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Cloning and Characterization of a Glutamine Transport Operon of *Bacillus stearothermophilus* NUB36: Effect of Temperature on Regulation of Transcription

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We cloned and sequenced a fragment of the *Bacillus stearothermophilus* NUB36 chromosome that contains two open reading frames (ORFs) whose products were detected only in cells of cultures grown in complex medium at high temperature. The nucleotide sequence of the two ORFs exhibited significant identity to the sequence of the *glnQ* and *glnH* loci of the glutamine transport system in enteric bacteria. In addition, growth response to glutamine, sensitivity to the toxic glutamine analog γ-L-glutamylhydrazide, and glutamine transport assays with parental strain NUB3621 and mutant strain NUB36500, in which the ORF1 coding segment in the chromosome was interrupted with the *cat* gene, demonstrated that *glnQ* and *glnH* encode proteins that are active in the glutamine transport system in *B. stearothermophilus*. The inferred promoter for the *glnQH* operon exhibited a low homology to the −35 and −10 regions of the consensus promoter sequences of *Bacillus subtilis* and *Escherichia coli* genes. In addition, the inferred promoter for the *glnQH* operon also exhibited a low homology with the consensus promoter sequence deduced from the sequences of the promoters of nine different genes from *B. stearothermophilus*. Transcription of the *glnQH* operon was activated in a nitrogen-rich medium at high temperature and inhibited under the same conditions at low temperature. Transcription of the *glnQH* operon was partially activated in a nitrogen-poor medium at low temperature. The region upstream from *glnQ* contains sequences that have a low homology with the nitrogen regulator I-binding sequences and the nitrogen-regulated promoters of enteric bacteria. The effect of temperature on the regulation of the *glnQH* operon is discussed.

Coultate and Sundaram (5) reported that the molar growth yield (49) of a prototrophic strain of *Bacillus stearothermophilus* progressively decreases at higher growth temperatures. The molar growth yields of this strain appear to be inversely related to the growth rate and high temperature. At higher temperatures, a larger proportion of the glucose carbon remains incompletely utilized in the medium, mostly as acetate, and energy production is uncoupled from respiration (5). Using another strain of *B. stearothermophilus*, de Vrij et al. (7) found that the efficiency of energy transduction was decreased at high temperature. Other temperature-induced alterations of metabolic activities were also reported during growth of other strains of *B. stearothermophilus*. These include use of alternative catabolic pathways (19) and changes in catabolic repression mechanisms (42), de novo synthesis of enzymes (42), and transport of amino acids (7, 42). In addition, cultures of *B. stearothermophilus* grown at or higher than their optimal temperature for growth generally reached cell densities that were lower than the cell densities of cultures grown under the same conditions at lower temperatures (56, 62). Thus, temperature may induce a reversible shift of metabolic activities in some strains of *B. stearothermophilus*.

We reported that cells of *B. stearothermophilus* NUB36 subjected to a temperature shift-up or shift-down of 15°C within the normal temperature range of growth enter a transient adaptation period of apparent metabolic instability before exponential growth at the new temperature (59). The de novo synthesis of a unique set of cellular proteins coincides with the transient adaptation period. The synthesis of at least four new proteins (proteins H1 through H4) was detected after a shift to a high temperature, and the synthesis of at least five new proteins (proteins L1 through L5) was detected after a shift to a low temperature. To determine whether the H proteins were active in the physiology of growth at high temperature, we cloned and characterized the gene that encodes membrane protein H2. We report here the findings of this study.

MATERIALS AND METHODS

Bacterial strains and plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *B. stearothermophilus* cultures were grown in modified Luria-Bertani (LB) medium (4), minimal glucose (MG) medium (4), and MG medium supplemented with 0.1% Bacto-Casamino Acids (Difco Laboratories, Detroit, Mich.) (MGCA medium). MG medium that did not contain NH_4NO_3 and nitrilotriacetate (nitrogen-free MG medium) was used in some experiments. Chloramphenicol and tetracycline were used at 10 and 5 μg/ml, respectively. When required, MG medium was supplemented with 20 μg of L-methionine per ml.

Cells from an overnight LB plate (11 to 14 h at 60°C) were used to inoculate a 300-ml, triple-baffled shake flask containing 20 ml of medium, and the culture was grown for 2 h (LB and MGCA media) or 3 h (MG medium and LB medium containing chloramphenicol and tetracycline) at the appropriate temperature (starter culture).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli JM109	Host for all in vitro-generated recombinant plasmids	Our collection (60)
B. subtilis BR151	Host for construction of pIF711-H*cat*C2.7	Our collection (61)
B. stearothermophilus		
NUB3621	Hsr$^-$ Hsm$^-$ Rif^r	4
NUB36105	*rfm-1 met-2*	55
NUB36500[b]	NUB3621 *glnQ*::*cat*	This study
Plasmids		
pGEM-7Zf(+)	Ap^r; *lacZ'*-with multiple cloning sites	Promega Corp.
pLW05	Cm^r	58
pTHT15	Tc^r	58
pLW06	Tc^r; 0.5-kb *Eco*RI fragment deleted from pTHT15	This study
pIF710	Ap^r; 1.4-kb *Eco*RI-*Ava*I fragment was deleted from pBR322 and replaced with synthetic polylinker	I. Fotheringham
pIF711	Ap^r Tc^r; pIF710 with pLW06 inserted into *Eco*RI site	I. Fotheringham
pH2-E2.0	Ap^r; pGEM-7Zf with 2.0-kb *Eco*RI chromosomal DNA fragment in *Eco*RI site	This study
pH2-C2.5	Ap^r; pGEM-7Z with 2.5-kb *Cla*I chromosomal DNA fragment in *Cla*I site	This study
pH2-HC1.3	Ap^r; pGEM-7Zf with *Hin*dIII fragment (contains 1.3-kb *Hin*dIII-*Cla*I fragment) from pH2-C2.5 in *Hin*dIII site	This study
pH2-H*cat*C2.7	Ap^r Cm^r, recessed 3' termini of 1.4-kb *Eco*RI fragment (contains *cat* gene) from pLW05 were filled in by using DNA polymerase I large fragment (Klenow), and engineered fragment was inserted into *Bal*I site of 1.3-kb *Hin*dIII-*Cla*I segment of *Hin*dIII fragment of pH2-HC1.3	This study
pIF711-H*cat*C2.7	Ap^r Cm^r; *Hin*dIII fragment (contains 1.3-kb *Hin*dIII-*Cla*I with *cat* gene in *Bal*I site) from pH2-H*cat*C2.7 inserted into *Hin*dIII site of pIF711	This study

[a] Abbreviations: Hsr, host-specific restriction; hsm, host-specific modification; Rif^r, rifampin resistance; Cm^r, resistance to chloramphenicol; Tc^r, resistance to tetracycline; Ap^r, resistance to ampicillin.

[b] *cat* inserted into the *glnQH* gene by a double-crossover recombination event.

To prepare cultures for protoplast transformation, for [^{35}S]methionine labeling experiments, and for glutamine transport assays, cells from a starter culture were used to inoculate a 300-ml, triple-baffled nephelometer flask containing 20 ml of LB or MGCA medium. Cultures were grown at 60°C (transformation), at 65°C (pulse-labeling), or at 45 and 65°C (glutamine transport). To prepare cultures for the isolation of chromosomal DNA and cellular RNA, cells from a starter culture were used to inoculate a 500-ml, triple-baffled shake flask containing 100 ml of LB medium (DNA) or MG, MGCA, or LB medium (RNA). The cultures were grown at 60°C (DNA) or at 45 and 65°C (RNA). For the isolation of protein H2, cells from a starter culture were used to inoculate a 2,800-ml, triple-baffled Fernbach flask containing 500 ml of MGCA medium. The culture was grown at 65°C.

Starter cultures were grown at the same temperature as experimental cultures. The starting cell density in each experimental culture was 2×10^7 to 3×10^7 CFU/ml, and all the cultures were grown to the mid-exponential phase of growth (1×10^8 to 2×10^8 CFU/ml).

Bacillus subtilis cultures were grown in Bacto-Penassay broth and Bacto-Tryptose Blood Agar Base (Difco). Chloramphenicol and tetracycline were used at 5 μg/ml. *Escherichia coli* cultures were grown in LB medium (6). Solid medium contained 1.5% agar and was supplemented with 50 μg of ampicillin per ml, 0.05 mM thiamine, and 0.4 mM methionine as required. For the detection of Lac$^+$ colonies, 0.1 ml of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and 0.1 ml of 10 mM isopropyl-β-D-thiogalactopyranoside were spread on the surface of an LB plate and dried for 30 min at 37°C with the cover ajar. *B. subtilis* and *E. coli* cultures were grown at 37°C.

