since enzyme levels in other members of the genus are lowest when glutamate is present in the medium [14]. On the other hand, low GDH activities have been correlated with the presence of glucose as the carbon source, an effect observed in *B. subtilis* (see Discussion).

For all enzymes, specific activities found for nitrate-grown cells were similar under both aerobic and anaerobic growth conditions (Table 1). Thus, under the conditions of growth used in our study, there appears to be no relationship between oxygen tension and the levels of ammonia assimilation enzymes.

Properties of GS. Because GS from a thermophilic marine *Bacillus* sp. has not been studied in any detail, we examined some properties of the partially purified enzyme prepared from crude extracts of FE-1. During the course of purification, we found that the enzyme could not tolerate precipitation by ammonium sulfate, and this step was omitted from the purification procedure. Treatment of the streptomycin sulfate preparation at 80°C in the presence of substrates did not have a significant effect on GS activity while effectively removing 87% of total protein. At this temperature and in the presence of protein concentrations greater than 25 mg/ml, the enzyme was stable for at least 10 min. The final GS preparation was typically 13.2-fold purified, with a 16% yield; it was judged to be approximately 30–40% homogeneous on the basis of SDS polyacrylamide gel electrophoresis. The enzyme exhibited a characteristic native molecular weight of approximately 600,000, as determined by gel filtration (not shown), and SDS-polyacrylamide gel electrophoresis indicated a subunit molecular weight of approximately 50,000, a property of dodecameric GSI enzymes [14].

As has been observed for GS from other *Bacillus* spp., the FE-1 enzyme preferred Mn^{2+} as the divalent cation for both the reverse transferase and biosynthetic activities (data not shown), and all assays measured the Mn^{2+}-dependent activity. Reverse transferase activity was stimulated by K_2SO_4, with a 10-fold increase in activity observed when included in the assay (Table 2). This effect has previously been observed for GS from *B. subtilis* [2] and has indicated the dependence of the transferse activity on the ionic environment. In addition, FE-1 GS exhibited some tolerance to NaCl, with a 29% decrease in activity in the presence of 1 *M* NaCl (Table 2).

Table 2. Effect of salt additions on GS activity from *Bacillus* sp. FE-1

	Relative specific activity[a]
No addition	100
+0.5 M NaCl	92
+1 M NaCl	71
+50 mM LiCl	93
+100 mM LiCl	90
+200 mM LiCl	67
+1 M LiCl	10
−0.4 M K_2SO_4	10

[a] The Mn^{2+}-dependent reverse transferase activity of GS from FE-1 was measured in the presence of the salts shown, as described in Methods. The standard assay included 0.4 mM K_2SO_4. Results are expressed as percentage of activity with added salt relative to the control assayed without added salt; 100% activity corresponds to a preparation having a specific activity of 2.4 μmol/min/mg protein.

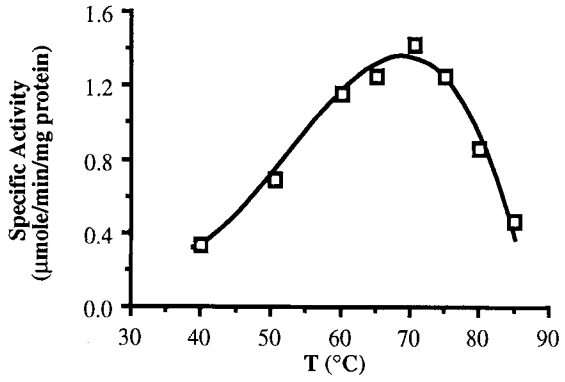

Fig. 1. Activity versus temperature profile for GS from *Bacillus* sp. FE-1. The Mn^{2+}-dependent reverse transferase activity was measured as described in Materials and Methods at the temperatures shown. Each point is the average of duplicate analysis.

