GLUTAMINE-REQUIRING MUTANTS OF BACILLUS SUBTILIS

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Received November 3, 1977

SUMMARY

Two glutamine-requiring (Gln^-) mutants of Bacillus subtilis SMY were deficient in glutamine synthetase activity in vitro. The Gln^- mutants sporulated poorly unless glutamine was provided at high concentrations. The differential rate of histidase synthesis following induction was 4- to 6-fold higher in the Gln^- mutants than in wild-type cells. In addition, glucose repression of utilization of alternative carbohydrates appeared to be partially relieved in the Gln^- mutants.

INTRODUCTION

In Bacilli, NH_4^+ is incorporated into cellular metabolites by the combined action of glutamine synthetase (GS) and glutamate synthase (1, 2). Glutamate dehydrogenase, an additional NH_4^+ assimilation enzyme present in gram-negative bacteria, is not usually present in significant amounts in Bacilli (2,3; S. Fisher, unpublished results), although such an enzyme activity may be present under certain environmental conditions (C. Elmerich and J.-P. Aubert, personal communication). GS from B. subtilis, unlike the analogous enzyme of gram-negative bacteria, is not known to undergo any modification in vivo or in vitro (1).

In gram-negative bacteria the expression of genes involved in nitrogen assimilation has been shown to be tightly regulated in response to the availability of NH_4^+ and other nitrogen-containing compounds (4). There is strong

Abbreviations: glutamine synthetase, GS; histidine-utilization operon, hut.

ISSN 0006-291X

evidence that GS and other proteins that modify its structure and activity play a central role in this regulation (4). The ability of B. subtilis to alter the expression of genes involved in NH_4^+ assimilation in response to NH_4^+ limitation has not been demonstrated, although GS activity increases 6-fold in response to NH_4^+ limitation (1).

Since spore formation in Bacilli is inhibited by the simultaneous presence of certain carbon, nitrogen and phosphorous sources, it has been proposed that one or more catabolites containing these elements (e.g., glutamine or a glutamine derivative [5]) may regulate the initiation of sporulation (6). Reysset and Aubert have proposed that GS may act as a positive regulator for genes required for sporulation since most glutamine-requirers isolated in B. megaterium were deficient in sporulation (7). To learn more about the interrelationships of GS, sporulation and cellular metabolism, we have isolated glutamine-requiring (Gln^-) mutants of B. subtilis, a sporulating organism with a known genetic system. The mutations we have studied have pleiotropic effects, causing conditional asporogeny and altering carbohydrate metabolism and the expression of histidase. Other Gln^- mutants with different properties have recently been isolated from B. subtilis by Dean and Aronson (8) and by Bott et al. (9).

MATERIALS AND METHODS

Bacterial Strains: Wild-type B. subtilis SMY was obtained from J. Segall. Strains QB 943 (pyrD trpC2 thyA thyB ilvA1) and QB 922 (gltA292 trpC2) were from F. Kunst. The derivatives of other strains are described in the text. Genotype symbols are those of Young and Wilson (10). The notations Aux^- and Gln^- indicate an undefined auxotrophy and glutamine requirement, respectively.

Media and Growth: Cells were grown in TSS medium unless stated otherwise. TSS medium contained 0.05 M Tris pH 7.5, 40 µg/ml $FeCl_3$-Na_3 Citrate, 2.5 mM K_2HPO_4 and 0.02% $MgSO_4 \cdot 7H_2O$. The carbon source (0.5% final concentration) was glucose or Na_3·Citrate. The nitrogen source was NH_4Cl (0.2%) and/or glutamine (0.2%). DSM medium (11), a minimal medium (MSS;12) and 121 salts (13) have been previously described. 121D medium contained 121 salts, 0.5% glucose, 2.5 mM K_2HPO_4, 0.1% casamino acids and 40 µg/ml $FeCl_3$-Na_3 Citrate.

Isolation of Mutants: To isolate glutamine-requiring mutants of strain SMY,

heavily mutagenized cells were grown in 121D medium containing glutamine (200 µg/ml) and then transferred to 121D medium lacking glutamine but containing 500 µg/ml of the 19 other amino acids. After one mass doubling, streptomycin (3 mg/ml) was added. One hour later the cells were harvested, washed, and plated on MSS medium containing 40 µg/ml glutamine. The survivors ($1/10^4$) were screened for glutamine requirers by replicating onto MSS plates lacking glutamine.