Cultures were grown on a gyratory shaker (190 to 200 rpm), and growth was monitored by using a Klett-Summerson colorimeter with a no. 42 blue filter (*B. stearothermophilus*) or a Bausch and Lomb Spectronic 20 colorimeter at 500 nm (*B. subtilis*) or 550 nm (*E. coli*).

Isolation of DNA. The large-scale isolation of plasmid DNA from *E. coli* was by the alkaline lysis procedure described by Sambrook et al. (46) followed by purification by precipitation with polyethylene glycol (3). The isolation of plasmid DNA from small-scale cultures of *B. stearothermophilus* and *E. coli* and of *B. subtilis* was as described by Sambrook et al. (46) and Rodriguez and Tait (43), respectively. Plasmid DNA for sequencing, isolated from small-scale cultures, was purified by a modification of the procedure described by Sambrook et al. (46). The DNA pellet was dissolved in 90 μl of 10 mM Tris HCl–1 mM EDTA buffer, pH 8.0 [TE (pH 8.0)], containing RNase (20 μg/ml). The solution was incubated for 30 min at 37°C. Fifty microliters of 8 M ammonium acetate was added, and the solution was held for 5 min in ice. The precipitate was removed by centrifugation at 12,000 × *g* for 10 min at 5°C. The supernatant fluids were transferred to a fresh microcentrifuge tube, mixed with 90 μl of isopropanol, and held for 30 min in ice. The precipitated plasmid DNA was recovered by centrifugation, and the pellet was dried by using a SpeedVac concentrator (Savant Instruments, Inc., Farmingdale, N.Y.). The pellet was suspended in 80 μl of 5 M NaCl and mixed with 30 μl of a 30% solution of polyethylene glycol 6000 in 1.5 M NaCl, and the mixture was held for 30 min in ice to precipitate the DNA. The DNA was collected by centrifugation, and the pellet was suspended in 100 μl of TE (pH 8.0). The DNA solution was extracted with phenol-chloroform-isopentanol (50:48:2). The aqueous layer was transferred to a fresh microcentrifuge tube, 10 μl of 3 M sodium acetate was added, and the DNA was precipitated with 300 μl of 100% ethanol. The precipitate was collected by centrifugation, washed with

200 μl of 70% ethanol, and dried. The plasmid DNA was dissolved in TE (pH 8.0).

Chromosomal DNA was isolated from *B. stearothermophilus* by the procedure described by Rodriguez and Tait (43) for the isolation of chromosomal DNA from *B. subtilis* except that sucrose in the SET buffer was replaced with 10% lactose and the cells were incubated with lysozyme for 10 min at 60°C.

Isolation of RNA. RNA was isolated from *E. coli* by using the procedure described by Duvall et al. (10) and from *B. stearothermophilus* by a modification of this procedure. *B. stearothermophilus* cells were collected by centrifugation and converted to protoplasts (4). The protoplasts were collected by centrifugation (58), suspended in 1 ml of 10 mM Tris HCl buffer (pH 8) containing 1,000 U of RNasin RNase inhibitor per ml, and held for 10 min in ice. The lysed protoplasts were extracted twice with phenol and once with chloroform, and the RNA was precipitated with ethanol. RNA was dissolved in 100 μl of 40 mM Tris HCl buffer (pH 7.9)–10 mM NaCl–6 mM $MgCl_2$–0.1 mM $CaCl_2$. The RNA solution was incubated with 100 U of RQ1 DNase per ml for 1 h at 37°C, extracted with phenol-chloroform-isopentanol, and precipitated with ethanol. The RNA pellet was dissolved in water, quantitated by A_{260}, and stored at −70°C.

Transformation. Protoplasts of *B. stearothermophilus* were transformed as described by Wu and Welker (58), and transformation of *E. coli* was accomplished by the procedure described by Perbal (40). Competent *B. subtilis* cells were prepared as described by Bott and Wilson (2), and transformation was as described by Sullivan et al. (51).

DNA hybridizations. Restriction fragments that were electrophoresed through 1% agarose slab gels were Southern blotted (50) onto nitrocellulose membranes (MicronSep; Micron Separations Inc., Westborough, Mass.). DNA-DNA hybridizations were done as described by Sambrook et al. (46). For ^{32}P-labeled oligonucleotide probes, hybridization was at 45°C (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), and the filters were washed at 62°C (6× SSC), and for ^{32}P-labeled DNA fragments, the hybridization and washing conditions were as described by Sambrook et al. (46). For colony hybridizations, the procedures described by Sambrook et al. (46) for transferring colonies from a master plate to a nitrocellulose filter (BA85; Schleicher & Schuell, Keene, N.H.) (method 1, p. 1.93) and for hybridization with labeled probes (method 1, p. 1.98) were used.

Kodak X-Omat film with an intensifying screen was used for autoradiography at −70°C.

RNA hybridizations. RNA (15 μg per well) was fractionated by electrophoresis in a 1.3% agarose–1.1 M formaldehyde gel as described by Meinkoth and Wahl (30). The RNA was transferred to a nitrocellulose filter and probed with labeled DNA as described by Meinkoth and Wahl (30). The size of the mRNA was estimated from the mobilities of the mRNA relative to those of the RNAs in a 0.24- to 9.5-kb RNA ladder (GIBCO BRL, Research Products Div., Gaithersburg, Md.).

Radiochemical labeling of oligonucleotides and DNA fragments. Oligonucleotides were synthesized by the Northwestern University Biotechnology Research Service Facility by using an Applied Biosystems 380B nucleic acid synthesizer. The 5′ end of an oligonucleotide was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (46). Plasmid DNAs were digested with the appropriate restriction enzymes, and DNA fragments were resolved and recovered from low-melting-temperature agarose gels as described by Sambrook et al. (46). Labeled DNA fragments, plasmid DNA, and oligonucleotides were purified by precipitation with ethanol as described by Sambrook et al. (46). DNA fragments and plasmid DNA were labeled with [γ-^{32}P]dATP by nick translation (46).

Protein electrophoresis. Two-dimensional gel electrophoresis was carried out as described previously (59), and one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried as described by Laemmli and Favre (25). To identify proteins and molecular weight protein standards separated by SDS-polyacrylamide gel electrophoresis, the gels or a vertical segment of a gel were stained with Coomassie brilliant blue as described by Sambrook et al. (46).

Primer extension mapping. An oligonucleotide primer that was complementary to the sequence encoding the amino acid sequence at positions 1 to 8 of ORF1 was synthesized. Primer extension analyses were carried out as described by Sambrook et al. (46). Labeled primer (5×10^5 cpm) was mixed with 5, 10, or 20 μg of total RNA. The denatured hybridization mixture was incubated for 12 h at 40°C. The annealed hybridization mixture was incubated with 40 U of avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, Wis.) for 2 h at 42°C. Analysis of the extended products was carried out by electrophoresis with 8% polyacrylamide–urea gels.

DNA sequencing. Sequencing of DNA was by the dideoxynucleotide chain termination method (47) with [α-^{35}S]dATP. Sequencing reactions were performed with the T7 DNA polymerase sequencing system (Promega). The DNA was sequenced in both strands by the strategy of Henikoff (15). Plasmids pH2-E2.0 and pH2-C2.5 were digested with *Sph*I and *Xho*I to generate the appropriate 3′ and 5′ overhangs. The plasmids were sequentially digested with exonuclease III and S1 nuclease to generate a nested series of overlapping clones with deletions. The start points of the deletions were separated, on the average, by about 200 bp. The fragments were ligated with T4 ligase, and the deletion plasmids were amplified in *E. coli* and purified.

