JOURNAL OF BACTERIOLOGY, Aug. 1999, p. 5042–5050 Vol. 181, No. 16
0021-9193/99/$04.00+0

Sensing of Nitrogen Limitation by *Bacillus subtilis*: Comparison to Enteric Bacteria

PING HU,[1]† TERRANCE LEIGHTON,[2] GALINA ISHKHANOVA,[1] AND SYDNEY KUSTU[1]*

Department of Plant and Microbial Biology[1] and Department of Molecular and Cell Biology,[2] University of California, Berkeley, California 94720

Received 4 February 1999/Accepted 11 June 1999

Previous studies showed that *Salmonella typhimurium* apparently senses external nitrogen limitation as a decrease in the concentration of the internal glutamine pool. To determine whether the inverse relationship observed between doubling time and the glutamine pool size in enteric bacteria was also seen in phylogenetically distant organisms, we studied this correlation in *Bacillus subtilis*, a gram-positive, sporulating bacterium. We measured the sizes of the glutamine and glutamate pools for cells grown in batch culture on different nitrogen sources that yielded a range of doubling times, for cells grown in ammonia-limited continuous culture, and for mutant strains (*glnA*) in which the catalytic activity of glutamine synthetase was lowered. Although the glutamine pool size of *B. subtilis* clearly decreased under certain conditions of nitrogen limitation, particularly in continuous culture, the inverse relationship seen between glutamine pool size and doubling time in enteric bacteria was far less obvious in *B. subtilis*. To rule out the possibility that differences were due to the fact that *B. subtilis* has only a single pathway for ammonia assimilation, we disrupted the gene (*gdh*) that encodes the biosynthetic glutamate dehydrogenase in *Salmonella*. Studies of the *S. typhimurium gdh* strain in ammonia-limited continuous culture and of *gdh glnA* double-mutant strains indicated that decreases in the glutamine pool remained profound in strains with a single pathway for ammonia assimilation. Simple working hypotheses to account for the results with *B. subtilis* are that this organism refills an initially low glutamine pool by diminishing the utilization of glutamine for biosynthetic reactions and/or replenishes the pool by means of macromolecular degradation.

How cells perceive and respond to nutrient limitation are basic questions in microbial physiology. We have posed the first question with respect to nitrogen limitation in enteric bacteria because nitrogen metabolism has a number of simplifying features. Among the important simplifications is that most compounds derive nitrogen by secondary transfers from only two central intermediates, the amino acids glutamate and glutamine. Because a decrease in growth rate is the most direct indication of nutrient limitation, we examined the correlation between nitrogen-limited growth and the pool sizes of these two central intermediates in *Salmonella typhimurium*. The results indicated that *Salmonella* apparently perceives extracellular nitrogen limitation as a decrease in the intracellular concentration of the glutamine pool (21). Similar results were obtained for *Escherichia coli* and *Klebsiella pneumoniae* (35a), and hence the conclusion appears to generalize for enteric bacteria.

To investigate whether lowering the intracellular glutamine pool was a general response to nitrogen limitation in the bacteria, we assessed this response in *Bacillus subtilis. B. subtilis*, a low-GC gram-positive bacterium, is phylogenetically distant from the proteobacteria (20), the group to which enteric bacteria belong, and has the capacity to sporulate under certain conditions of nutrient deprivation including nitrogen limitation (6, 34, 35). In addition, *B. subtilis* differs from enteric bacteria in at least two other important regards. First, it has only a single pathway for assimilation of ammonia into glutamate, the so-called glutamine synthase (GS)/glutamate synthase (GOGAT) cycle (Fig. 1), whereas enteric bacteria have this pathway and an additional one, the reductive amination of 2-oxoglutarate catalyzed by biosynthetic glutamate dehydrogenase (7, 21, 36). Second, regulation of GS in *B. subtilis* is very different from that in enteric bacteria. Transcription of the *glnA* gene of *B. subtilis*, which encodes GS, is controlled negatively by the products of the *glnR* and *tnrA* genes, and GS is not known to be posttranslationally modified (10, 12, 37, 49). By contrast, transcription of the *glnA* gene of enteric bacteria is controlled by the positive regulatory element NtrC in conjunction with the σ^{54} holoenzyme form of RNA polymerase, and GS is covalently modified by adenylylation (21, 45). The signals that control the function of the GlnR and TnrA repressors of *B. subtilis* have not been identified (12, 36, 37, 49). Although it has been postulated that both *glnA* transcription and covalent modification of GS in enteric bacteria are controlled by a ratio of glutamine to 2-oxoglutarate, in vivo evidence for this is limited (30, 44).

To achieve nitrogen limitation in *B. subtilis*, we grew it on poor nitrogen sources in batch culture and in ammonia-limited continuous culture, as we had done previously for enteric bacteria (21). We made use of a wild-type strain of *B. subtilis* 168 that grows well on minimal medium in the absence of glutamate or tricarboxylic acid cycle intermediates and a minimal medium that allows good growth of this strain in the absence of such supplements (34, 35). In addition, we studied *glnA* mutant strains that required glutamine for optimal growth on ammonia to see whether we could detect a decrease in their internal glutamine pools. Such strains of *S. typhimurium* simulate nitrogen limitation internally even when grown on high concentrations of ammonia. In the aggregate, our results indicate that *B. subtilis* initially depletes its glutamine pool upon nitrogen limitation but seems to have a mechanism(s) for refilling it. Apparently as a consequence of the latter, the clear correlation

* Corresponding author. Mailing address: 111 Koshland Hall, UC Berkeley, Berkeley, CA 94720-3102. Phone: (510) 643-9308. Fax: (510) 642-4995. E-mail: kustu@nature.berkeley.edu.

† Present address: diaDexus, LLC, Santa Clara, CA 95054.

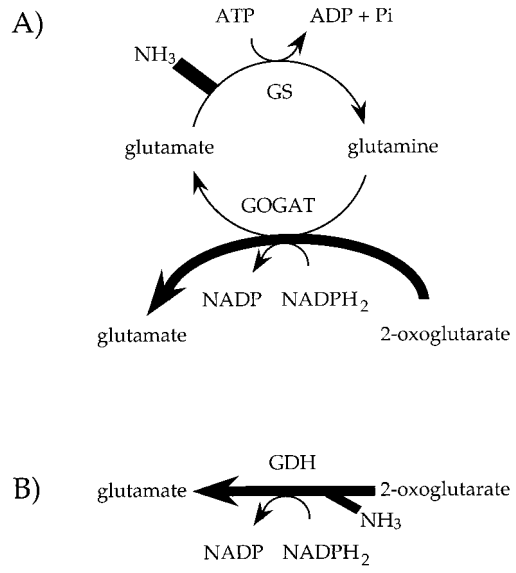

FIG. 1. Pathways for assimilation of ammonia in *B. subtilis* (only pathway A) and enteric bacteria (pathways A and B). (A) GS/GOGAT cycle. One mole of ammonia is assimilated into glutamate for each turn of the cycle (heavy arrows). A portion of the glutamine is withdrawn for biosynthesis. Because GS has a high affinity for ammonia and ATP hydrolysis is coupled to glutamate synthesis, this pathway operates efficiently even at low ammonia concentrations. (B) Biosynthetic glutamate dehydrogenase (GDH) pathway. Because GDH has a relatively low affinity for ammonia and the reaction it catalyzes is reversible, this pathway operates efficiently only at high ammonia concentrations. GS catalyzes synthesis of glutamine from the glutamate generated by GDH (not shown).

observed between the size of the glutamine pool and growth rate in enteric bacteria is far less obvious in *B. subtilis*.