Glutamine auxotrophs, including strains SF022, SF070, SF071 and SF073, grew well on glucose+glutamine+NH_4Cl medium, but did not grow on glucose+NH_4Cl or glucose+glutamate (0.006%)+NH_4Cl medium. Other mutants, whose glutamine requirement could be partially spared by glutamate were isolated, but were not investigated further. The gln mutations of the four mutants studied were transferred into the parental genetic background by transforming (11) the auxotrophic strain SF9 (see below) with saturating amounts of DNA. These Aux^+ Gln^- strains (SF22, SF70, SF71 and SF73) were used for all subsequent studies.

To obtain an auxotrophic mutant of SMY needed to backcross gln mutations into the parental strain, mutagenized cells of SMY were treated with streptomycin as above, plated on DSM medium, and screened for inability to grow on MSS plates. The phenotype of one strain (SF9) was designated Aux^-. Glutamine is not the nutrient required by SF9, but the auxotrophy has not been characterized further. A prototrophic transformant of SF9 was called SF10 and was used as the wild-type strain in all subsequent studies.

RESULTS

GS activity in crude extracts of the backcrossed Aux^+ Gln^- strains was analyzed using an assay (to be published elsewhere) that measures the conversion of [^{14}C]-glutamate to [^{14}C]-glutamine. Wild-type extracts synthesized 366 nmoles of [^{14}C]-glutamine per hr per mg protein. No synthesis of glutamine above background levels was observed with extracts of any Gln^- mutant. The background was sufficiently high, however, that we can only conclude that the Gln^- mutants had less than 10% of the GS activity seen in wild-type extracts.

The Gln^- mutants reverted to Gln^+ at frequencies [10^{-8}(SF22 and SF70) and 10^{-7}(SF71 and SF73)] consistent with the Gln^- phenotype being due to a single mutation. By crossing the Gln^- strains with each other and obtaining recombination indices (R.I.) for the gln mutations, we concluded that all four gln mutations are closely linked (R.I. = 0.001-0.19). The pairs of mutations gln-22 and gln-70 (R.I. = 0.001) and gln-71 and gln-73 (R.I. < 0.002) may be identical.

The gln mutation of SF22 was introduced into the motile strain QB 943

Table 1

DEPENDENCE OF SPORE FORMATION ON

GLUTAMINE IN RESUSPENSION MEDIUM[1]

Strain	Glutamine Conc. (%)	CFU/ml at T_0 (= A)[2]	Heat Resistant CFU/ml at T_{20} (= B)[3]	Sporulation Frequency (%) = (B/A x 100)
SF10	0	2.8×10^8	2.9×10^8	103
	0.005	2.8×10^8	3×10^8	107
	0.05	2.8×10^8	2.1×10^8	75
	0.2	2.8×10^8	2.8×10^8	100
SF73	0	1.8×10^8	$<10^5$	< 0.1
	0.005	1.8×10^8	$<10^5$	< 0.1
	0.05	1.8×10^8	8×10^7	44
	0.2	1.8×10^8	1.3×10^8	72
SF22	0	1×10^8	$<10^5$	< 0.1
	0.005	1×10^8	$<10^5$	< 0.1
	0.05	1×10^8	2×10^7	20
	0.2	1×10^8	5.8×10^7	58

1) Mutant and wild-type cells were grown to a density of 2×10^8 cells per ml in DSM medium supplemented with 0.15% glutamine, harvested and resuspended in Sterlini-Mandelstam medium (18) without added KH_2PO_4. Glutamine was added as indicated.

2) Colony-forming units per ml at time of resuspension.

3) Colony-forming units per ml at 20 hr. after resuspension that survived heating to 80° - 85°C for 10 min.

by transformation. The resultant strain, SF227 (pyrD thyA thyB ilvA1 gln-22), was transduced to Gln^+ with phage PBS1 (14) grown on QB 9221, a Trp^+ derivative of QB 922. The gln-22 mutation was 94% cotransduced with thyA and 25% cotransduced with gltA292. Analysis of recombinant classes suggested gln-22 - thyA - gltA292 as the order of the markers.