Amino-terminal sequencing. Cells from 1,500 ml of culture were collected by centrifugation, and cellular proteins were isolated as described by Wu and Welker (59). A cellular protein sample, isolated from 2×10^{10} to 4×10^{10} cells, was loaded onto a preparative SDS-polyacrylamide gel (12 by 14 by 1.5 cm), and the proteins were resolved by one-dimensional electrophoresis. A 2-cm horizontal segment of the gel that contained proteins with molecular masses between 23 and 30 kDa was excised from the gel and macerated in 0.05% SDS as described by Kalkkinen (20). The gel was removed by centrifugation at 3,000 × *g* for 10 min followed by filtration through 0.45-μm-pore-size membrane filters. Filtered protein solutions from eight gels were combined, and the proteins were concentrated and washed with 100 ml of 0.05% SDS by use of an Ultrafiltration cell (Amicon Div., W. G. Grace and Co., Beverly, Mass.) with a PM10 Diaflo ultrafilter. The protein solution was further concentrated by using a Centricon-10 microconcentrator (Amicon). The proteins were precipitated with 3 volumes of cold (−20°C) acetone. The precipitate was collected by centrifugation at 12,000 × *g* for 20 min and dissolved in isoelectric focusing sample solution (59). A sample of ^{35}S-labeled cellular proteins (59) was added to the unlabeled cellular protein sample to make identification of protein H2 easier. The proteins were resolved by two-dimensional gel electrophoresis. The proteins were electroblotted onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.) by using the procedure described by Matsudaria (29). The H2 protein

spot was cut out of each of seven of these membranes, and the NH_2-terminal sequence was determined by the Northwestern University Biotechnology Research Service Facility by using an Applied Biosystems 477A protein sequencer.

In vitro transcription-translation. The protein products of the cloned inserts in plasmids pH2-C2.5, pH2-HCl.3, and pH2-E2.0 were identified by using a prokaryotic DNA-directed translation kit from Amersham Corp. (Arlington Heights, Ill.). Proteins produced from the plasmids (2.5 μg per reaction) were labeled with [L-^{35}S]methionine according to the instructions of the manufacturer, with a 30-min incubation and a 5-min (cold) methionine chase reaction. Samples containing 6×10^4 cpm were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis. Molecular mass protein standards (10 to 100 kDa) from BioRad Laboratories, Inc. (Richmond, Calif.) were used as size standards.

Isolation of *cat* gene insertion mutants. A mutant allele of ORF1 was constructed in vitro by inserting a 1.4-kb DNA fragment that contains the *cat* gene from pLW05 into the *Bal*I site of ORF1. The engineered plasmid (pIF711-H*cat*C2.7) was used to transform protoplasts of *B. stearothermophilus* NUB3621 to chloramphenicol resistance (Chl^r) and tetracycline resistance (Tet^r). Several Chl^r Tet^r transformants were purified by single-colony isolations, and the presence of pIF711-H*cat*C2.7 in each transformant was verified by restriction analyses. A starter culture of NUB 3621 (pIF711-H*cat*C2.7), grown in LB medium that contained chloramphenicol and tetracycline at 50°C, was diluted to a cell density of approximately 10^7 CFU/ml, and a sample (0.1 ml) was spread onto the surface of a plate containing LB medium with chloramphenicol (LBC). The plate was incubated overnight at 50°C. The cells from the master plate were velveteen replicated onto a fresh LBC plate, and the plate was incubated for 12 h at 65°C. The growth cycle was repeated nine more times. Cells on the surface of the LBC plate were suspended in 5 ml of LBC medium, washed, and diluted in the same medium. Samples were plated on LBC plates, and the plates were incubated overnight at 50°C. Colonies were transferred to the same medium with sterile toothpicks to form a master plate. After incubation at 50°C, the master plate was replicated onto the appropriate media to score for the tetracycline-sensitive phenotype. Colony hybridization, using labeled pIF711, a 0.9-kb *Cla*I-*Eco*RI fragment, and a 1.1-kb *Eco*RI-*Cla*I fragment as the probes, was used to verify the absence of plasmid DNA and the configuration of the insert in the Chl^r Tet^s isolates. Approximately 70 to 80% of the Chl^r Tet^s isolates did not contain vector DNA sequences, and 8 to 12% had the *cat* gene inserted into ORF1 by a double-crossover recombination event.

Glutamine transport assays. The transport of glutamine was measured by using a modification of the procedure described by Masters and Hong (28). Exponential-phase cells grown in MGCA medium at 45 and 65°C were collected by centrifugation at 2,000 × *g* for 5 min at room temperature. The cells were washed three times with nitrogen-free MG medium and suspended to a cell density of 1×10^9 to 2×10^9 CFU/ml in the same medium that contained 20 μg of chloramphenicol per ml. L-[G-^{3}H]glutamine (35 Ci/mmol) (Amersham) was added to a final concentration of 0.5 mM. The cells were incubated for 5 min with shaking at 45 or 65°C. The cells were collected on a 0.45-μm-pore-size membrane filter and dried, and the radioactivity was measured with a liquid scintillation spectrometer.

Materials and routine procedures. Restriction endonucleases were obtained from several sources (GIBCO BRL; Promega; and New England BioLabs, Beverly, Mass.) and were used as specified by the manufacturers. RNasin RNase inhibitor, RQ1 DNase, T7 Polymerase Sequencing System, and exonuclease III were obtained from Promega; RNase was obtained from Sigma Chemical Corp. (St. Louis, Mo.); S1 nuclease and T4 DNA ligase were obtained from GIBCO BRL; and DNA polymerase I large fragment (Klenow) was obtained from Insertional Biotechnologies, Inc., New Haven, Conn. [α-^{32}P]dATP (3,000 Ci/mmol) and [γ^{32}P]ATP (4,000 Ci/mmol) were obtained from ICN Biomedicals, Inc., Costa Mesa, Calif.; [α-^{35}S]dATP (1,094 Ci/mmol) was from Dupont, NEN Research Products, Boston, Mass.; and L-[G-^{3}H]glutamine (35 Ci/mmol) was from Amersham. Restriction analyses and cloning techniques were as described by Sambrook et al. (46). Agarose gel electrophoresis was performed as described by Wu and Welker (58). For the growth response experiments, approximately 50 filter paper disks (Schleicher & Schuell no. 740-E; 1/4-in. [ca. 0.63-cm] diameter) were placed in a sterile petri dish containing 10 ml of L-glutamine or γ-L-glutamylhydrazide (γ-GH; 100 μg/ml) (ICN Biomedicals), 20 μg of L-histidine per ml, or 40 μg of L-arginine per ml. Excess liquid was removed, and the disks were dried in a forced-air incubator at 50 to 60°C.

Computer analysis of DNA sequences. DNA sequence analysis was performed by using the University of Wisconsin Genetics Computing Group DNA Analysis program, and sequences were compared by using the TFastA program (39) with the most recent version of the DNA and protein data bases and the Stem-Loop program to find inverted repeats within a sequence.

Nucleotide sequence accession number. The ORF1-ORF2 (*glnQH*) sequence was submitted to the Genetic Sequence Data Bank (GenBank) and was assigned the accession number M61017.

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5' CAAGTTAATAAATATTATGGTGATTT 3'
   G C C G C C C C
   A         A
   G         G
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FIG. 1. Sequence of oligonucleotides synthesized from the amino acid sequence at positions 6 to 14.

RESULTS

Cloning and nucleotide sequence of the gene encoding protein H2. The strategy for identifying the *B. stearothermophilus* gene encoding the H2 protein was to use as a hybridization probe a synthetic oligonucleotide designed on the basis of a portion of the NH_2-terminal sequence of the H2 protein. Concentrated protein H2 was transferred from two-dimensional gels to polyvinylidene difluoride membranes, and 22 residues of amino acid sequence from the NH_2 terminus were obtained as described in Materials and Methods (parentheses indicate positions at which amino acid assignments were uncertain): Met Ile Tyr Phe (His) Gln Val Asn Lys Tyr Tyr Gly Asp Phe (His) Val Leu Ile Asp Ile Asn Leu.

A mixture of oligonucleotides, each 26 bases long, was synthesized from the amino acid sequence at positions 6 to 14. That the positions have more than one possible base reflects the degeneracy of the code (Fig. 1).

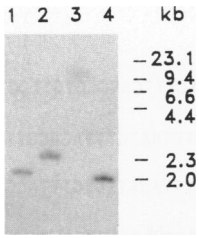

FIG. 2. Autoradiograph showing hybridization of an oligonucleotide probe to restriction endonuclease-digested chromosomal DNA. Chromosomal DNA from *B. stearothermophilus* NUB36105 *rfm-1 met-2* was digested with *Hin*dIII (lane 1), *Pst*I (lane 3), *Eco*RI (lane 4), and *Cla*I (lane 2) and hybridized to a ^{32}P-labeled oligonucleotide probe. DNA fragment sizes in kilobase pairs are shown on the right.