MATERIALS AND METHODS

Growth conditions for *B. subtilis* and *S. typhimurium*. The minimal medium (34, 35) was modified from Neidhardt medium (32) to optimize growth of *B. subtilis*. The important modifications were (i) increasing the morpholinepropanesulfonic acid (MOPS) buffer from 40 to 80 mM and raising the initial pH to 7.6 to provide more buffering capacity; (ii) setting the concentrations of magnesium and manganese to 0.4 and 0.1 mM, respectively, those suggested by Hageman et al. (18); (iii) decreasing the calcium concentration to 0.14 mM, 10% of that suggested by Hageman et al. (18); and (iv) removing NaCl. The *B. subtilis* strain fails to use either MOPS or Tricine as carbon or nitrogen source (25a), and consequently this medium serves well for our purposes.

S. typhimurium was grown on the MOPS medium of Neidhardt et al. (32) at 37°C, and *B. subtilis* was grown on modified MOPS medium (34, 35) at the same temperature. Minimal media were supplemented with glucose or glycerol (0.2 to 0.4%) as the carbon source and the nitrogen sources indicated (at 10 mM total nitrogen). They were supplemented with glutamine (5 mM) and/or other amino acids as necessary to satisfy auxotrophies. Cultures used as inocula were grown to the late exponential phase on the same medium. Luria broth (LB), nutrient broth, or tryptose blood agar base agar plates (Difco) were used for routine maintenance purposes; when required, the following antibiotics were added at the concentrations indicated: chloramphenicol, 10 μg/ml; ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; and tetracycline, 10 μg/ml.

Upshift of *B. subtilis* cells from nitrogen limitation to sufficiency. *B. subtilis* cells were grown on minimal medium with glucose or glycerol (0.2%) as the carbon source and *N*-acetylglucosamine (10 mM) as the sole nitrogen source. When the cells had entered early exponential phase (optical density at 650 nm [OD_{650}] of 0.1 to 0.2), NH_4Cl, or arginine was added to a final concentration of 10 mM (NH_4Cl) or 2.5 or 5 mM (arginine). As the cultures continued to grow, samples were taken for analysis.

Growth in ammonia-limited continuous culture. A New Brunswick Scientific BioFloC30 chemostat with a culture volume of 325 ml was used for continuous culture. The reservoir medium contained 0.2% glucose as the carbon source and 2 mM NH_4Cl as the nitrogen source. These concentrations were similar to those used by Dawes and Mandelstam (6) for ammonia-limited continuous cultures of *B. subtilis*, and the excess of glucose over NH_4Cl, which minimizes autolytic tendencies (22), was similar to that used by Pierce et al. (34, 35) in ammonia-limited batch cultures. An exponential-phase culture of *B. subtilis* (OD_{650} of ~0.8; 180 ml) grown in the same medium with 10 mM NH_4Cl was used to inoculate the chemostat. The culture was aerated rapidly with stirring at >500 rpm and maintained constant pH. Dilution rates were adjusted as shown in Table 3. Samples were taken to determine (i) optical density of the culture; (ii) amount of ammonia remaining in the medium (Ammoniak kit [Sigma Chemical Co., St. Louis, Mo.]); detection limit, (~20 μM); (iii) β-galactosidase activity; and (iv) glutamate and glutamine pool sizes. The samples were stored at −80°C until they were analyzed.

To determine the percentage of spores in chemostat cultures, fresh samples were diluted with LB medium and spread on LB or tryptose blood agar base agar plates for cell counts. Samples were also heated at 80°C for 20 min or extracted with chloroform and then diluted and spread to determine the number of spores. In addition to this quantitative method for assessing sporulation, samples were examined microscopically for the appearance of refractility. Refractile spores were seen microscopically when the incidence of sporulation was 0.5% or higher but not when the incidence was ≤0.1%.

The *S. typhimurium gdh* strain SK3121 (Table 1) was grown in ammonia-limited continuous culture as described previously (21).

Plasmid and strain constructions. Plasmid pSF14, which carries the *glnR* and *glnA* genes of *B. subtilis* and the region upstream of the transcriptional start site necessary for nitrogen regulation (14, 17, 40), was provided by H. J. Schreier. Plasmid pHJS57, which carries just the *glnA* gene of *B. subtilis* (37), was a gift from A. L. Sonenshein. Genetic manipulations of *B. subtilis* were performed as described by Harwood and Cutting (19).

The promoter for the *glnRA* operon of *B. subtilis* was subcloned from pSF14 to pDH32, a *B. subtilis* integration vector with a promoterless *lacZ* gene, in the following several steps. The *Dra*I-*Sac*I fragment from pSF14, which begins at position −84 with respect to the *glnR* transcriptional start and carries the promoter and part of the *glnR* gene, was cloned into the *Hin*cII-*Sac*I sites of a pBluescript II SK vector (Stratagene) to yield pGH1. The *Xho*I-*Sac*I fragment from pGH1, which carries the entire *glnR* promoter insert, was then cloned into the *Sal*I and *Sac*I sites of pTZ19 to yield pGH2, and the *Hin*dIII-*Eco*RI fragment from pGH2 that carries the *glnR* promoter insert was recloned into pBluescript to yield pGH3. Plasmid pGH3 was digested with *Hpa*I, which cleaves at position +75 with respect to the *glnR* transcriptional start (55 bp downstream of the translational start), and *Sma*I; the larger fragment was religated to delete the portion of *glnR* after position +75 (plasmid pGH4). The *Bam*HI-*Kpn*I fragment of pGH4, which carries the *glnR* promoter insert (positions −84 to +75), was cloned into pTZ19 to yield pGH5. The *Eco*RI-*Bam*HI fragment of pGH5, which carries the *glnR* promoter insert, was then cloned into pDH32, which carries only *Eco*RI and *Bam*HI as cloning sites, to yield the requisite *lacZ* fusion (plasmid pGH6). Finally, pGH6 was linearized with *Sca*I and transformed into *B. subtilis* to yield a *glnRA* promoter (*glnRAp*)-*lacZ* transcriptional fusion integrated at the *amyE* locus (16, 47). In this cloning, the *Dra*I and *Hpa*I sites were in the *glnRAp* insert whereas all other sites were in the vectors.

Integration of a *Salmonella glnA-lacZ* fusion at the *put* locus of *S. typhimurium* has been described elsewhere (21). This fusion was transferred to other strains by phage P22-mediated transduction. Plasmid pGln14, which carries Δ*glnA14*::Spc^r (2), was obtained from Susan Fisher and was used to construct strain B134, which carries the deletion-insertion on the chromosome (Table 1, footnote *e*).

Isolation and growth of leaky *glnA* mutants. Isolation of leaky *glnA* mutants of *B. subtilis* (Table 1) was accomplished in two steps: (i) isolation of glutamine auxotrophic strains and (ii) selection of revertant strains that could grow with NH_4Cl as the sole nitrogen source in the absence of glutamine.