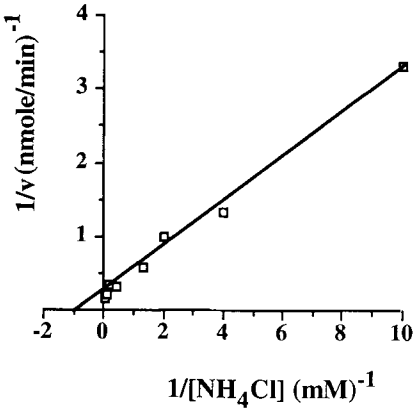

Fig. 2. Effect of NH_4Cl concentration on reaction velocity for GS from *Bacillus* sp. FE-1. Assays were done under standard conditions, the Mn^{2+}-dependent forward activity was measured as described in Materials and Methods, except that NH_4Cl concentration was varied. Each point is the average of duplicate analyses. Reactions were initiated by the addition of partially purified GS at a final concentration of 32 μg/ml.

On the other hand, only 10% of enzyme activity remained when 1 M LiCl was added to the assay mixture (Table 2). These properties differed from the *B. subtilis* enzyme, which is inhibited approximately 50% in the presence of 1 M NaCl or 1 M LiCl ([1] and Schreier, unpublished observation). Optimal reverse transferase activity was found between 65° and 75°C (Fig. 1); the Arrhenius plot derived from the data shown in Fig. 1 exhibited a sudden drop above 75°C (not shown). Such a drop may be due to enzyme inactivation, although other explanations are possible.

Hyperbolic kinetics were observed for ammonia, and the reciprocal plot (Fig. 2) indicated an apparent K_M of 1.0 mM for that substrate. This value is similar to the K_M for ammonia for the GS from *B. stearothermophilus* [18] and suggests that GS in strain FE-1 plays a major role in ammonia assimilation under nitrogen-limiting conditions.

Discussion

Members of the genus *Bacillus* comprise at least three groups distinguishable on the basis of routes used for ammonia assimilation [14]. The presence of GDH, GOGAT, and GS in FE-1 includes this organism in the group represented by *B. licheniformis*, which produce all three enzymes. Unlike *B. licheniformis*, however, GDH activity in FE-1 did not vary with the nitrogen source used for growth, and in this respect the organism appears to resemble *B. subtilis*, a member of the group that utilizes the GS/GOGAT pathway for assimilation and glutamate production during growth. In these organisms, GDH activity is barely detectable when glucose is present in the medium and serves in a catabolic capacity when glutamate is the carbon source [8]. Thus, even though CAA may provide a source of glutamate under all cultural conditions examined for FE-1, the presence of glucose could explain the absence of variations in GDH levels. This apparent absence of regulation as well as the fact that GS levels were elevated under nitrogen-limiting conditions suggests that FE-1 utilizes the GS/GOGAT pathway for assimilation under these conditions. Furthermore, the high affinity for ammonia determined for GS makes it likely that this enzyme is predominant in ammonia

assimilation in FE-1, especially since the ammonia concentration in pore fluids from where FE-1 was isolated was measured at 3–4 mM [10].

The regulation of GS levels obtained for FE-1 is similar to that observed for other members of the genus. In *B. subtilis*, regulation has been found to occur primarily at the level of transcription, and there is no evidence for post-translational control in any *Bacillus* spp. [4, 14, 15]. However, two kinetically different GS species having distinct regulatory properties have been described for the extreme thermophile *Bacillus caldolyticus* [19]. While we cannot rule out this possibility for FE-1, the substrate saturation profile determined for ammonia was similar to the *B. stearothermophilus* enzyme [18] and did not yield biphasic kinetic patterns characteristic of the enzymes from *B. caldolyticus* [19]. Furthermore, preliminary Southern analyses have indicated the presence of only one structural gene for GS in this organism (Schreier, unpublished).

The results presented here, to our knowledge, are the first describing the ammonia assimilatory enzymes in a thermophilic marine *Bacillus* sp. Differences in the regulation of GS, GOGAT, and GDH in FE-1 as well as the properties of GS compared with other *Bacillus* sp. may reflect, in part, the manner by which the organism has adapted to its environment, which is rich in ammonia. Further studies on these pathways will allow us to describe the role these enzymes, and others, play in nitrogen metabolism in this organism. Moreover, a comparison of the mechanisms involved in the control of GS expression in FE-1 may shed light on transcriptional control in a thermophilic organism.

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