Chromosomal DNA from *B. stearothermophilus* NUB 36105 *rfm-1 met-2* was digested with *Hin*dIII, *Pst*I, *Eco*RI, or *Cla*I. The restriction fragments were resolved by agarose gel electrophoresis, and Southern blot hybridizations were performed by using the ^{35}P-labeled oligonucleotide as a probe. The probe hybridized with single 2.1-, 18.0-, 2.0-, and 2.5-kb fragments in the *Hin*dIII, *Pst*I, *Eco*RI, and *Cla*I digests, respectively (Fig. 2). These results indicate that there is only one copy of the gene that encodes protein H2.

Chromosomal DNA was digested with *Eco*RI, and DNA fragments of the appropriate size were eluted from an agarose gel and ligated into *Eco*RI-digested and dephosphorylated plasmid pGEM-7Zf. The ligation mixture was transformed into *E. coli* JM109. Recombinant colonies were picked and screened by in situ colony hybridization, with the oligonucleotide used as a probe. The plasmids of three clones that gave strong positive signals were isolated and mapped by restriction analyses. The plasmid in each isolate carried a 2.0-kb *Eco*RI insert that hybridized with the oligonucleotide probe. The plasmid was designated pH 2-E2.0. The 2.5-kb *Cla*I fragment was cloned by the same procedure except that the recombinant colonies were probed with a 1.1-kb *Eco*RI-*Cla*I segment of the 2.0-kb *Eco*RI fragment in plasmid pH2-E2.0. The plasmids of three clones that gave strong positive signals contained the 2.5-kb *Cla*I fragment. The plasmid was designated pH2-C2-5. A partial restriction map of the region encompassed by the two fragments is shown in Fig. 3. The 2.0-kb *Eco*RI and 2.5-kb *Cla*I fragments both contained a 1.1-kb *Eco*RI-*Cla*I segment that hybridized with the oligonucleotide probe.

The sequencing strategy is shown in Fig. 3. The nucleotide sequence of 2.23 kb of DNA was determined as described in Materials and Methods and is shown in Fig. 4.

Features of the coding region. The region sequenced contained two open reading frames (ORF) (Fig. 3 and 4). ORF1 is 726 nucleotides long, beginning with a UUG start codon at position 39 and ending with two UAA stop codons at position 765. The deduced N-terminal amino acid sequence (22 residues) is identical to that of protein H2 determined by amino acid sequencing except at positions 1 and 18. At position 1, Met (AUG) was replaced with Leu (UUG), and at position 18, Tyr was replaced with Lys in the predicted sequence. Alternative start codons GUG, UUG, and AUU are frequently used in prokaryote genes; all are single-base changes from AUG (38). Thus, an alternative reading of the first codon would account for the replacement of Met with Leu. The lack of agreement at position 18 can be explained as an error in the determination of the protein sequence. ORF1 encodes a peptide of 242 amino acids with a predicted molecular mass of 27.4 kDa. The molecular mass of the H2 protein estimated by two-dimensional gel electrophoresis was found to be 27 kDa (59). Thus, ORF1 may encode the H2 protein.

ORF2 is 786 nucleotides long, beginning with an AUG (Met) start codon at position 811 and ending with a UAA stop codon at position 1596. The start codon of ORF2 is 40 nucleotides from the last stop codon of ORF1. ORF2 encodes a peptide of 262 amino acids with a predicted molecular mass of 28.7 kDa.

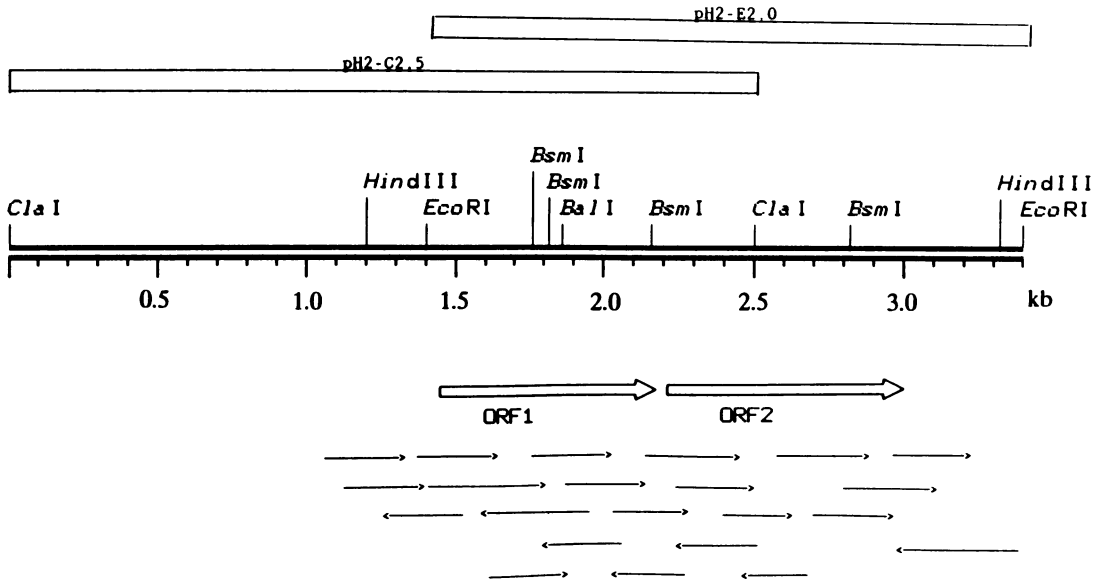

FIG. 3. Partial restriction map and sequencing strategy of the chromosomal region that contains the gene encoding protein H2. The two open bars represent the fragments cloned in pH2-C2.5 and pH2-E2.0. Relevant restriction sites are indicated. The thin arrows indicate the directions and extents of the DNA regions sequenced. The large arrows represent the lengths and positions of ORF1 and ORF2.

