A High Frequency of *Bacillus subtilis* Glutamine Auxotrophs Revert to Cold Sensitivity

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A large number of spontaneous conditional revertants of *Bacillus subtilis* glutamine auxotrophs were isolated by plating cells on minimal medium and incubating them at various temperatures. No heat-sensitive revertants were found among 2000 scored; however, revertants with a cold-sensitive glutamine requirement were quite common. Growth of the latter class ceased immediately upon transfer to the restrictive temperature implying that glutamine synthetase activity was affected. Mapping data are consistent with a genetic location in or near *glnA*, the structural gene for glutamine synthetase, for all cold-sensitive revertants tested. These revertants are likely to have second site suppressor mutations in *glnA* since many have altered levels or kinetics of glutamine synthetase activity or a changed pattern of feedback inhibition.

INTRODUCTION

The regulation of enzymes involved in assimilation of ammonia and other nitrogen sources in certain Gram-negative bacteria has been extensively studied (for review, see Tyler, 1978). By contrast, the control of nitrogen metabolism in bacilli is as yet poorly understood. There appear to be significant differences, however, in nitrogen metabolism between Gram-negative organisms, such as *Escherichia coli*, and bacilli. Most *Bacillus* species lack detectable glutamate dehydrogenase (EC 1.4.1.4) (Hong *et al.*, 1959). Consequently, the concerted activity of glutamate synthase (EC 1.4.1.13) and glutamine synthetase (GS; EC 6.3.1.2) are pivotal for nitrogen metabolism whether the ammonia concentration of the medium is high or low. Kane & Deshpande (1979) have reported that *Bacillus subtilis* contains detectable glutamate dehydrogenase active only during the late-exponential phase of growth. Secondly, some *Bacillus* species, including *B. subtilis*, lack a glutamyl-tRNA synthetase (Wilcox & Nirenberg, 1968). Glutamyl-tRNA^{Gln} is formed by first esterifying glutamate to tRNA^{Gln} then amidating the glutamate to form glutamyl-tRNA^{Gln}. The glutamine formed by GS is used therefore mainly or entirely as an amido donor in biosynthetic reactions. Finally, there is no direct evidence for *in vivo* adenylation of GS in *B. subtilis* (Deuel *et al.*, 1970; Gancedo & Holzer, 1968) as has been described by Ginsburg & Stadtman (1973) and Gancedo & Holzer (1968) for Gram-negative organisms.

In addition to the differences noted above, there is the possibility that GS may be involved in the regulation of sporulation (Elmerich & Aubert, 1972; Reyssset & Aubert, 1975). Overall, there appear to be a number of unique aspects to the regulation of nitrogen metabolism in bacilli. All of the glutamine auxotrophs previously isolated contained some GS activity,

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especially when Mn^{2+} was employed as the activating cation in assays (Dean *et al.*, 1977; Dean & Aronson, 1980). The inability to isolate mutants devoid of activity implied that the enzyme had an indispensable function, at least for the range of selective conditions used. Conditional revertants were isolated, therefore, in order to study possible pleiotropic regulatory properties of this enzyme. While no high temperature-sensitive revertants were found, there was a high frequency of cold-sensitive revertants. The physiology of these mutants has been studied in order to understand the basis for the cold-sensitivity.

METHODS

Bacterial strains and cultivation. The strains of *Bacillus subtilis* used in this study are listed in Table 1. Glutamine auxotrophs were isolated from JH641 or JH642 by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Adelberg *et al.*, 1965) or ethyl methanesulphonate (EMS) (Ito & Spizizen, 1971). Following mutagenesis, glutamine auxotrophs were enriched by penicillin treatment (Dean *et al.*, 1977) of cultures resuspended in a minimal salts medium (Schaeffer *et al.*, 1965), or in minimal medium supplemented with all amino acids (each at $50 \mu g ml^{-1}$) except glutamine or with 0.1% (w/v) nutrient broth. Appropriate transformations were then performed to introduce the mutant alleles into isogenic backgrounds (Dean *et al.*, 1977). Spontaneous revertants of mutant DRD2 were obtained by spreading cells on minimal agar lacking glutamine followed by incubation at $30^\circ C$. Colonies to be tested were streaked twice to give single colonies under selective conditions to assure purity.