We first attempted to isolate leaky *glnA* mutant strains of *B. subtilis* by the positive selection procedure of Kustu and McKereghan (25), which was initially used in *S. typhimurium* and was then successfully employed to isolate glutamine auxotrophs of *B. subtilis* (7). Selection is for growth on D-histidine as the histidine source in a strain carrying a stable lesion in the histidine biosynthetic operon. The nitrogen source is NH_4Cl. In *Salmonella*, the selection is known to be based on the fact that a decrease in the glutamine pool leads to physiological derepression of a transport system for D-histidine and that transport rather than racemization limits use of D-histidine to satisfy histidine auxotrophy (24, 25). To employ the selection in *B. subtilis*, we first introduced the *hisA82*::Tn*917* lesion into our wild-type strain by phage PBS1-mediated transduction from donor strain 1A626 (Table 1), with selection for erythromycin resistance. The resulting strain, B278, was then used as the parental strain for selection of spontaneous D-histidine utilizers. Of the small colonies picked after 3 days of incubation, 12 grew normally on minimal medium containing L-histidine when it was supplemented with glutamine. Of these 12, only 1 had a lesion in *glnA* (see below). This strain, B13, was a glutamine auxotroph (that is, it did not grow detectably on NH_4Cl in liquid culture in the absence of glutamine or of glutamate or a source of glutamate such as proline); since glutamine is the obligatory precursor of glutamate in *B. subtilis* (Fig. 1), glutamate spares the glutamine requirement. The lesion in B13 was localized to *glnA* by three means: (i) it could be transferred to strain B134 (Δ*glnA14*::Spc^r) by transformation to growth on proline plus NH_4Cl, and other growth phenotypes (Table 6) were also inherited; (ii) it could be complemented by plasmid pHJS57, which carries just *glnA*; and (iii) it was cloned, and the sequence change in *glnA* was determined. (Nine of the twelve strains that grew optimally with glutamine also grew optimally with glutamate. Based on Western blotting for glutamine synthetase, at least three or four of these were not derepressed for *glnA* expression, and hence these nine strains were not studied further. Two of the twelve strains that grew optimally with

TABLE 1. Bacterial strains used

Strain	Relevant genotype	Source	Parent		Reference
			Recipient	Donor	
B. subtilis					
168 Wild type	*trp*$^+$	T. Leighton			35
1A626	*hisA82*::Tn*917 trpC2*	*Bacillus subtilis* Genetic Stock Center			48
1A174	*glnA* (G243S) *ilvC1 pheA1 trpC2*	*Bacillus subtilis* Genetic Stock Center			8
B13	*glnA* (P306H) *hisA82*::Tn*917*		B278[a]		This study
B23	*amyE*::[Φ(*glnRAp-lacZ*)-Cam^r]		168[b]		This study
B28	*glnA* (P306H) *hisA82*::Tn*917* *amyE*::[Φ(*glnRAp-lacZ*)-Cam^r]		B13[c]	B23	This study
B32	*glnA* (P306H) *amyE*::[Φ(*glnRAp-lacZ*)-Cam^r]		B28[c]	B23	This study
B40	*glnA* (G243S, Y308C) *ilvC1 pheA1 trpC2*		1A174[d]		This study
B134	Δ*glnA14*::Spc^r		168[e]		This study
B169	*glnA* (G243S, Y308C)		B134[c]	B40	This study
B275	*glnA* (G243S)		B134[c]	1A174	This study
B276	*glnA* (G243S, Y308C) *amyE*::[Φ(*glnRAp-lacZ*)-Cam^r]		B169	B23	This study
B277	*glnA* (G243S) *amyE*::[Φ(*glnRAp-lacZ*)-Cam^r]		B275[c]	B23	This study
B278	*hisA82*::Tn*917*		168[f]	1A626	This study
S. typhimurium					
SK711	*gdh-51 zch-1463*::Tn*10* (Tet^r)[g]				28
SK2979	Wild type				21
SK2980	Δ(*glnA-ntrB-ntrC*)*60*				21
SK2983	*glnA85*				21
SK2986	*glnA88*				21
SK3041	*putPA1303*::[Kan^r-Φ(*glnA′-′lacZYA*)]				21
SK3080	*gdh-51 zch-1463*::Tn*10*		SK2979[h]	SK711	This study
SK3094	*gdh-51* Δ(*glnA-ntrB-ntrC*)*60 zch-1463*::Tn*10*		SK2980[h]	SK711	This study
SK3117	*glnA424 putPA1303*::[Kan^r-Φ(*glnA′-′lacZ*)]			21	
SK3121	*gdh-51 zch-1463*::Tn*10* *putPA1303*::[Kan^r-Φ(*glnA′-′lacZ*)]		SK3080[i]	SK3041	This study
SK3131	*glnA424 gdh-51 zch-1463*::Tn*10*		SK3094[i]	SK3117	This study
SK3146	*glnA53 glnA425 gdh-51 zch-1463*::Tn*10*		Spontaneous mutation[j]		This study
SK3154	*glnA53 glnA425*		SK2980[i]	SK3146	This study
SK3157	*glnA53 glnA425 gdh-51 zch-1463*::Tn*10* *putPA1303*::[Kan^r-Φ(*glnA′-′lacZ*)]		SK3146[i]	SK3041	This study
SK3160	*glnA53 glnA425* *putPA1303*::[Kan^r-Φ(*glnA′-′lacZ*)]		SK3154[i]	SK3041	This study
SK3163	*glnA424 gdh-51 zch-1463*::Tn*10* *putPA1303*::[Kan^r-Φ(*glnA′-′lacZ*)]		SK3131	SK3041	This study

[a] Spontaneous mutation; selection for growth on minimal salts agar plates with D-histidine as the sole histidine source.

[b] Integration. The promoter region of the *B. subtilis glnRA* operon necessary for regulation (−104 to +55) was cloned into plasmid pDH32 (a *B. subtilis* integration vector with a promoterless *lacZ* gene). The resulting plasmid was linearized and transformed into *B. subtilis* 168 cells with selection for chloramphenicol resistance. The *glnRAp-lacZ* fusion was integrated at the *amyE* locus and was transferred to other strains by chromosomal transformation.

[c] Chromosomal transformation with selection for Cam^r, His$^+$, or growth on NH_4Cl as the sole nitrogen source.

[d] Spontaneous selection was for growth on minimal salts agar plates with NH_4Cl as the sole nitrogen source.

[e] Integration. Plasmid pGln14 (2, 49), which carries the *B. subtilis glnA* gene with its *Eco*RI fragment replaced by a spectinomycin resistance cassette, was linearized and transformed into *B. subtilis* 168 cells with selection for spectinomycin resistance. The disrupted *glnA* gene was integrated into the chromosome by homologous recombination.

[f] Transduction with phage PBS1.

[g] The *gdh-51* allele is 90% linked by P22-mediated transduction to *zch-1463*::Tn*10*.

[h] P22-mediated transduction with selection for tetracycline resistance. Candidates were used as donors to strain SK3062 (Δ*gltB824*), and transductants were screened for glutamate auxotrophy. If the candidate carried the *gdh-51* mutation, 90% of the transductants were glutamate auxotrophs, because both pathways for synthesis of glutamate were inactivated.

[i] Phage P22-mediated transduction with selection for Kan^r or growth on NH_4Cl as the sole nitrogen source.

[j] SK3095 (*glnA53 gdh-51*) was used as the parent strain, selecting for growth on minimal salts agar plates with NH_4Cl as the sole nitrogen source.

glutamine were glutamine auxotrophs with lesions in *glnR* [apparently *glnR** lesions] [37, 39].)

From the glutamine auxotrophs B13 and 1A174 (8) and the glutamine auxotroph SK3095 (*glnA53 gdh-51 zch-1453*::Tn*10*) of *S. typhimurium*, we selected partial revertants that could grow on ammonia, which included leaky *glnA* mutant strains. The lesions in strains 1A174 and B40, which was derived from 1A174, were localized to *glnA* by the three means described above. The *glnA* region of all mutant strains of *B. subtilis* was cloned by PCR, and its sequence was determined to identify mutations. For strains B32, B277, and B276, mutations resulted in the following amino acid substitutions in GS: P306H, G243S, and both G243S and Y308H, respectively (19a). The GS activities of these strains in crude cell extracts with Mg^{2+} as the divalent cation were <0.005, <0.005, and 0.018 μmol/min/mg of protein, respectively, whereas that of the wild-type strain was 0.022 μmol/min/mg of protein (19a).