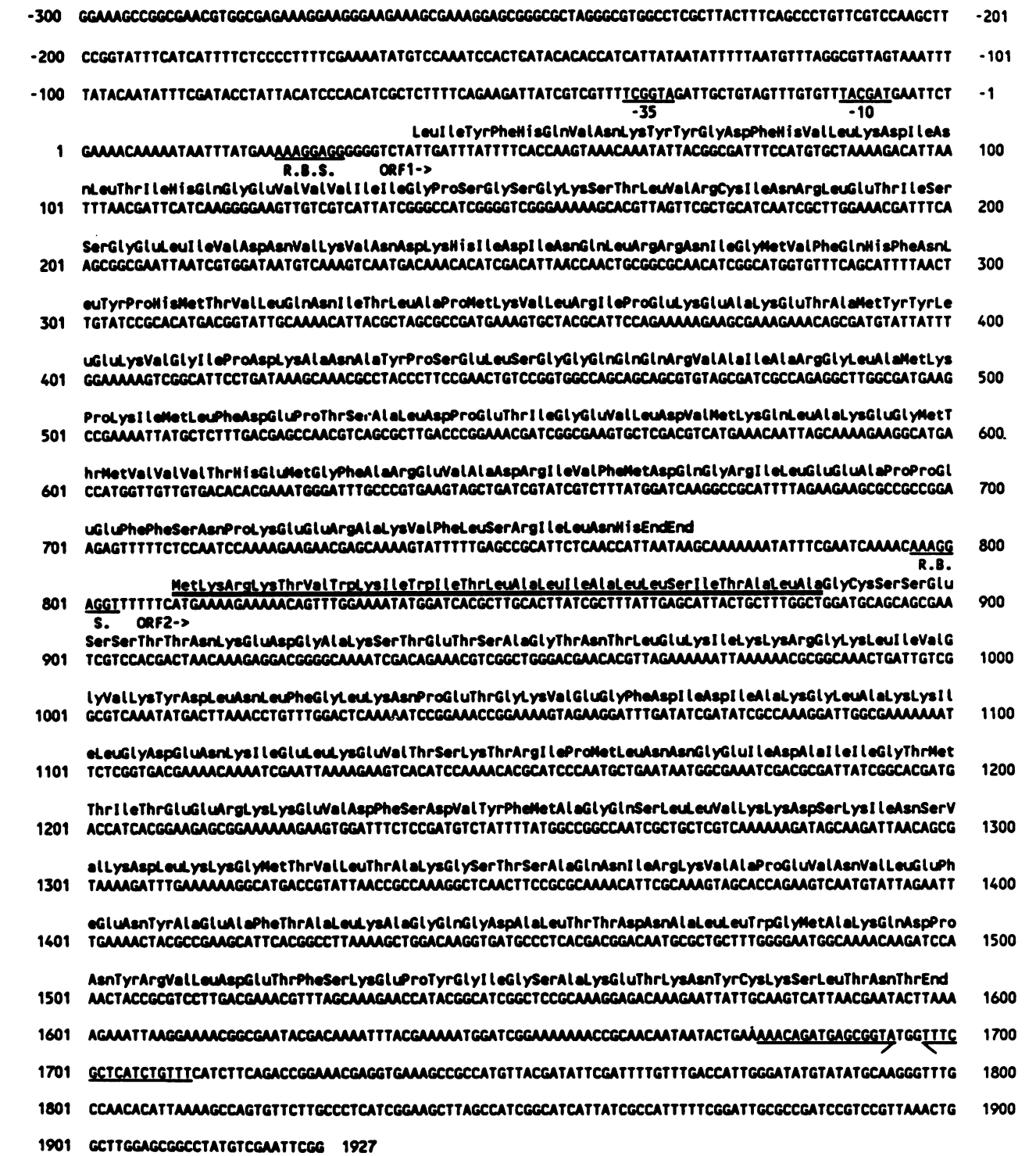

FIG. 4. Nucleotide sequence of the chromosomal region that contains the gene encoding protein H2. The likely ribosome-binding sites (R.B.S.) and promoter −35 and −10 sequences are underlined. Potential terminator sequences are underlined with arrows. The transcription start site is indicated with an asterisk and numbered as position 1. The predicted amino acid sequences of ORF1 and ORF2 are given in the three-letter code. A probable signal peptide sequence for the protein encoded by ORF2 is underlined with a heavy bar.

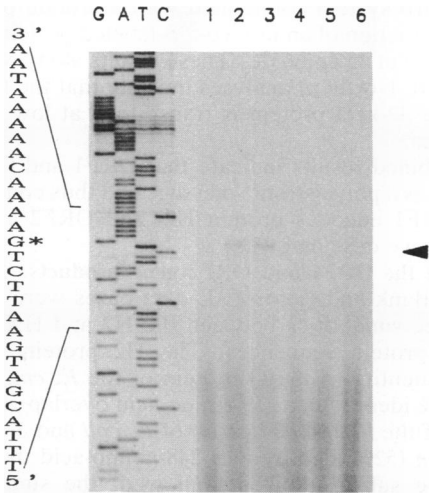

FIG. 5. Primer extension analyses of ORF1 and ORF2 transcription. RNA was isolated from exponential-phase cultures of *B. stearothermophilus* NUB36105 *rfm-1 met-2* grown in MGCA medium at 65°C (lane 1, 5, μg of RNA; lane 2, 10 μg of RNA; lane 3, 20 μg of RNA) or 45°C (lane 4, 20 μg of RNA). Controls were yeast RNA (lane 5, 20 μg) and no RNA (lane 6). Hybridization was as described in Materials and Methods. Also shown are dideoxynucleotide sequence reactions (lanes G, A, T, and C) carried out with the same primer that was used for the reverse transcriptase reaction and with plasmid pH2-HCl.3 as the template. The position of the transcription start site is indicated with an asterisk on the inferred sequence (Fig. 4). The position of the extended primer is indicated by an arrowhead.

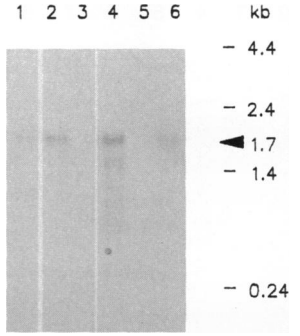

FIG. 6. Northern blot analysis of RNA from ORF1 and ORF2. RNAs from cells of *B. stearothermophilus* NUB36105 *rfm-1 met-2* grown in MG (lanes 1 and 2), MGCA (lanes 3 and 4), and LB (lanes 5 and 6) media at 45°C (lanes 1, 3, and 5) and 65°C (lanes 2, 4, and 6) were fractionated on a denaturing agarose gel, blotted to nitrocellulose filters, and probed with ^{32}P-labeled 1.1-kb *Eco*RI-*Cla*I fragment. Each lane came from the same agarose gel. Sizes of the RNA standards in kilobase pairs (KB) and the ORF1-ORF2 transcript (arrowhead) are indicated on the right.

The sequence of the region 339 bp upstream from the UUG start codon of ORF1 was determined. Three UAA stop codons are located at positions −107, −124, and −133. In vitro transcription-translation experiments, to be described later, revealed that a third ORF is located upstream of ORF1. Thus, one of the stop codons may be the termination of this ORF.

Features of the noncoding regions. Probable ribosome-binding sites are 9 and 7 bp upstream from the start codons of ORF1 and ORF2, respectively. These sites, consisting of 8 (ORF1) or 9 (ORF2) nucleotides complementary to the 3′ end of *B. stearothermophilus* 16S rRNA (9), have predicted ΔG° of −19 and −21.2 kcal/mol (1 cal = 4.164 J), respectively (54). Strong complementarity between the mRNA and the 16S rRNA was in the range (−14 to −22 kcal/mol) reported for genes of mesophilic bacilli (12). Duplex stabilities, calculated by use of the improved free-energy parameters described by Freier et al. (11), were −13.3 and −15.4 kcal/mol for the ribosome-binding sites of ORF1 and ORF2, respectively.

Primer extension analyses were used to identify the transcription start site for ORF1 and ORF2. Total RNA, isolated from cultures of *B. stearothermophilus* grown in MGCA medium at 45 and 65°C, was hybridized with the ^{32}P-labeled oligonucleotide that was complementary to the sequences encoding the amino acid sequence at positions 1 through 8 of ORF1. A single transcription start site was mapped at the position of a guanine (Fig. 5) that is 38 bp upstream from the translation start site of ORF1 (Fig. 4). Examination of the DNA sequence upstream from this site revealed the −35 (positions −38 through −33) and −10 sequences (positions −13 through −8) of the probable promoter: TCGGTA–19 bp–TACGAT. An extended product was not detected in the hybridization mixture with total RNA isolated from cells grown at 45°C.

A 16-bp-long palindrome, hyphenated by 3 bp and located 78 bp downstream from the UAA stop codon of ORF2, could act as a transcription terminator (44).

Transcription of ORF1 and ORF2. RNA isolated from cells of *B. stearothermophilus* grown in MG, MGCA, or LB medium at low and high temperatures was fractionated on a denaturing agarose gel and probed with the 1.1-kb *Eco*RI-*Cla*I fragment that contains all of ORF1 and part of ORF2 (Fig. 6) or the 0.9-kb *Cla*I-*Eco*RI fragment that contains only part of ORF2 (data not shown). A single 1.7-kb RNA species was detected in RNA isolated from cells grown in MGCA and LB media at 65°C but not in RNA from cells grown at 45°C (Fig. 6). The size of the mRNA transcript is about the size of the mRNA expected if ORF1 and ORF2 are transcribed as a polycistronic message, beginning at the transcription start site and ending at the potential transcription terminator (1.7 kb). A 1.1-kb RNA species was detected in the same RNA samples when they were probed with the 1.2-kb *Cla*I-*Hin*dIII fragment that contains the probable ORF upstream from ORF1 (data not shown). The 1.1-kb RNA species was detected in cells grown in MG, MGCA, and LB media at 45 and 65°C. These results indicate that temperature and the composition of the medium do not affect the transcription of this ORF. Thus, this ORF serves as a convenient internal control for the Northern (RNA) analyses shown in Fig. 6. These results, along with those obtained with primer extension experiments, indicate that ORF1 and ORF2 constitute an operon that is transcriptionally activated in a nitrogen-rich medium at high temperature and inhibited under the same conditions at low temperature.

The 1.7-kb mRNA species was detected in RNA isolated from cells grown in MG medium at 45 and 65°C. However, the amount of the 1.7-kb mRNA detected at 45°C was less than the amount detected at 65°C. The significance of these results will be discussed below.