Conditional revertants were spontaneously occurring variants and were isolated as follows. A single colony of the appropriate glutamine auxotroph growing on tryptose blood agar (Difco) plus 1 mg glutamine ml^{-1} was used to inoculate a flask containing Penassay broth (Difco) plus glutamine and grown to the late-exponential phase at $37^\circ C$. Cells were collected by centrifugation, resuspended in an equal volume of minimal medium (Spizizen, 1958), and about 10^8 were plated on minimal medium lacking glutamine. The plates were incubated at either $45^\circ C$ or $30^\circ C$ for 2–7 d. All revertants studied sporulated well and were stored as spores in sterile silica gel.

Preparation of crude extracts. Crude extracts were prepared from cells (50 ml) grown in minimal medium plus 2 mg glutamine ml^{-1} to the late-exponential phase at $45^\circ C$. Cells were harvested by centrifugation and washed twice with 50 mM-imidazole/HCl (pH 7.0). The cell pellet was resuspended in 2–3 ml of the same buffer containing 100 μg lysozyme ml^{-1} and incubated at $37^\circ C$ for 30–60 min. To ensure complete lysis and reduced viscosity, extracts were placed on ice and sonicated for 30–60 s in a Branson sonicator fitted with a microtip using 1 s pulses.

Enzyme assay. Glutamine synthetase (GS) was assayed by the glutamyl transferase method as described previously (Dean *et al.*, 1977). The reaction mixture contained 50 mM-imidazole/HCl (pH 7.0), 10 mM-ATP, 100 mM-glutamate, 40 mM-hydroxylamine and either 40 mM-MgSO₄ or 10 mM-MnCl₂. The reaction, in a final volume of 2 ml, was initiated by the addition of crude extract and terminated by the addition of ferric chloride reagent (Elliot, 1955). For spot assays of individual colonies, a single colony was scraped off with a toothpick and resuspended in 150 μl of the above reaction buffer (Mn^{2+} -stimulated) supplemented with lysozyme and cetyltrimethylammonium bromide, each at 100 $\mu g ml^{-1}$. After incubation at $37^\circ C$ for 30 min, the reaction was terminated by the addition of ferric chloride reagent and the amount of colour formed was estimated visually. Results of GS assays are expressed as nmol γ -glutamyl hydroxamate formed min^{-1} (mg protein)⁻¹. All values are the average of at least two determinations. Protein was assayed by the Lowry method.

Transformation. Transformation was done as described by Anagnostopoulos & Spizizen (1961) or Bott & Wilson (1968). Transformations were performed by treating competent *B. subtilis* cultures with 0.1 to 0.001 μg DNA ml^{-1} purified from donor cells according to the procedure of Marmur (1961).

Table 1. *Bacterial strains*

Strain	Genotype	Source
JH641	<i>pheA1 trpC2 ilvC</i>	J. Hoch
JH642	<i>pheA1 trpC2</i>	J. Hoch
168 I ⁻ Thy ⁻	<i>trpC2 thyA thyB</i>	J. Hoch
DRD2	<i>pheA1 trpC2 ilvC glnA100</i>	Dean <i>et al.</i> (1977)
DRD101	<i>pheA1 trpC2 ilvC gln^{cs}</i>	EMS mutagenesis of DRD2 (Dean, 1979)
DRD4	<i>pheA1 trpC2 glnA103</i>	Dean <i>et al.</i> (1977)
DRD7	<i>pheA1 trpC2 glnA5</i>	EMS mutagenesis of JH642 (Dean, 1979)
DRD17	<i>pheA1 trpC2 glnA108</i>	NTG mutagenesis of JH642 (Dean, 1979)
DRD41	<i>pheA1 trpC2 glnA201</i>	EMS mutagenesis of JH642 (Dean, 1979)
DL10	<i>pheA1 trpC2 ilvC gln^{cs}</i>	From DRD2 (this study)
DL12, 13, 14, 15	<i>pheA1 trpC2 ilvC gln⁺</i>	From DRD2 (this study)
DL4, 5, 11	<i>pheA1 trpC2 gln^{cs}</i>	From DRD4 (this study)

Table 2. *Relative numbers of revertants of glutamine auxotrophs when selected at different temperatures*

About 10^8 cells of each parent were plated on minimal medium lacking glutamine (see Methods) and incubated at 30 °C or 45 °C for 2–7 d. The average of the number of colonies arising on duplicate plates was determined. In addition, revertants arising at 45 °C were tested for the Gln^{cs} phenotype.