Determination of enzyme activities. GS activities of wild-type and mutant strains of *B. subtilis* were determined as described by Dean et al. (8) except that

TABLE 2. Growth, pool sizes, and *glnRAp-lacZ* expression of *B. subtilis*[a] batch cultures on different nitrogen sources

Carbon source	Nitrogen source[b]	Doubling time (min)	Mean pool size (nmol/mg [dry wt] ± error)		β-Galactosidase[c] (U/ml/OD_{650})
			Glutamate	Glutamine	
0.2% glucose	Glutamine	45			110
	Arginine	48	520[d]	72[d]	360
	Ammonium chloride	62	360 ± 20	27 ± 1	790
	Proline	72	—[e]	22 ± 4	3,220
	GABA	74	400 ± 20	24 ± 2	3,630
	Urea	84	370 ± 20	29 ± 3	3,570
0.2% glycerol	Glutamine	48			150
	Arginine	48	390[d]	53[d]	420
	Ammonium chloride	60	290 ± 30	20 ± 1	810
	Proline	74	—	19 ± 3	3,000
	GABA	75	400 ± 20	18 ± 2	3,280
	Urea	80	320 ± 20	16–36[f]	3,120–1,090[f]
	N-Acetylglucosamine	395	130 ± 5	21 ± 1	550

[a] Wild-type strain B23, which carries a *glnRAp-lacZ* transcriptional fusion at the *amyE* locus.
[b] Concentrations of the nitrogen sources: glutamine, arginine, and urea, 5 mM; ammonium chloride, proline, *N*-acetylglucosamine, and GABA, 10 mM.
[c] Slope of β-galactosidase activity versus optical density of the culture, which is the differential rate of synthesis of β-galactosidase from *glnRAp*.
[d] Value for cells at an OD_{650} of 0.7 and the lowest of the three values observed during exponential growth. Pool concentration progressively declined for cells at an OD_{650} of between 0.1 and 0.9.
[e] —, Cells excreted glutamate to the medium when grown on 10 mM proline as the sole nitrogen source.
[f] The glutamine pool of the samples increased during growth, and thus we report a range. Similarly, the differential rate of β-galactosidase synthesis decreased over the range indicated.

cells were broken by sonication and the assay temperature was 37°C. β-Galactosidase activity was determined by the method of Miller (29), and protein concentration was determined by the method of Bradford (4). Polyclonal antiserum directed against *B. subtilis* GS was obtained from A. L. Sonenshein.

Measurement of amino acid pool size (no-harvest protocol). All strains used to measure amino acid pools were histidine prototrophs. Cell suspension (0.2 volume) was added directly to ice-cold methanol (0.8 volume) so that the cell membrane would be disrupted immediately with minimum disturbance of prior physiological state (21). Aspartic acid and α-aminoadipic acid were added as internal standards to correct for losses during subsequent manipulations. Generally, α-aminoadipic acid was used for calculations, because the aspartate pool of *B. subtilis* was substantial in some cases. After lyophilization, samples were stored at −80°C and were then prepared for analysis as described elsewhere (21). Amino acids were derivatized with *o*-phthaldialdehyde, and derivatives were separated on a reversed-phase high-pressure liquid chromatography column (4.6 by 100 mm; C_{18}; Rainin model 80-OPA-C3), using the conditions described previously (21). Amounts of amino acids were determined by fluorescence; the limit of detection is 2 to 3 pmol (26).

To check for the presence of amino acids in media, cell suspensions were rapidly filtered (0.2-μm-pore-size Millipore filters), and the first medium to be collected was analyzed. Glutamate was detected in the culture medium when cells were grown on proline as the nitrogen source, presumably due to leakage or excretion. No significant amount of glutamine was observed in the culture medium under these conditions, and little of either amino acid was detected in the medium when cells were grown on any of the other nitrogen sources used.

Errors for the glutamate and glutamine pool concentrations in Tables 2, 5, and 6 are the maximum difference between individual values for an experiment and the average value that is presented. Values were determined for cells at three points during exponential growth (OD_{650} of between 0.12 and 0.75). Errors for the glutamate pool concentration were rounded up to the nearest 5, whereas those for the glutamine pool concentration were rounded up to the nearest unit.

RESULTS

Glutamine pools of *B. subtilis* grown on different nitrogen sources in batch culture. As we had for *S. typhimurium* (21), we used two criteria for nitrogen-limited growth of *B. subtilis*: first, that the doubling time of the culture was longer than that on glutamine or arginine, the optimal nitrogen sources for *Bacillus*; and second, that *glnA* expression was elevated (12, 36). We took the latter as an indication that slowing of growth was due to nitrogen limitation rather than another limitation or an inhibitory effect of which we were unaware. *B. subtilis* B23, a derivative of wild-type strain 168 carrying a fusion of the *glnRA* promoter to *lacZ* (*glnRAp-lacZ*) at the *amyE* locus, was grown on modified MOPS medium (35) with glucose or glycerol (0.2%) as the carbon source and glutamine, arginine, NH_4Cl, proline, γ-aminobutyric acid (GABA), urea, or *N*-acetylglucosamine (10 to 20 mM nitrogen) as the nitrogen source (13). Glutamine and arginine, the best nitrogen sources for *B. subtilis*, yielded doubling times of ~48 min on both carbon sources (Table 2); ammonia, GABA, proline, and urea yielded doubling times of ~60 to 85 min. Commensurate with doubling times, expression from *glnRAp* was higher on the other nitrogen sources than on glutamine and arginine. *N*-Acetylglucosamine, which could be catabolized only with glycerol as the carbon source, yielded a doubling time of 395 min, but expression from *glnRAp* (550 U) was almost as low as that on arginine (420 U) and was well below that on proline, GABA, or urea (≥3,000 U in each case). The latter provided the first indication that *N*-acetylglucosamine was not simply a limiting nitrogen source.

As described previously (21) (see also Materials and Methods), samples were removed from each culture at three times during exponential growth (OD_{650} of 0.12 to 0.75) and assayed for glutamate and glutamine pools. The data were averaged and are presented as single points in Table 2. The glutamate pool size was very high with arginine as the nitrogen source (520 or 390 nmol/mg [dry weight] with glucose or glycerol, respectively, as the carbon source). Although the pool size decreased somewhat with ammonia as the nitrogen source (360 or 290 nmol/mg [dry weight] with glucose or glycerol, respectively, as the carbon source), it increased again with proline (data not shown) and remained at least as high with GABA or urea as it was with ammonia. Because doubling times on proline, GABA, and urea were longer than on ammonia and *glnA* expression was higher, nitrogen limitation did not appear to correlate with a decrease in the glutamate pool size. The glutamate pool was markedly lower on *N*-acetylglucosamine than on other nitrogen sources (130 nmol/mg [dry weight]).