In vitro transcription-translation analyses of the gene products of ORF1 and ORF2. In vitro DNA-directed protein synthesis experiments were carried out using the plasmids pH2-C2.5, pH2-HCl.3, and pH2-E2.0. The proteins were resolved by one-dimensional SDS-polyacrylamide gel electrophoresis. The results are shown in Fig. 7. A polypeptide

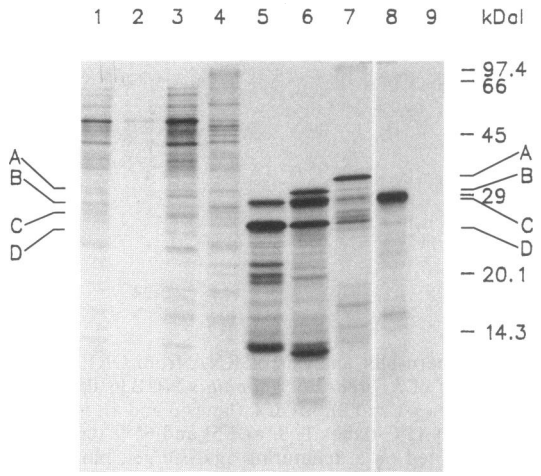

FIG. 7. In vitro transcription-translation of ORF1-ORF2. Plasmids pH2-HCl.3, pH2-E2.0, and pH2-C2.5 were used to direct polypeptide synthesis in a coupled transcription-translation system. Proteins from cells of an exponential-phase culture of *B. stearothermophilus* NUB36105 *rfm-1 met-2* grown in MGCA medium at 65°C were labeled with [^{35}S]methionine as described by Wu and Welker (59). Cellular proteins were separated into membrane and cytosol fractions (59). The ^{35}S-labeled proteins, isolated from cells (lanes 1 to 4) and from transcription-translation reaction mixtures (lanes 5 to 9), were resolved by one-dimensional SDS-polyacrylamide gel electrophoresis, and the gel was subjected to fluorography and autoradiography. Lane 1, total cellular proteins; lane 2, membrane wash fluids; lane 3, cytosol fraction; lane 4, membrane fraction; lane 5, plasmid pH2-HCl.3 containing the 1.3-kb *Hin*dIII-*Eco*RI fragment (ORF1); lane 6, plasmid pH2-E2.0 containing the 2.0-kb *Eco*RI fragment (ORF1 and ORF2); lane 7, plasmid pH2-C2.5 containing the 2.5-kb *Cla*I fragment (ORF1 and the ORF upstream from ORF1); lane 8, pAT153 vector; lane 9, DNA control. The lettered lines on the left and right sides indicate the positions of the 32-kDa protein (A), the 30-kDa protein (protein HX) (B), β-lactamase (C), and protein H2 (D). Each lane came from the same polyacrylamide gel. The positions of the molecular mass protein standards in kilodaltons are indicated on the right.

having a molecular mass of about 27 kDa (position D) was detected when the 1.3-kb *Hin*dIII-*Cla*I fragment (contains ORF1 and part of ORF2) was the template (lane 5). The 27-kDa protein was detected in the total-cellular-protein sample (lane 1) and in the membrane protein fraction (lane 4). In addition, two-dimensional gel electrophoresis analyses revealed that the 27-kDa protein comigrated with the H2 protein that was detected in an in vivo-^{35}S-labeled membrane fraction (data not shown). Protein H2 (position D) and a 30-kDa protein (position B) were detected when the 2.0-kb *Eco*RI fragment (contains ORF1 and ORF2) was the template (lane 6). No polypeptide with a molecular mass of about 30 kDa was detected in the total-cellular-protein sample (lane 1), the membrane wash fraction (lane 2), the cytosol fraction (lane 3), or the membrane protein fraction (lane 4). The experiments described in the following section revealed that the 30-kDa protein is processed in vivo, resulting in a 26-kDa protein.

A 32-kDa protein (position A) and protein H2 (position D) were detected when the 2.5-kb *Cla*I fragment (contains ORF1 and the probable ORF upstream from ORF1) was used as the template (lane 7). We were not able to locate the 32-kDa protein in the cytosol fraction (lane 3) or in the membrane fraction (lane 4). However, two-dimensional gel electrophoresis revealed that the 32-kDa protein synthesized in the in vitro system comigrated with a protein present in the cytosol fraction of an in vivo-^{35}S-labeled protein fraction of cells grown at 45 and 65°C. These results along with those obtained with Northern analyses indicate that the ORF that encodes the 32-kDa protein is transcribed at low and high temperatures.

The combined results indicate that ORF1 and ORF2 are transcribed as a polycistronic message and thus constitute an operon. ORF1 encodes protein H2, and ORF2 encodes a protein that we designate HX.

Nature of the ORF1 and ORF2 gene products. The most recent GenBank and GenEMBL data bases were searched for sequence similarities between the H2 and HX proteins and other protein sequences. The H2 protein exhibited significant identity to the Q protein of the *E. coli glnHPQ* operon (58% identity in a 239-amino-acid overlap) and to the P protein of the *hisJQMP* operon of *E. coli* and *Salmonella typhimurium* (52% identity in a 248-amino-acid overlap). If conservative substitutions are allowed, the similarity increases to 84 to 87% in this alignment. The HX protein exhibited some identity to the H protein of the *E. coli glnHPQ* operon (28% identity in a 198-amino-acid overlap), the J protein of the *S. typhimurium hisJQMP* operon (27% identity in a 215-amino-acid overlap), and the lysine-arginine-ornithine-binding protein encoded by the *S. typhimurium argT* gene (29% identity in a 154-amino-acid overlap). Conservative substitutions increase the similarity to 73 to 84%.

In enteric bacteria, the H protein is a periplasmic glutamine-binding protein and the Q protein is a membrane-bound protein. Both are essential for glutamine transport (*glnHPQ* operon) (35). Periplasmic binding proteins J (*hisJ*) and LAO (*argT*) are involved in histidine transport (17) and lysine-arginine-ornithine transport, respectively, and both proteins interact with the membrane-bound P protein encoded by *hisP* (16). Thus, the H2 protein and the HX protein may have similar functions in *B. stearothermophilus*, as do the Q and H proteins of the glutamine permease operon, the P and J proteins of the histidine permease operon, and the P protein of the histidine permease operon and the LAO protein active in arginine transport in enteric bacteria. The periplasmic glutamine-binding (protein H) and histidine-binding (protein J) proteins contain an NH_2-terminal 22-amino-acid leader peptide that is removed by the signal peptidase during transport into the periplasmic space (17, 35). The HX protein contains a characteristic NH_2-terminal 25-amino-acid signal peptide (Fig. 4; several positively charged amino acid residues followed by a hydrophobic amino acid core and a COOH-terminal alanine [45]) that may be removed during transport to the exterior side of the membrane. Thus, a processed HX protein would be composed of 237 amino acids giving a molecular mass of approximately 26 kDa. However, a 26-kDa protein was not detected in an in vivo-^{35}S-labeled cellular protein sample isolated from cells grown at 65°C that was analyzed by two-dimensional gel electrophoresis (59). The predicted pI for a processed HX protein would be approximately 8.6. Since standard two-dimensional gel electrophoresis does not resolve basic proteins (36), the basic proteins in a ^{35}S-labeled cellular protein sample isolated from cells grown at 45 and 65°C were resolved by using two-dimensional nonequilibrium pH gradient electrophoresis (37). A visual comparison of the two autoradiograms revealed a 26-kDa protein spot in cells grown at 65°C but not in cells grown at 45°C (data not shown). The results of these studies indicate that the signal peptide of the 29- to 30-kDa HX protein is removed in vivo during transport across the membrane.

To determine whether proteins H2 and HX are active in amino acid transport, the ability of prototrophic strain NUB3621 to utilize glutamine, histidine, or arginine as a sole nitrogen source was determined. Cells of cultures grown in MGCA medium were collected by centrifugation, washed once, and suspended in nitrogen-free MG medium. A sample was spread over the surface of a nitrogen-free MG plate. After being dried, filter paper disks that contained glutamine, histidine, or arginine were placed on the surface of the seeded plate. The plates were incubated for 48 h at 45 or 65°C. Growth was detected around the glutamine disk at 65°C but not at 45°C. Growth was detected around the histidine disk at 45 and 65°C, and no growth was detected around the arginine disk at either temperature. Parental strain NUB3621 did not respond to any of the other amino acids tested in a manner identical to that observed with glutamine. Thus, the utilization of glutamine as a sole nitrogen source and the transcription of the ORF1-ORF2 operon occur at 65°C but not at 45°C.