Parent	No. of revertants		Percentage of 45 °C revertants with Gln ^{cs} phenotype
	At 30 °C	At 45 °C	
DRD2	22	47	50
DRD4	0	55	58
DRD7	220	280	NT
DRD17	76	120	17
DRD41	ca 700	ca 500	NT

NT, Not tested.

RESULTS

Isolation of revertants

Spontaneously occurring glutamine-independent revertants were isolated from five glutamine auxotrophs altered in *glnA*. Of approximately 2000 revertants isolated from three of these strains (DRD2, DRD17 and DRD41) in 12 independent selections at 30 °C, none were found to have a temperature-sensitive glutamine requirement when scored at 45 °C. In contrast, all three strains from which revertants were isolated at 45 °C yielded cold-sensitive glutamine auxotrophs at a high frequency.

The apparent reversion frequency was dependent on the temperature at which the revertants were selected (Table 2). In most, but not all cases, selection at the higher temperature resulted in a higher apparent reversion frequency. Although the number of revertants isolated from a particular auxotroph at a particular temperature varied among experiments, the ratio of the reversion frequencies at 30 °C to 45 °C for a particular auxotroph was generally reproducible. Three independent selections for spontaneous revertants of DRD4 at 45 °C yielded an average reversion frequency of 7×10^{-7} .

The mutation conferring glutamine auxotrophy in strain DRD2 (*glnA100*) also resulted in a 6- to 15-fold overproduction of GS protein (Dean *et al.*, 1977). The Mn²⁺-stimulated GS activity of several revertants isolated at 45 °C was determined by spot assays of single colonies (see Methods) and a majority (10/16) retained the high activity of the parental strain. When selection was performed at 30 °C, however, nearly all of the revertants examined (30/32) had Mn²⁺-stimulated GS levels similar to the wild-type strain, JH641 (Table 4). In both experiments, several colonies were used to start cultures in liquid medium and the GS activity estimated by the spot assay was verified by the more quantitative assay. Both experiments were performed with revertants pooled from four independent selections to preclude the possibility that random fluctuations in the frequency of different revertant types might bias the results.

Growth studies

On shifting cultures growing in minimal medium with ammonium sulphate as the sole nitrogen source from 45 °C to 30 °C, cultures of the wild-type strain JH641 continued to grow, but cultures of the cold-sensitive revertant DRD101 ceased growth immediately (Fig. 1). Addition of glutamine or glutamate permitted growth of DRD101 at the lower temperature (data not shown). The immediate effect on growth after shifting cold-sensitive revertants (others behaved in the same way as DRD101) to 30 °C is consistent with a defect in GS activity rather than in GS synthesis. If the latter were the only alteration in these mutants, then

Fig. 1. Effect of a temperature shift on the growth of strains in minimal medium with $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source. Cultures growing at 45°C were shifted to 30°C at 4 h. Absorbance at 610 nm was measured in a Beckman DB-G spectrophotometer. Strains: $\blacktriangle$, JH641; $\bullet$, DRD101; $\circ$, DRD2.

Table 3. *Genetic mapping of glnA revertants*

Transformation crosses were performed as described in Methods.