Since most GS mutants isolated by Aubert and coworkers were asporogenous (7), we examined the ability of our Gln^- mutants to sporulate. The Gln^- mutants

Table 2

DIFFERENTIAL RATE OF HISTIDASE SYNTHESIS

Medium	SF10 (WT)	SF22 (Gln^-)	SF221 (Gln^+)	SF222 (Gln^+)	SF70 (Gln^-)	SF71 (Gln^-)	SF73 (Gln^-)	SF731 (Gln^+)
Glucose+ Glutamine	0.002	0.0015					0.002	
Glucose+ Histidine+ Glutamine	0.022	0.105	0.019	0.015	0.12	0.15	0.10	0.018
Citrate+ Histidine+ Glutamine	0.38	0.21			0.23	0.21	0.20	

Histidase was assayed by the method of Hartwell and Magasanik (19). Exponentially growing cells in TSS medium (C source as indicated) that had been induced for 1 hr. with histidine were made permeable by the addition of 0.01% cetyltrimethyl ammonium bromide and 0.05% sodium deoxycholate, mixed for 5 sec. and stored on ice (20). One unit of histidase activity was the amount of enzyme required to produce 27.2 nmoles of urocanic acid per ml of culture per hr. at 30°C, pH 9.4. The differential rate of histidase synthesis was the increase in histidase activity per unit increase in culture turbidity (measured with Klett-Summerson colorimeter). The numbers given are the average of 2-6 determinations in each case.

failed to sporulate in the absence of glutamine or with glutamine present at the level of an amino acid supplement (0.005%) (Table 1). When glutamine was present at 0.05% or 0.2%, however, the Gln^- mutants sporulated at nearly wild-type levels. Gln^+ revertants and transformants sporulated normally in the presence or absence of glutamine. Thus the apparent asporogeny of our mutants is conditional and can be relieved by a high concentration of glutamine.

To see if our mutants were altered in the regulation of a nitrogen utilization operon, we examined the expression of histidase in the Gln^- mutants. The differential rate of histidase synthesis in uninduced mutant cells in glucose+glutamine medium did not differ significantly from wild-type levels. In induced cells in the same medium this rate was 4- to 6-fold higher than that

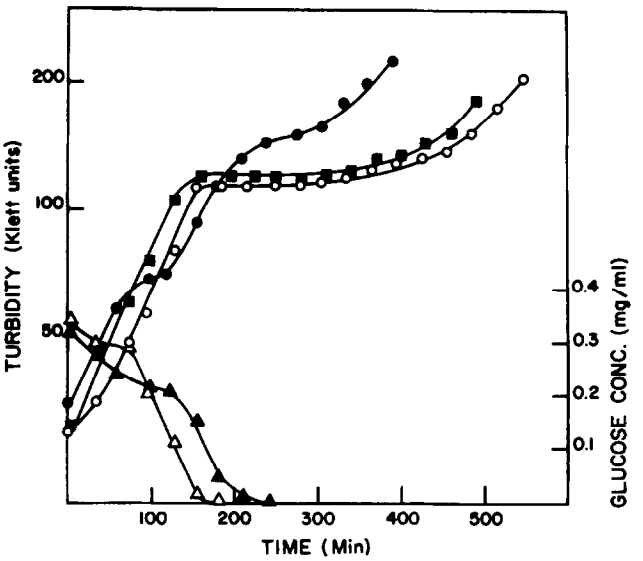

Figure 1. Cells in exponential growth in glucose+glutamine medium were harvested and resuspended in TSS medium containing 0.04% glucose, 0.2% citrate and 0.2% glutamine. Growth at 37°C was monitored with a Klett-Summerson colorimeter. To determine glucose concentrations, 1 ml culture samples were boiled for 10 min, centrifuged to remove debris and assayed with the Worthington Ultramicro-Glucostat Kit.

Symbols: Growth, SF10 -o-o-; SF22 -●-●-, SF222 -■-■-; Glucose concentrations, SF10 -Δ-Δ-, SF22 -▲-▲-.

observed in wild-type cells (Table 2). This increased rate of histidase synthesis in the Gln^- mutants was apparently due to the gln mutation since a Gln^+ revertant of SF22 (called SF221) and Gln^+ transformants of SF22 and SF73 (SF222 and SF731) all synthesized histidase at the wild-type rate in glucose+glutamine +histidine medium (Table 2). The differential rate of histidase synthesis in the Gln^- mutants in citrate+glutamine+histidine medium was at most 2-fold greater than in the mutants in glucose+glutamine+histidine medium and did not reach the maximal level of expression seen in wild-type cells in citrate+glutamine+histidine medium (Table 2).