In enteric bacteria, transcription of the *glnHPQ* operon is activated by nitrogen-limiting conditions (24, 57). When *B. stearothermophilus* was grown in a nitrogen-rich medium, transcription of the ORF1-ORF2 operon was inhibited at 45°C and activated at 65°C. To determine whether cells grown in a nitrogen-rich medium at high temperature were actually starved for nitrogen, RNA was isolated from cells grown in MGCA medium supplemented with 100 μg of glutamine per ml or in MG medium supplemented with 1% Bacto-Casamino Acids. A single 1.7-kb ORF1-ORF2 transcript was detected in the RNAs of cells grown in supplemented media at 65°C (data not shown). These results indicate that the transcription of the ORF1-ORF2 operon was not inhibited under conditions of nitrogen and glutamine excess at high temperature.

Interruption of ORF1. To demonstrate that the ORF1-ORF2 operon is active in glutamine transport, we isolated a mutant (NUB36500) in which ORF1 was interrupted with a DNA segment that contained the *cat* gene. To confirm that the *cat* gene was inserted by a double-crossover recombination event into the ORF1 coding region in mutant strain NUB36500, the chromosomal DNA of this strain was cleaved with *Eco*RI, *Hin*dIII, and *Cla*I. The DNA fragments were separated by agarose gel electrophoresis, and Southern blot hybridizations were performed with the 0.9-kb *Cla*I-*Eco*RI fragment (contains 0.5 kb of ORF2) as a probe. The results are shown in Fig. 8. The probe hybridized to a 2.1-kb *Hin*dIII fragment in parental strain NUB3621 (lane 1) and to a 3.6-kb *Hin*dIII fragment in NUB36500 (lane 2). The probe hybridized to a 2.0-kb *Eco*RI fragment in the parental strain (lane 3) and to a 1.6-kb fragment in NUB36500 (lane 4). These latter results were expected, since a new *Eco*RI site had been introduced on each side of the DNA fragment that contains the *cat* gene. After digestion with *Hin*dIII and *Eco*RI, the probe hybridized to a 1.9-kb fragment in the parental strain (lane 5) and to a 1.4-kb fragment in NUB36500 (lane 6). The probe hybridized to a 0.9-kb fragment (lanes 7 and 8) and a 0.8-kb fragment (lanes 9 and 10) in both the parental strain and NUB36500 when their DNAs were digested with *Eco*RI and *Cla*I and with *Hin*dIII and *Cla*I, respectively. These hybridization analyses confirmed that the DNA fragment containing the *cat* gene had inserted into the *Bal*I site of ORF1 in the chromosome. The location of the *cat* gene in ORF1 was also verified by Southern blot analyses with a 1.1-kb *Eco*RI-*Cla*I fragment (contains ORF1 and 0.3 kb of ORF2) and a 1.4-kb *Eco*RI fragment that contained the *cat* gene as probes (data not shown).

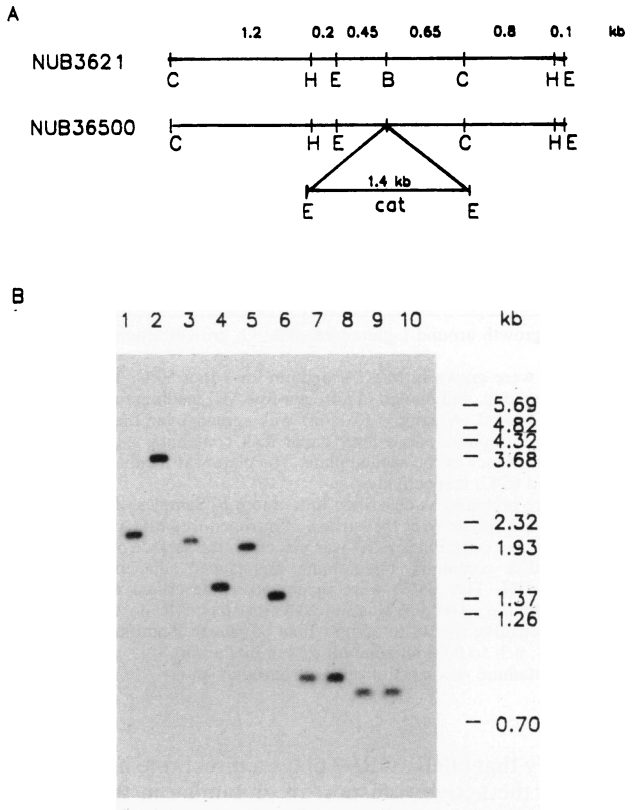

FIG. 8. Southern blot analysis of the *cat* gene insertion into ORF1. (A) Partial restriction map of the chromosomal region that contains ORF1-ORF2 in parental strain NUB3621 and mutant strain NUB36500. Relevant restriction sites are indicated, and the map positions (in kilobase pairs [kb]) are from Fig. 3. (B) Chromosomal DNAs from the parental strain (lanes 1, 3, 5, 7, and 9) and NUB36500 (lanes 2, 4, 6, 8, and 10) were digested with *Hin*dIII (lanes 1 and 2), *Eco*RI (lanes 3 and 4), *Hin*dIII and *Eco*RI (lanes 5 and 6), *Eco*RI and *Cla*I (lanes 7 and 8), and *Hin*dIII and *Cla*I (lanes 9 and 10), and the fragments were separated by agarose gel electrophoresis. Southern blot hybridizations were performed using ^{32}P-labeled 0.9-kb *Cla*I-*Eco*RI as a probe. Each lane came from the same agarose gel. Fragment sizes in kilobase pairs (kb) are shown on the right.

Strain NUB36500, which contains the *cat* gene inserted into ORF1, did not utilize L-glutamine as a sole nitrogen source and was resistant to the toxic glutamine analog γ-GH (28) at 65°C, whereas the parental strain utilized L-glutamine and was sensitive to γ-GH under the same conditions (Table 2). As expected, the parental strain did not utilize L-glutamine as a sole nitrogen source at 45°C. The parental strain in media that contained alternative sources of nitrogen at 65°C was resistant to γ-GH. The parental strain grown in MG medium at 65°C was sensitive to γ-GH, whereas NUB36500 was resistant to γ-GH under the same conditions. Finally, the sensitivity of the parental strain to γ-GH was significantly decreased when 50 μg of L-glutamine per ml was added to MG medium. These combined results indicate that γ-GH competes with glutamine for the proteins of the glutamine transport system.

The parental strain grown in MG medium at 45°C was slightly sensitive to γ-GH. The results of these studies and of the Northern analyses indicate that the ORF1-ORF2 operon is weakly transcribed in minimal medium at low temperature.

TABLE 2. Growth characteristics of parental strain NUB3621 and mutant strain NUB36500 to L-glutamine and γ-GH

Characteristics	Medium	Response by strain[a]:			
		NUB3621		NUB36500	
		45°C	65°C	45°C	65°C
Utilization of L-glutamine as sole source of nitrogen[b]	Nitrogen-free MG	NG	G	NG	NG
Sensitivity to γ-GH[c]	Nitrogen-free MG	—[d]	S	—[d]	—[d]
	MG	SS	S	R	R

[a] NG, no growth around L-glutamine disk; G, growth around L-glutamine disk.

[b] Cultures were grown in MGCA medium for 3 h at 50°C. The cells were washed, suspended, and diluted in nitrogen-free MG medium to a cell density of 3×10^7 CFU/ml. A sample (0.1 ml) was spread over the surface of a nitrogen-free MG plate, and a filter paper disk containing L-glutamine was placed on the surface of the seeded plate. The plate was incubated for 48 and 24 h at 45 and 65°C, respectively.

[c] Cells were prepared as described in footnote *b*. Samples of a diluted cell suspension were spread over the surface of nitrogen-free MG and MG plates. A filter paper disk containing γ-GH was placed on the surface of the plates. A filter paper disk containing L-glutamine was placed adjacent to the disk containing γ-GH. The plates were incubated as described in footnote *b*. Growth around the γ-GH disk was scored as resistance (R; no zone of growth inhibition), sensitive (S; 10- to 15-mm zone of growth inhibition), or slightly sensitive (SS; 0.3- to 0.5-mm zone of growth inhibition).

[d] —, L-Glutamine was not utilized as a nitrogen source.

To verify that ORF1-ORF2 plays a direct role in glutamine transport, the active transport of glutamine in the parental and mutant strains was measured. The results are shown in Table 3. Strain NUB36500 was defective in the transport of glutamine. Cells of the parental strain grown at 65°C efficiently transported glutamine at 45 and 65°C. However, cells of the parental strain grown at 45°C did not transport glutamine at 45 or 65°C. The combined results indicate that the ORF1-ORF2 operon encodes a glutamine transport system.