Donor	Recipient	Phenotype of Thy^+ transformants			Percentage cotransfer*
		Gln $^+$	Gln $^-$	Gln cs	
DRD2 (<i>glnA100</i>)	168 I $^-$ Thy $^-$	116	9	—	7.2
DRD101 (<i>gln^{cs}</i>)	168 I $^-$ Thy $^-$	423	0	50	10.6
DL5 (<i>gln^{cs}</i>)	168 I $^-$ Thy $^-$	63	0	9	12.5
DL10 (<i>gln^{cs}</i>)	168 I $^-$ Thy $^-$	264	2	16	6.4
DL12 (<i>gln⁺</i>)	168 I $^-$ Thy $^-$	242	0	—	0

* Percentage of Thy^+ transformants that acquired the Gln $^-$ or Gln cs phenotype.

pre-existing enzyme should have been able to sustain growth for some period after the shift-down.

Genetic mapping of revertants

Two types of transformation crosses were performed to determine the genetic location(s) of the lesions conferring the Gln cs phenotype. Genetic linkage to *thyA* was tested by preparing DNA from several revertant strains and using this DNA to transform strain 168 I $^-$ Thy $^-$ to Thy^+ . Thy^+ transformants were selected at 45°C , to suppress *thyB⁺* transformants (Neuhard *et al.*, 1978), in the presence of glutamine and scored for the Gln $^-$ phenotype at both 30°C and 45°C . If the lesion responsible for the Gln cs phenotype were closely linked to the parental *glnA* marker, *gln^{cs}* should show the same linkage to *thyA* as does *glnA*. If the *gln^{cs}* lesion were unlinked to the *glnA*–*thyA* region, however, none of the *thyA⁺* transformants would be expected to be *gln^{cs}* (or *gln⁺* in the case of prototrophic revertants). This cross was performed for three cold-sensitive revertants and one prototrophic revertant (Table 3). The results were consistent with a location for *gln^{cs}* at or near *glnA*, since the percentage cotransfer values were similar to those previously found for *glnA* and *thyA* (Dean *et al.*, 1977).

Since the markers tested mapped in the *glnA* region, we used another genetic test to map the lesion more precisely. DNA was prepared from strain JH641 (*glnA⁺*) and used to transform strain DL11 (*gln^{cs}*) to glutamine prototrophy at 30°C . The resulting glutamine

Table 4. Growth rate and GS activity of revertants

Strain	Temp.*	Specific GS activity†		Percentage activity remaining in presence of 5 mM inhibitor			Mean doubling time‡ (min)
		With Mg ²⁺	With Mn ²⁺	Alanine	Glycine	Glutamine	
JH641	—	10.5	7.6	54	79	26.7	46
DRD2 (<i>glnA100</i>)	—	3.9	45.7	100	99	89	—
DRD101 (<i>gln^{cs}</i>)	37 °C	18.3	32.8	96	97	94	75
DL10 (<i>gln^{cs}</i>)	45 °C	28.2	36.1	100	99	83	60
DL12 (<i>gln⁺</i>)	30 °C	6.5	4.2	NT	NT	NT	NT
DL13 (<i>gln⁺</i>)	30 °C	7.1	4.8	NT	NT	NT	NT
DL14 (<i>gln⁺</i>)	30 °C	5.0	3.3	NT	NT	NT	NT
DL15 (<i>gln⁺</i>)	30 °C	2.1	22.3	NT	NT	NT	NT
DRD4 (<i>glnA103</i>)	—	5.1	12.7	94	100	100	—
DL4 (<i>gln^{cs}</i>)	45 °C	5.4	22.7	54	80	NT	64
DL5 (<i>gln^{cs}</i>)	45 °C	3.3	12.9	68	86	NT	ca 150
DL11 (<i>gln^{cs}</i>)	45 °C	3.9	43.7	75	100	100	80

NT, Not tested.

* Temperature at which revertant was isolated.

† Specific activities are expressed as nmol γ -glutamyl hydroxamate formed min⁻¹ (mg protein)⁻¹.

‡ Growth rate was measured at 45 °C in a minimal medium lacking glutamine (see legend to Fig. 1).

prototrophs would be expected to carry the wild-type *glnA*⁺ allele. Thirteen transformants were purified by two isolation streakings under selective conditions. DNA was prepared from each of these strains and used to transform strain DRD4 (the parent of DL11) to glutamine prototrophy at 45 °C. If the *gln^{cs}* gene were unlinked to the *glnA*⁺ locus, then the DNA from the donors should transform DRD4 to either *gln⁺* or *gln^{cs}* at approximately equal frequencies. Nearly all transformants (940 of 942) from the above crosses were *gln⁺* indicating very close linkage between the *glnA103* mutation in strain DRD4 and the *gln^{cs}* lesion in DL11. As a control, strain DRD4 was transformed with DNA from DL11 (*gln^{cs}*) and all transformants were *gln^{cs}*.