Although the glutamine pool was more than 2.5-fold lower on ammonia than on arginine (increase in doubling time of ~13 min), there was no further decrease in this pool on other poor nitrogen sources including *N*-acetylglucosamine. Hence,

TABLE 3. Residual ammonia, pool sizes, and *glnRAp-lacZ* expression of *B. subtilis*[a] grown in ammonia-limited continuous culture[b]

Sample	Interval[c] (h)	Dilution rate[d] (h^{-1})	OD_{650}	Ammonia[e] (mM)	Pool size (nmol/mg [dry wt])		β-Galactosidase sp act (Miller units)
					Glutamate	Glutamine	
1	2.5	0.72	0.28	1.0	280	22	770
2	6		0.35	1.0	290	24	660
3	18		0.36	0.9	260	20	700
4	3	0.63	0.48	0.6	300	25	710
5	5.5		0.58	0.1	270	22	990
6	8		0.61	<0.02	330	2.9	4,760
7	16	0.57	0.58	<0.02	350	7.5	4,760
8	18.5		0.57	<0.02	330	8.4	4,840
9	22.5		0.57	<0.02	540	13	4,820
10	17	0.72	0.41	0.6	280	25	810
11	20.5		0.40	0.7	280	24	770
12	24		0.41	0.6	260	21	810
13	18	0.68	0.46	0.4	280	25	820
14	21.5		0.55	0.1	300	29	2,830
15	18	0.37	0.57	<0.02	390	6.9	4,090
16	21.5		0.58	<0.02	390	6.4	4,040

[a] Wild-type strain B23, which carries a *glnRAp-lacZ* transcriptional fusion at the *amyE* locus.
[b] The carbon source was glucose, and the chemostat was inoculated with 180 ml of culture in mid-exponential phase as described in Materials and Methods.
[c] Time that the culture had been at a particular rate before the sample was taken.
[d] Dilution rates of 0.72, 0.63, 0.57, 0.68, and 0.37 h^{-1} correspond to doubling times of 57, 66, 73, 61 and 112 min, respectively.
[e] Residual ammonia in the medium.

nitrogen limitation did not appear to correlate with a drop in the glutamine pool.

Having observed two peculiarities in the behavior of cells grown on *N*-acetylglucosamine as the nitrogen source (low expression from *glnRAp* and a low glutamate pool) we wanted to test further whether *N*-acetylglucosamine was simply a limiting nitrogen source or whether it inhibited growth on better nitrogen sources such as arginine or ammonia (21). When cells were grown on *N*-acetylglucosamine, addition of arginine or NH_4Cl did not result in an increase in the growth rate for more than 2 h. Moreover, even when the cells had fully adapted to the presence of both nitrogen sources, the doubling time on a mixture of arginine (2.5 or 5 mM) and *N*-acetylglucosamine (5 mM) or of NH_4Cl (5 mM) and *N*-acetylglucosamine (5 mM) was 60 or 70 min, respectively, whereas the corresponding doubling time in the absence of *N*-acetylglucosamine was 48 or 60 min. Thus, *N*-acetylglucosamine appeared to inhibit growth on the two preferred nitrogen sources.

Glutamine pools of *B. subtilis* in ammonia-limited continuous culture. To overcome potential complications of using different compounds to achieve nitrogen limitation, including the complication that most yielded carbon skeletons as well as ammonia or other nitrogen-containing intermediates, we turned to the use of ammonia-limited continuous culture. When the dilution rate of an ammonia-limited chemostat was decreased sufficiently for *B. subtilis* to deplete ammonia from the medium completely (Table 3, sample 6), the glutamine pool dropped sevenfold and *glnRAp-lacZ* expression was elevated about sixfold. (There was no decrease in the glutamate pool.) However, surprisingly, when the dilution rate was decreased slightly more (samples 7 to 9), the glutamine pool rose to about half of the maximum value seen in the chemostat, despite the fact that residual ammonia in the medium remained undetectable and *glnRAp-lacZ* expression remained maximal. When the culture was cycled by again increasing the dilution rate (samples 10 to 12), all parameters returned to those characteristic of nitrogen sufficiency (samples 1 to 3). Upon a second decrease in the dilution rate (samples 15 and 16), ammonia was depleted from the medium, *glnRAp-lacZ* expression rose to the maximum value seen in the chemostat, and the glutamine pool again dropped, although only some fourfold. Thus, the glutamine pool decreased to a value between the lowest and highest values seen when the dilution rate was decreased the first time.

To observe the largest decrease in the glutamine pool upon the first lowering of the dilution rate of the culture (sevenfold in the experiment above), a sample had to be taken before the culture had adapted. In the two experiments performed, the decrease in the pool size was smaller once the culture had adapted to the low dilution rate. In a single experiment in which the culture was started at a low dilution rate and allowed to adapt, we saw no change in pools or *glnRAp-lacZ* expression when the dilution rate was increased. We have no explanation for this. Even after 40 h at the lowest dilution rate used (0.37), the degree of sporulation was <3% (see Materials and Methods), comparable to values reported by Dawes and Mandelstam at this dilution rate (6). At high dilution rates, the degree of sporulation was <0.1% (data not shown).

To eliminate the possibility that the unexpected instability of the glutamine pool in *B. subtilis* at low dilution rates was due to the fact that it had only a single pathway for ammonia assimilation, we examined the behavior of a *gdh* mutant strain of *S. typhimurium*, SK3121, in an ammonia-limited chemostat. As was true for wild-type *S. typhimurium* (21), the glutamine pool decreased >10-fold at dilution rates low enough to result in depletion of ammonia from the medium (Table 4, samples 1, 2, 8, 9, 10, and 11). The glutamate pool decreased <1.6-fold under these conditions, and *glnA* expression was maximal. By contrast to the case for *B. subtilis*, the glutamine pool remained low (below our limit of reliable detection) until the dilution rate was increased. Thus, results for the *gdh* mutant strain of *Salmonella* provided no evidence for fluctuation of the glutamine pool at low dilution rates in an organism employing only the GOGAT cycle for ammonia assimilation. We note parenthetically that *glnA* expression decreased little in the *gdh* mutant strain of *Salmonella* at high dilution rates, a result different from that for the wild-type strain (21). As observed by others (27, 41, 42, 46), both glutamine and glutamate pools of *S. typhimurium* were markedly lower than those of *B. subtilis* even under conditions of ammonia sufficiency.

TABLE 4. Residual ammonia, pool sizes, and *glnA-lacZ* expression of an *S. typhimurium gdh* mutant strain[a] grown in ammonia-limited continuous culture[b]

Sample	Interval[c] (h)	Dilution rate[d] (h^{-1})	Ammonia[e] (mM)	OD_{650}	Pool size (nmol/mg [dry wt])		β-Galactosidase sp act (Miller units)
					Glutamate	Glutamine	
1	20	0.37	<0.02	0.45	32	<0.5	20,200
2	42		<0.02	0.41	45	<0.5	20,800
3	22	0.6	0.4	0.36	51	7.2	12,300
4	44		0.3	0.41	49	6.2	9,800
5	22	0.67	0.9	0.26	47	6.4	10,800
6	44		1.2	0.25	50	6.1	10,700
7	18	0.56	0.3	0.27	43	6.5	16,700
8	22	0.42	<0.02	0.44	40	<0.5	19,200
9	44		<0.02	0.45	42	<0.5	20,400
10	22	0.37	<0.02	0.50	41	<0.5	19,800
11	40		<0.02	0.50	40	<0.5	19,100

[a] Strain SK3121, which carries a *glnA-lacZ* fusion at the *put* locus.
[b] About 40 ml of culture in early exponential phase was inoculated into the chemostat as described in reference 21. The carbon source was glucose.
[c] Time that the culture had been at a particular dilution rate before the sample was taken.
[d] Dilution rates of 0.37, 0.6, 0.67, 0.56, and 0.42 h^{-1} correspond to doubling times of 112, 69, 62, 74, and 99 min, respectively.
[e] Residual ammonia in the medium.

Glutamine pools in *glnA* mutant strains of *B. subtilis*. Leaky *glnA* mutant strains (glutamine bradytrophs) of *S. typhimurium* are able to grow on ammonia as the sole nitrogen source but grow optimally only when glutamine is added (21, 25). When they are grown on ammonia alone, their internal glutamine pools are low and *glnA* expression is maximal. The growth behavior of such mutant strains allowed us to calibrate growth rate as a function of glutamine pool size and thereby demonstrate that decreases in the glutamine pool observed in wild-type *Salmonella* under nitrogen-limiting conditions were, in fact, sufficient to account for slow growth (21).