To see if overproduction of histidase was due to a general defect in glucose repression, we examined the effect of glucose on utilization of citrate in the Gln^- mutants. Wild-type cells in a diauxic growth experiment showed an

initial period of growth on glucose that was followed by a plateau after the glucose was exhausted (Figure 1). Growth presumably resumed only after the proteins needed to transport and metabolize citrate had been synthesized. By contrast, Gln^- mutants (e.g., SF22) grew to a higher cell density during the initial period of growth and showed a greatly diminished plateau period. This altered pattern of diauxic growth was due to the gln mutation since Gln^+ transformants of SF22 (Figure 1) and SF73 (data not shown) showed wild-type patterns of diauxic growth. Similar diauxic growth patterns have been observed for the glucose-repressed carbon sources inositol, sorbitol, and maltose (data not shown).

DISCUSSION

The glutamine-requiring mutants of B. subtilis SMY described here have mutations linked to thyA that cause deficiencies in GS activity in vitro and alterations in diauxic growth patterns and histidase expression. Bott et al. (9) have isolated glutamine-requiring strains of B. subtilis 168 which have mutations that are also linked to thyA. Dean and Aronson (8) have isolated mutants of B. subtilis that are likely to have lesions in the structural gene for GS. The mutations in these strains also map near thyA. Expression of histidine and arginine degradation operons in the latter mutants under conditions of N limitation is normal, however (D. Dean, personal communication). Preliminary immunological experiments with our Gln^- mutants suggest the presence of mutant proteins that cross-react with antibody to wild-type GS. While such a result is consistent with our Gln^- mutants carrying GS structural gene mutations, it does not rule out the possibility that they are mutated in a gene regulating GS synthesis and/or modification.

The asporogeny of our mutants appears to be due to their high glutamine requirement for sporulation as has been observed previously for other amino acid auxotrophs (15). Our results, however, do not contradict the notion

that GS is a positive regulator of sporulation, since we cannot rule out the possibility that other GS mutants of B. subtilis would fail to sporulate even in the presence of glutamine or that high internal glutamine concentrations in the Gln^- mutants are needed to allow GS to carry out a regulatory role in sporulation.

Two mechanisms can be invoked to explain the overproduction of histidase in glucose-containing medium and the altered diauxic growth patterns in the Gln^- mutants. According to one model wild-type GS plays a direct regulatory role in glucose repression of the utilization of both carbon and nitrogen sources. An alternative explanation is that while GS plays a direct regulatory role in histidase expression, the altered diauxic growth pattern is an indirect result of the gln mutation. Since the TCA cycle is the link between carbon source dissimilation and NH_4^+ assimilation and since, in B. subtilis, several enzymes of the TCA cycle are repressed by the simultaneous presence of glucose and glutamate (16), it would be reasonable for GS, the key enzyme in NH_4^+ assimilation, to play a role in this repression. In a cell deficient in GS the TCA cycle might be derepressed. Such an event would allow glutamine to be used as an energy source even in the presence of glucose. This would result not only in a greater production of cell mass in the initial diauxic growth phase in the Gln^- mutants, but would also facilitate subsequent expression of glucose-repressed genes by providing energy immediately after glucose is exhausted.

The partial derepression of histidase synthesis in the Gln^- mutants is less easily explained. In K. aerogenes there is strong evidence that GS is responsible for the stimulation of hut operon expression during nitrogen limitation (4). In B. subtilis, however, (17), there is no evidence that hut operon expression is at all affected by nitrogen limitation. Nonetheless, it would be logical for hut expression to respond to the availability of both carbon and nitrogen sources. A regulatory complex that included GS and some component of glucose metabolism and that was able to control coordinately

the TCA cycle and pathways that feed it would be particularly important for the economy of the cell. Such a system could presumably be implicated in the regulation of sporulation as well. It is interesting to note that an early blocked sporulation mutant is also partially relieved of catabolite repression of histidase (21).

ACKNOWLEDGEMENT

We thank J. Felton, M. Malamy, M. Schaechter and A. Wright for their interest and helpful criticism, K. Bott for informing us of his results before publication, and F. Kunst and J. Hoch for supplying strains and advice on mapping. This work was supported by a research grant (GM19168) from the National Institute of General Medical Sciences.

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