Biochemical studies

The genetic results were consistent with the hypothesis that the cold-sensitive revertants were second site mutations in the GS structural gene. It is difficult to establish this close linkage unambiguously due to the paucity of suitable genetic markers in the *glnA* region of the chromosome and the lack of a routine procedure for complementation analysis in *B. subtilis*. For these reasons we characterized the GS activity of several revertants biochemically (Table 4). The Mg²⁺-stimulated activities in revertants were generally lower than those observed for the wild-type strain JH641. All cold-sensitive revertants examined had Mn²⁺-stimulated activities as high, or higher, than the *gln* parent strain and higher than that observed for strain JH641, consistent with the results obtained using the spot assay described above. In contrast, prototrophic revertants isolated at 30 °C had Mn²⁺-stimulated activities that were similar to that of strain JH641 or were intermediate between the activities observed for JH641 and the auxotrophic parent.

There was no obvious correlation between the amount of GS activity observed in crude extracts and the growth rate measured at 45 °C in the absence of glutamine (Table 4). It is possible that the lack of correlation is due to alterations in the affinity of GS for one or more of its substrates (Dean *et al.*, 1977). This altered affinity may not be apparent in assays of crude extracts performed with saturating substrate concentrations. At least one revertant contained GS with altered kinetic properties (see below).

Feedback inhibition of GS activity in crude extracts from several revertants by alanine, glycine or glutamine was also measured (Table 4). In contrast to the auxotrophic parent

strain, GS activity of revertants of DRD4 was inhibited by alanine and/or glycine. Results for strain DL11 suggest that the physical sites on the GS protein responsible for feedback inhibition by alanine and glycine may be separate since the GS activity of this strain was partially inhibited by alanine but not by glycine.

Growth of strains DL5 and DL11, but not of any other strains tested, was strongly inhibited by addition of 10 mM-alanine to minimal medium. This inhibition was reversed by simultaneous addition of glutamine (data not shown). These two strains may possess relatively low levels of GS activity *in vivo* (as judged by their growth rates in the absence of glutamine) but near normal levels of feedback inhibition of alanine, and thus be more susceptible to metabolite imbalance. This interpretation is consistent with earlier kinetic studies (Deuel & Stadtman, 1970; Deuel & Prusiner, 1974) that show that *B. subtilis* GS is regulated by feedback inhibition rather than by an adenylation-deadenylation system. The amount of feedback inhibition by alanine, glycine or glutamine was the same irrespective of whether the assay was performed at 25 °C or 45 °C for any strain tested.

DISCUSSION

Several hundred glutamine auxotrophs have been isolated after treatment of *B. subtilis* with a variety of mutagens (or spontaneously) and using several enrichment techniques (Dean, 1979). The inability to find mutants totally devoid of GS activity implied that there was selective pressure to sustain some level of GS antigen, perhaps related to pleiotropic regulatory functions of this enzyme. All of the mutations were mapped close to *thyA* and very close to each other on the basis of recombination indices. Since some of the mutants studied have GS activities with altered properties (electrophoretic, kinetic or temperature stability), it is likely that many have alterations in the structural gene although some could be altered in a closely linked regulatory gene.

All of the revertants conferring cold sensitivity mapped in the *thyA-glnA* region of the *B. subtilis* chromosome, and for at least one revertant the mutation was very closely linked to *glnA*, consistent with the view that most, if not all, of the revertants examined may have secondary alterations in *glnA* that suppress the original auxotrophic mutation. This conclusion is supported by the observation that GS from many of the revertants, in contrast to the *gln* parent, was inhibited by alanine and/or glycine (Table 4). A revertant that did not regain the ability to be feedback-inhibited (DL10) had altered kinetic properties of its GS in response to an increasing concentration of sodium glutamate. The $V_{\max}$ appeared to be altered when assays were done at either 25 °C or 45 °C. Other revertants were not tested for altered kinetic properties.