Growth defects in the four *Salmonella glnA* mutant strains and the accompanying decreases in the glutamine pool, but not the glutamate pool, are documented in Table 5. When the *gdh-51* lesion (28) was present in these strains and hence they had only the GOGAT cycle for synthesis of glutamate, their growth rates on ammonia were dramatically lower (Table 5) and, in fact, two of the strains failed to grow (i.e., they became outright glutamine auxotrophs). For the two *glnA gdh* strains of *Salmonella* that retained the ability to grow on ammonia, glutamine pools were very low (Table 5). The glutamate pool in these strains decreased <2-fold even at a doubling time as long as 300 min.

We made comparable measurements for leaky *glnA* mutant strains of *B. subtilis*, which also have only the GOGAT cycle for ammonia assimilation. These strains grew as rapidly as the congenic wild type on glutamine as the nitrogen source or on glutamine and NH_4Cl as nitrogen sources (Table 6). As had been observed previously for other *glnA* strains of *B. subtilis* (12, 36), *glnRAp-lacZ* expression was very high for these strains (5,300 to 7,000 U/ml/OD_{650}) when they were grown on glutamine or glutamine and NH_4Cl as nitrogen sources, whereas expression in the wild-type strain was minimal under these conditions (~140 U/ml/OD_{650}).

All three of the *glnA* mutant strains could grow on ammonia if proline was also provided as a source of glutamate with glucose or glycerol as the carbon source (Table 6). However, all of the mutant strains grew more slowly than the wild type on ammonia plus proline (doubling times of 60 to 160 min), whereas the wild type retained its optimal growth rate (doubling time of 48 min). All three mutant strains had lower glutamine pools than did the wild type, and the two faster-growing strains, B276 (G243S, Y308C) and B277 (G243S), expressed *glnRAp-lacZ* at very high levels (6,000 to 7,500 U/ml/OD_{650}). The slowest-growing mutant strain, B32 (P306H), expressed *glnRAp-lacZ* at about the same level as the wild type on this mixture of nitrogen sources (~700 U/ml/OD_{650}). The low *glnRAp-lacZ* expression in strain B32 was reminiscent of

TABLE 5. Growth, pool sizes, and *glnA-lacZ* expression of *S. typhimurium glnA* and *glnA gdh* mutants

Strain[a]	Relevant genotype	Doubling time (min)[b]	Avg[c] pool size (nmol/mg [dry wt]) ± error		β-Galactosidase[d] (U/ml/OD_{650})
			Glutamate	Glutamine	
SK3041	Wild type	48	69 ± 5	12 ± 1	5,600
SK3117	*glnA424*	60	62 ± 5	5 ± 1	26,000
SK3160	*glnA425glnA53*	72	70 ± 5	4.5 ± 1	28,000
SK2983	*glnA85*	105	61 ± 5	1 ± 1	22,000[e]
SK2986	*glnA88*	131	59 ± 5	0.6 ± 1	27,000[e]
SK3121	*gdh-51*	60	62 ± 5	12 ± 2	8,500
SK3163	*glnA424 gdh-51*	96	58 ± 5	<0.5	25,000
SK3157	*glnA425 glnA53 gdh-51*	300	33 ± 5	<0.5	20,000

[a] All strains carried a *glnA-lacZ* fusion at the *put* locus.
[b] Cultures were grown with glucose as the sole carbon source and NH_4Cl as the sole nitrogen source.
[c] Average of three determinations made during exponential growth.
[d] Differential rate.
[e] Value determined by Ikeda et al. (21).

TABLE 6. Growth, glutamine pool sizes, and *glnRAp-lacZ* expression of *B. subtilis glnA* mutants

Carbon source	Strain[a]	Nitrogen source(s)[b]	Doubling time (min)	Avg[c] glutamine pool size (nmol/mg [dry wt]) ± error	β-Galactosidase[d] (U/ml/OD_{650})	No. of experiments
Glucose	B23 (wild type)	Glutamine + NH_4Cl	48		155	2
	B276 (G243S, Y308C)		45		6,000	1
	B277 (G243S)		48		7,000	2
	B32 (P306H)		49		5,700	2
	B23 (wild type)	Glutamine	48		130	2
	B276 (G243S, Y308C)		48		5,300	2
	B277 (G243S)		48		6,500	2
	B32 (P306H)		48		5,400	2
	B23 (wild type)	Proline + NH_4Cl	48	30 ± 4	670	3
	B276 (G243S, Y308C)		60	11 ± 2	7,500	3
	B277 (G243S)		120	2.8 ± 1	6,000	3
	B32 (P306H)		160	3.0 ± 1	620	3
	B23 (wild type)	Proline	60	13 ± 3	3,300	2
	B276 (G243S, Y308C)		70	4 ± 1	6,500	2
	B277 (G243S)		165	2.5 ± 2	4,800	2
	B32 (P306H)		No growth			
	B23 (wild type)	NH_4Cl	65	25 ± 5[c]	800	4
	B276 (G243S, Y308C)		125	40 ± 6[c]	4,300	8
	B277 (G243S)		No growth			
	B32 (P306H)		No growth			
Glycerol	B23 (wild type)	Proline + NH_4Cl	48	35 ± 3	700	1
	B276 (G243S, Y308C)		59	12 ± 2	7,000	2
	B277 (G243S)		130	3.5 ± 1	6,000	1
	B32 (P306H)		150	2 ± 1	750	1
	B23 (wild type)	Proline	60	12 ± 3	3,400	1
	B276 (G243S, Y308C)		65	4 ± 1	6,500	2
	B277 (G243S)		180	2.5 ± 1	2,700	1
	B32 (P306H)		No growth			

[a] All strains carried a *glnRAp-lacZ* transcriptional fusion at the *amyE* locus.
[b] Cultures were grown with glucose or glycerol as the carbon source and the indicated nitrogen source(s). Inocula were grown with the indicated nitrogen sources plus glutamine (5 mM).
[c] Average of three determinations made during exponential growth. Pools of glutamate for strains B23 and B276 grown with NH_4Cl alone were 290 and 273 nmol/mg (dry weight), respectively. The glutamate pool for cells grown with proline is not reported because these cells excrete glutamate.
[d] Differential rate.

that in the wild-type strain grown on *N*-acetylglucosamine as the sole nitrogen source (Table 2), and we can only speculate that it may be related to slow growth (doubling time of 150 min or longer).

The two mutant strains that grew fastest on ammonia plus proline, B276 and B277, could also grow on proline as the sole nitrogen source. Again, their doubling times were longer than that of the wild-type strain on proline and their glutamine pools were lower than that of the wild type. Expression of *glnRAp-lacZ* was high in all of the strains including the wild type.

Only the mutant strain that grew fastest on proline, B276, was also able to grow on ammonia as the sole nitrogen source. Surprisingly, its glutamine pool was greater than that of the wild-type strain despite its low growth rate (doubling time of ~125 min). Expression of *glnRAp-lacZ* was clearly greater than that in the wild-type strain (4,300 U/ml/OD_{650} for the mutant versus 800 for the wild type). Both the glutamine pool and *glnRAp-lacZ* expression are reminiscent of those in the wild-type strain as it adapted to low dilution rates in an ammonia-limited chemostat (Table 3, samples 7 to 9 and samples 5 and 16). Results of a number of additional experiments, in which growth and washing of cultures used as inocula were varied, indicated (data not shown) (i) that the glutamine pool size of B276 was at least as high as that of B23 (wild type) with NH_4Cl as the sole nitrogen source (NH_4Cl also used as sole nitrogen source for inocula); (ii) that the glutamine pool size of B276 could be up to four- to fivefold higher than that of B23 when cultures used for inocula were supplemented with glutamine (5 mM), whether or not they were subsequently washed; (iii) that the glutamate pool size of B276 was normal (see also Table 6, footnote *c*); and (iv) that *glnRAp-lacZ* expression in B276 was much greater than that in B23.