To determine the role of *glnQH* in the physiology of growth at high temperature, mutant strain NUB36500 was subjected to temperature shifts from 45 to 65°C and from 65 to 45°C (59). After a temperature shift-up, the lag period before exponential growth at 65°C was 2.0 to 2.5 times longer than the lag period observed with the parental strain (56). In a temperature shift-down experiment, the mutant responded in the same manner as the parental strain under the same conditions. Cultures of mutant strain NUB36500 grown in LB medium at 65°C exhibited a final cell density of 7×10^9 to 9×10^9 CFU/ml. A culture of the parental strain grown under the same conditions exhibited a cell density of 2×10^8 to 4×10^8 CFU/ml. The mutant strain and the parental strain grown in LB medium at 45°C exhibited final cell densities of 7×10^8 to 9×10^8 CFU/ml. The rate of growth of the mutant strain at 45 and 65°C was identical to the rate of growth of the parental strain under the same conditions. These results indicate that the *glnQH* operon plays an important role in the physiology of growth at high temperature.

TABLE 3. Transport of glutamine by parental strain NUB3621 and mutant strain NUB36500

Growth temp	Glutamine transport (nmol/10^9 CFU)[a] by strain:			
	NUB3621		NUB36500	
	45°C	65°C	45°C	65°C
45°C	<0.01	<0.01	<0.01	<0.01
65°C	3.9 + 0.2	5.1 + 0.1	<0.01	<0.01

[a] Values are the averages ± standard deviations for three experiments.

DISCUSSION

We cloned and sequenced a fragment of *B. stearothermophilus* NUB36 chromosomal DNA that contains two ORFs. ORF1 and ORF2 encode membrane proteins H2 and HX, respectively. The two genes are transcribed as a polycistronic message and thus constitute an operon. The ORF1-ORF2 operon is transcribed in cells grown in complex media at 65°C but not in cells grown at 45°C under the same conditions. The H2 and HX proteins exhibit significant identity to membrane proteins of enteric bacteria that are active in glutamine transport. In addition, growth response to glutamine, sensitivity to the toxic glutamine analog γ-GH, and glutamine transport assays with the parental strain NUB3621 and mutant strain NUB36500, in which the ORF1 coding segment in the chromosome was interrupted with the *cat* gene, demonstrated that proteins H2 and HX were active in glutamine transport. Thus, we tentatively designate protein H2 as the membrane-bound protein (*glnQ*) and protein HX as the glutamine-binding protein (*glnH*). However, the identities of these proteins and the presence of other proteins that are active in the transport of glutamine must be established by biochemical and genetic techniques.

The *glnQH* transcript originated downstream from a sequence (TCGGTA–19 bp–TACGAT) that had a 3- of 6-base match at the −35 region and a 4- of 6-base match at the −10 region of a consensus promoter sequence (−35 region, TTGACA; −10 region, TATAAT) recognized by the σ^A holoenzyme form of *B. subtilis* RNA polymerase (14, 31) and of *E. coli* genes (13, 14). There were 19 bp between the −35 and −10 regions, which is in good agreement with the 17- to 19- and 15- to 20-bp spacers of *B. subtilis* and *E. coli* promoters, respectively. In addition, there was no significant homology to the promoters recognized by the minor sigma holoenzyme forms of the *B. subtilis* and *E. coli* RNA polymerases (8). A computer program (MacTargsearch) described by Mulligan et al. (32) was used to search the *E. coli* promoter file provided with the program for the occurrence of sites with a sequence and spacing similar to those of the *glnQH* promoter. A relatively low similarity score (30.8%) indicates that the *glnQH* promoter has a poor homology to the consensus promoter sequence of *E. coli*.

A consensus promoter sequence, deduced from the nucleotide sequences of the promoters of nine different genes from *B. stearothermophilus* (1, 18, 21–23, 26, 33, 52, 53), is TTGACt/c–17 to 20 bp–TATTa/cT. The highly conserved positions (occurring in more than 75% of the cases) are indicated by boldface capital letters, well-conserved positions (occurring in 50 to 75% of the cases) are indicated by capital letters, and weakly conserved positions (occurring in less than 50% of the cases) are indicated by lowercase letters. A diagonal line indicates that the position can contain either residue in 50% of the cases. The *glnQH* promoter sequence has a 2- of 6-bp match at the −35 region and a 4- of 6-bp match at the −10 region of the consensus promoter sequence. In conclusion, the *glnQH* promoter has a low degree of homology with the consensus promoter sequences of other prokaryote genes. Perhaps a unique form of RNA polymerase holoenzyme of *B. stearothermophilus* would recognize the *glnQH* promoter.

Enteric bacteria respond to nitrogen deprivation by in-

creasing the expression of a number of operons that function in the utilization of ammonia and alternative sources of nitrogen (27). Promoters of these nitrogen-regulated (Ntr) operons have a consensus sequence, CTGGC/TAC/TA/G [N_4] TTGCA, that is between 6 and 11 bp upstream from the transcription start site. The sensitivity of Ntr promoters to activation by the nitrogen regulator I (NR_I) protein depends on the presence of binding sites for NR_I, usually located more than 100 bp upstream from the transcription start site (27). NR_I-binding sites have the sequence GCAC [N_5] TG GTGC.

The *E. coli glnHPQ* operon has two different types of promoter in the 5'-flanking region of *glnH* (34). The upstream and downstream promoters have a sequence that is similar to the consensus promoter sequence of other *E. coli* genes. The downstream promoter also has identity to Ntr promoters. This promoter (CTGGCACG [N_4] TTTCA) has a 12- of 13-bp match with the consensus Ntr promoter that is 11 bp from the transcription start site. The upstream promoter plays a role in the constitutive expression of the *glnHPQ* operon, and the downstream promoter plays a role in the inducible expression of the *glnHPQ* operon under glutamine-deprived, nitrogen-limiting conditions. The sequence of the NR_I-binding site, located 115 bp upstream from the *glnH* transcription start site, is identical to the consensus NR_I sequence. Promoters of other Ntr genes generally lack the −35 and −10 regions characteristic of genes of enteric bacteria (27).

In a nitrogen-poor medium (MG medium), transcription of the *glnQH* operon of *B. stearothermophilus* is partially activated at low temperature. These results suggest that the *glnQH* operon is driven by a weak Ntr promoter. The *glnQH* operon of *B. stearothermophilus* has one promoter, but the −10 region (positions −11 to −27) contains the sequence CTGTAGTT [N_4] TTTAC, which has a 6- of 13-bp match with the consensus Ntr promoter. This sequence is located 10 bp upstream from the transcription start site. This sequence does not contain the elements GG–10 bp–GC, which are the most conserved in Ntr promoters (27). In addition, the region from positions −246 to −236 (GCTA [N_5] TG GCCT) has a 5- of 10-bp match with the consensus NR_I-binding site that is 231 bp upstream from the transcription start site.

The glutamine transport systems of *E. coli* (57) and *S. typhimurium* (24) are regulated by nitrogen availability. Transcription of the *glnHPQ* operon is activated in cultures grown in a nitrogen-poor medium and inhibited in cultures grown in a nitrogen-rich medium. In contrast, transcription of the *glnQH* operon of *B. stearothermophilus* was activated in a nitrogen-rich medium at high temperature and inhibited under the same conditions at low temperature. This indicates that the *glnQH* operon is transcriptionally regulated by temperature. However, since the *glnQH* promoter contains a sequence that has a low degree of homology with the consensus Ntr promoter of enteric bacteria, the operon may be weakly driven from this promoter under conditions of nutrient depletion and low temperature. Specific nutrients may act as activators or inhibitors of the transcription of the *glnQH* operon, and the levels of these nutrients may change with changes in temperature.

The regulation of the glutamine transport system of bacilli may be very different from the regulatory scheme reported for enteric bacteria (24, 57). Although there are no published studies on the glutamine transport system of *B. subtilis*, the regulation of nitrogen metabolism in this organism (48) differs from that reported for enteric bacteria (41).

Studies are now under way to investigate the regulation of expression from the *glnQH* promoter and the relationship between the inability to transport glutamine, the modified thermoadaptation response, and the increased cell yield exhibited by the mutant strain at high temperature.

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