The possibility that the mutations conferring a Gln⁺ or Gln^{cs} phenotype lie in a *glnA*-linked regulatory gene whose product affects GS activity, rather than in *glnA* itself, has not been disproved. However, there is no evidence for a GS cascade regulatory system in *B. subtilis* (Gancedo & Holzer, 1968; Deuel & Stadtman, 1970; Deuel & Prusiner, 1974) as there is in enteric bacteria, and it has been proposed that *B. subtilis* GS activity is regulated directly by feedback inhibition. Our previous results are consistent with this idea in that all 50 glutamine auxotrophs mapped showed similar linkage to *thyA* and were very closely linked to each other, but the data are not adequate to rule out a closely linked regulatory locus.

Cold-sensitivity in micro-organisms has been shown to be due to mutations in a wide variety of genes and to result from several different mechanisms. Perhaps the most familiar examples are alterations in components of the translational apparatus. Many alterations affecting ribosomal components confer a cold-sensitive phenotype (Jaskunas *et al.*, 1974). Cold-sensitive mutations affecting a tRNA-modifying enzyme (Brenchley & Ingraham, 1973) and RNA polymerase (Gragarov *et al.*, 1980) have also been described. It is possible, therefore, that the phenotypes of the revertants were due to indirect suppression of glutamine auxotrophy by secondary mutations in components of the translational or transcriptional

apparatus. This is highly unlikely since no genes involved in transcription or translation have been mapped in the *glnA* region of the *B. subtilis* chromosome (Henner & Hoch, 1980) and the revertants examined in this study were altered in GS activity, not synthesis, and displayed alterations in the GS protein as noted above.

Cold-sensitive metabolic defects have been noted by others. In some cases, cold-sensitivity is due to hypersensitivity to feedback inhibition at the restrictive temperature (O'Donovan & Ingraham, 1965; Abd-El-Al & Ingraham, 1969). In all *gln^{cs}* revertants examined, the level of feedback inhibition observed did not vary as a function of temperature. This observation is not conclusive since GS activity in these revertants may be hypersensitive to inhibition by metabolites that normally have little physiological effect, or to a particular combination of metabolites at the restrictive temperature. Nevertheless, it seems likely that the *gln^{cs}* phenotype is due to differential levels of enzyme activity *per se* at the permissive and restrictive temperatures, a conclusion supported by the observation that in at least one cold-sensitive revertant (DL11) the ratio of the GS activities measured at 25 °C and 45 °C is approximately twofold greater than that observed for the wild-type strain JH641 (data not shown). Cold-sensitivity due to inhibition of enzyme activity has been demonstrated in at least two other cases (Hoffman & Ingraham, 1970; Kohno & Roth, 1979).

The reason for the prevalence of cold-sensitive revertants was suggested by the 10- to 20-fold greater activity of GS when assayed at 45 °C versus 25 °C. The difference was reflected physiologically by the observation that the glutamine requirement of strain DRD2 is slightly leaky at 45 °C but not at 30 °C. It is possible that *B. subtilis* GS activity and/or stability is greater at higher temperatures at least in some of the *glnA* mutants. This stability could explain the higher apparent reversion frequency at 45 °C since at higher temperatures partial revertants would regain enough enzyme activity to allow growth, whereas at 30 °C the selection for a return to the wild-type or near wild-type protein conformation would be more stringent. The most likely explanation, although not the only possible one, is that most or all of the revertants studied represent second site mutations in *glnA* that suppress or partially suppress the original mutation.

Finally, it may be noted that the isolation of spontaneous revertants at 30 °C that regain both prototrophy and wild-type levels of GS activity (Table 4) supports our earlier conclusion (Dean *et al.*, 1977, Dean & Aronson, 1980) that the glutamine requirement and overproduction of GS protein in strain DRD2 (as well as some other glutamine auxotrophs) is due to a single mutation. The regulation of production of GS would thus involve the structural gene and possibly a closely linked regulatory region.

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