DISCUSSION

Despite a number of peculiarities in its responses, *B. subtilis*, like *S. typhimurium*, appears to perceive external nitrogen limitation as internal glutamine limitation, at least initially. The clearest evidence for this conclusion came from measurements of pool sizes of glutamate and glutamine for a wild-type strain of *B. subtilis* 168 grown in an ammonia-limited chemostat (Table 3). (Unlike the case for wild-type *S. typhimurium* (21), pools of this strain grown on different nitrogen sources in batch culture were not readily interpretable [Table 2 and Results].) Upon initial depletion of ammonia from the medium in an ammonia-limited chemostat (low dilution rate), wild-type *B. subtilis* decreased its glutamine pool 7.5-fold but did not decrease its glutamate pool. Unexpectedly, the glutamine pool was partially refilled with time, although *glnRAp-lacZ* expression remained maximal. On a subsequent cycle of ammonia depletion (cycling through high and then low dilution rates) the glutamine pool was again decreased, but only ~4-fold. Two simple hypotheses that might account for partial refilling of an initially low glutamine pool are (i) that *B. subtilis* decreases utilization of glutamine for biosynthetic reactions and thereby

propagates a single primary limitation into several secondary limitations and (ii) that *Bacillus* increases degradation of nitrogen-containing compounds, e.g., by proteolysis (3, 23, 31, 33). These hypotheses remain to be tested. Whether replenishment of the glutamine pool is related to the fact that *Bacillus* can sporulate upon nutrient limitation (6, 34, 35) is not clear, but it cannot be accounted for simply by massive sporulation in the chemostat because sporulation remained <3% even at the lowest dilution rate used.

Both wild-type *S. typhimurium* and a *gdh* mutant strain deplete their internal glutamine pools abruptly when ammonia is exhausted from the medium in an ammonia-limited chemostat (Table 4) (21). By contrast to the case for *Bacillus*, the pool remains low for as long as external ammonia remains undetectable, i.e., for as long as the low dilution rate required for complete depletion of ammonia is maintained. As is usually the case, cycling of the culture through a high and then a low dilution rate yielded exactly the same results as were obtained the first time. Since the *gdh* mutant strain has only one pathway for ammonia assimilation—the GOGAT cycle—as does *B. subtilis*, results with the *Salmonella gdh* strain indicate that the peculiar replenishment of the glutamine pool observed in *Bacillus* is not a consequence of its having only a single pathway for ammonia assimilation and glutamate synthesis.

The capacity of *B. subtilis* to modulate its glutamine pool in ways not seen in *S. typhimurium* or other enteric bacteria (35b) was apparently manifested in a leaky *glnA* mutant strain as well as the wild-type strain (Table 6). Although the three leaky *glnA* strains of *Bacillus* that we tested did deplete their glutamine pools in predictable ways when grown on a combination of ammonia and proline as nitrogen sources or on proline alone, the one strain able to grow on ammonia in the absence of other supplements, B276, had a higher glutamine pool than the wild type on this nitrogen source despite its long doubling time. We note in this connection that the leaky *glnA* strains generally showed both depletion of their internal glutamine pool concentrations and elevation of *glnA* expression when they were grown in the presence of an external source of glutamate, i.e., proline. Apparent replenishment of the glutamine pool in B276 occurred when this strain was grown on ammonia as sole nitrogen source, as it did in the wild-type strain in an ammonia-limited chemostat (Table 3). Whether this unexpected behavior in the absence of an external source of glutamate is related to the fact that most strains of *B. subtilis* require glutamate, or an amino acid such as proline that yields glutamate readily, to grow well in defined minimal medium (1, 6, 11, 34, 35) is unclear, as is the basis for the growth stimulation by glutamate.

In both *B. subtilis* and *S. typhimurium*, the *glnA* gene, which encodes GS, is transcribed at high levels when the glutamine pool is low. However, it is clear that a low glutamine pool is not required for high levels of *glnA* expression (e.g., results for wild-type *B. subtilis* grown on GABA or urea in batch culture [Table 2]), *glnA* mutant strain B276 grown on ammonia in batch culture [Table 6], and a *Salmonella gdh* mutant strain grown under ammonia-sufficient conditions in the chemostat [Table 4]). Schreier et al. (42), Deshpande et al. (9), and Fisher and Sonenshein (15) have all reported that *glnA* expression in *B. subtilis* can be high independent of a drop in the glutamine pool (12, 36). Potential roles of other metabolites in controlling *glnA* transcription in both *Bacillus* and *Salmonella* remain to be elucidated, as do the roles of GS itself and the TnrA product in *B. subtilis* (5, 12, 36, 38, 43, 49).

ACKNOWLEDGMENTS

We are very grateful to Susan Fisher, Hal Schreier, and Linc Sonenshein for gifts of materials and advice.

This work was supported by NIH grant GM38361 to S.K.

REFERENCES

1. **Aronson, J. N., J. F. Doerner, E. W. Akers, D. P. Borris, and M. Mani (ed.).** 1975. γ-Aminobutyric acid pathway of glutamate metabolism by *Bacillus thuringiensis*. American Society for Microbiology, Washington, D.C.
2. **Atkinson, M. R., and S. H. Fisher.** 1991. Identification of genes and gene products whose expression is activated during nitrogen-limited growth in *Bacillus subtilis*. J. Bacteriol. **173:**23–27.
3. **Bernlohr, R. W.** 1972. ^{18}Oxygen probes of protein turnover, amino acid transport, and protein synthesis in *Bacillus licheniformis*. J. Biol. Chem. **247:**4893–4899.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Brown, S. W., and A. L. Sonenshein.** 1996. Autogenous regulation of the *Bacillus subtilis glnRA* operon. J. Bacteriol. **178:**2450–2454.
6. **Dawes, I. W., and J. Mandelstam.** 1970. Sporulation of *Bacillus subtilis* in continuous culture. J. Bacteriol. **103:**529–535.
7. **Dean, D. R., and A. I. Aronson.** 1980. Selection of *Bacillus subtilis* mutants impaired in ammonia assimilation. J. Bacteriol. **141:**985–988.
8. **Dean, D. R., J. A. Hoch, and A. I. Aronson.** 1977. Alteration of the *Bacillus subtilis* glutamine synthetase results in overproduction of the enzyme. J. Bacteriol. **131:**981–987.
9. **Deshpande, K. L., J. R. Katze, and J. F. Kane.** 1981. Effect of glutamine on enzymes of nitrogen metabolism in *Bacillus subtilis*. J. Bacteriol. **145:**768–774.
10. **Deuel, T. F., A. Ginsburg, H. Yeh, E. Shelton, and E. R. Stadtman.** 1970. *Bacillus subtilis* glutamine synthetase. Purification and physical characterization. J. Biol. Chem. **245:**5195–5205.
11. **Donnellan, J. E., Jr., E. H. Nags, and H. S. Levinson.** 1964. Chemically defined synthetic media for sporulation and for germination and growth of *Bacillus subtilis*. J. Bacteriol. **87:**332–336.
12. **Fisher, S. H.** 1999. Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la difference! Mol. Microbiol. **32:**223–232.
13. **Fisher, S. H.** 1993. Utilization of amino acids and other nitrogen-containing compounds, p. 221–228. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
14. **Fisher, S. H., M. S. Rosenkrantz, and A. L. Sonenshein.** 1984. Glutamine synthetase gene of *Bacillus subtilis*. Gene **32:**427–438.
15. **Fisher, S. H., and A. L. Sonenshein.** 1984. *Bacillus subtilis* glutamine synthetase mutants pleiotropically altered in glucose catabolite repression. J. Bacteriol. **157:**612–621.
16. **Grandoni, J. A., S. B. Fulmer, V. Brizzio, S. A. Zahler, and J. M. Calvo.** 1993. Regions of the *Bacillus subtilis ilv-leu* operon involved in regulation by leucine. J. Bacteriol. **175:**7581–7593.
17. **Gutowski, J. C., and H. J. Schreier.** 1992. Interaction of the *Bacillus subtilis glnRA* repressor with operator and promoter sequences in vivo. J. Bacteriol. **174:**671–681.
18. **Hageman, J. H., G. W. Shankweiler, P. R. Wall, K. Franich, G. W. McCowan, S. M. Cauble, J. Grajeda, and C. Quinones.** 1984. Single, chemically defined sporulation medium for *Bacillus subtilis*: growth, sporulation, and extracellular protease production. J. Bacteriol. **160:**438–441.
19. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for *Bacillus*. John Wiley & Sons, New York, N.Y.

19a. **Hu, P.** Unpublished data.

20. **Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace.** 1998. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. **180:**366–376.
21. **Ikeda, T. P., A. E. Shauger, and S. Kustu.** 1996. *Salmonella typhimurium* apparently perceives external nitrogen limitation as internal glutamine limitation. J. Mol. Biol. **259:**589–607.
22. **Jolliffe, L. K., R. J. Doyle, and U. N. Streips.** 1981. The energized membrane and cellular autolysis in *Bacillus subtilis*. Cell **25:**753–763.
23. **Koch, A. L.** 1991. Quantitative aspects of cellular turnover. Antonie Leeuwenhoek **60:**175–191.
24. **Kustu, S. G., N. C. McFarland, S. P. Hui, B. Esmon, and G. F.-L. Ames.** 1979. Nitrogen control in *Salmonella typhimurium*: co-regulation of synthesis of glutamine synthetase and amino acid transport systems. J. Bacteriol. **138:**218–234.
25. **Kustu, S. G., and K. McKereghan.** 1975. Mutations affecting glutamine synthetase activity in *Salmonella typhimurium*. J. Bacteriol. **122:**1006–1016.

25a. **Leighton, T.** Personal communication.

26. **Lindroth, P., and K. Mopper.** 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence with o-phthaldialdehyde. Anal. Chem. **51:**1667–1674.
27. **Meers, J. L., D. W. Tempest, and C. M. Brown.** 1970. 'Glutamine(amide): 2-oxoglutarate amino transferase oxido-reductase (NADP)', an enzyme involved in the synthesis of glutamate by some bacteria. J. Gen. Microbiol. **64:**187–194.
28. **Miller, E. S., and J. E. Brenchley.** 1984. Cloning and characterization of

gdhA, the structural gene for glutamate dehydrogenase of *Salmonella typhimurium*. J. Bacteriol. **157:**171–178.
29. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Mura, U., P. B. Chock, and E. R. Stadtman.** 1981. Allosteric regulation of the state of adenylylation of glutamine synthetase in permeabilized cell preparations of *Escherichia coli*. J. Biol. Chem. **256:**13022–13029.
31. **Nath, K., and A. L. Koch.** 1971. Protein degradation in *Escherichia coli*. II. Strain differences in the degradation of protein and nucleic acid resulting from starvation. J. Biol. Chem. **246:**6956–6967.
32. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
33. **O'Hara, M. B., and J. H. Hageman.** 1990. Energy and calcium ion dependence of proteolysis during sporulation of *Bacillus subtilis* cells. J. Bacteriol. **172:**4161–4170.
34. **Pierce, J. A.** 1994. Ph.D. thesis. Stanford University, Stanford, Calif.
35. **Pierce, J. A., C. R. Robertson, and T. J. Leighton.** 1992. Physiological and genetic strategies for enhanced subtilisin production by *Bacillus subtilis*. Biotechnol. Prog. **8:**211–218.
35a. **Schmitz, R., and S. Kustu.** Unpublished data.
35b. **Schmitz, R., L. He, and S. Kustu.** Unpublished data.
36. **Schreier, H. J.** 1993. Biosynthesis of glutamine and glutamate and the assimilation of ammonia, p. 281–298. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
37. **Schreier, H. J., S. W. Brown, K. D. Hirschi, J. F. Nomellini, and A. L. Sonenshein.** 1989. Regulation of *Bacillus subtilis* glutamine synthetase gene expression by the product of the *glnR* gene. J. Mol. Biol. **210:**51–63. (Erratum, **226:**571, 1992.)
38. **Schreier, H. J., S. H. Fisher, and A. L. Sonenshein.** 1985. Regulation of expression from the *glnA* promoter of *Bacillus subtilis* requires the *glnA* gene product. Proc. Natl. Acad. Sci. USA **82:**3375–3379.
39. **Schreier, H. J., and C. A. Rostkowski.** 1995. *Bacillus subtilis glnR* mutants defective in regulation. Gene **161:**51–56.
40. **Schreier, H. J., C. A. Rostkowski, J. F. Nomellini, and K. D. Hirschi.** 1991. Identification of DNA sequences involved in regulating *Bacillus subtilis glnRA* expression by the nitrogen source. J. Mol. Biol. **220:**241–253.
41. **Schreier, H. J., T. M. Smith, and R. W. Bernlohr.** 1982. Regulation of nitrogen catabolic enzymes in *Bacillus* spp. J. Bacteriol. **151:**971–975.
42. **Schreier, H. J., T. M. Smith, T. J. Donohue, and R. W. Bernlohr.** 1981. Regulation of nitrogen metabolism and sporulation in *Bacillus licheniformis*, p. 138–141. *In* H. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
43. **Schreier, H. J., and A. L. Sonenshein.** 1986. Altered regulation of the *glnA* gene in glutamine synthetase mutants of *Bacillus subtilis*. J. Bacteriol. **167:** 35–43.
44. **Senior, P. J.** 1975. Regulation of nitrogen metabolism in *Escherichia coli* and *Klebsiella aerogenes*: studies with the continuous-culture technique. J. Bacteriol. **123:**407–418.
45. **Stadtman, E. R., U. Mura, P. B. Chock, and S. G. Rhee.** 1980. The interconvertible enzyme cascade that regulates glutamine synthetase activity, p. 41–59. *In* J. Mora and R. Palacios (ed.), Glutamine: metabolism, enzymology, and regulation. Academic Press, New York, N.Y.
46. **Tempest, D. W., J. L. Meers, and C. M. Brown.** 1970. Influence of environment on the content and composition of microbial free amino acid pools. J. Gen. Microbiol. **64:**171–185.
47. **Turner, R. J., Y. Lu, and R. L. Switzer.** 1994. Regulation of the *Bacillus subtilis* pyrimidine biosynthetic (*pyr*) gene cluster by an autogenous transcriptional attenuation mechanism. J. Bacteriol. **176:**3708–3722.
48. **Vandeyar, M. A., and S. A. Zahler.** 1986. Chromosomal insertions of Tn917 in *Bacillus subtilis*. J. Bacteriol. **167:**530–534.
49. **Wray, L. J., A. E. Ferson, K. Rohrer, and S. H. Fisher.** 1996. TnrA, a transcription factor required for global nitrogen regulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **93:**8841–8845.