CHARACTERIZATION OF GLUTAMINE REQUIRING MUTANTS OF BACILLUS SUBTILIS

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SUMMARY

Mutants of B. subtilis 168 which exhibited an absolute requirement for glutamine have been isolated and characterized. Of the two mutants studied in detail, one had normal levels of glutamine synthetase and sporulated normally, the other had reduced glutamine synthetase and was asporogenic. Both mutants were mapped close to the thy A region of the chromosome by PBS1 transduction.

A study of spontaneous revertants selected for glutamine prototrophy (or the sporulation character in the case of the asporogenic mutant) led to the conclusion that there is a relationship between the glutamine requirement and sporulation. However, the influence of glutamine could not be entirely explained by the catalytic properties of glutamine synthetase.

INTRODUCTION

The enzyme glutamine synthetase (GS) is known to play a pivotal role in nitrogen metabolism and the cellular regulation of many prokaryotic species. In Gram-negative bacteria, where the situation is best understood, GS is involved in the biosynthesis of enzymes which supply ammonia to the cells (1). In Bacilli, where GS is the only enzyme responsible for ammonia assimilation (2,3), previous studies made with B. subtilis (4,5,6) and B. megaterium (7,8) support the hypothesis that the enzyme may play a regulatory role both in nitrogen metabolism and sporulation. In particular, the direct analysis of B. megaterium mutants with

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ABBREVIATIONS

Glutamine synthetase ..... GS

Glutamine requirement .....gln^-

Asporogeny.....Sp^-

Sporogeneous.....Sp^+

Difco Nutrient Broth of Schaeffer.....NBS

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altered GS activity (8) stimulated our desire to study the biochemical and physiological effects of similar mutants in B. subtilis, a species amenable to genetic analysis. Two recent reports (9,10) suggest that other laboratories simultaneously began the same type of research. In this report we describe our isolation and preliminary characterization of glutamine requiring mutants of B. subtilis.

MATERIALS AND METHODS

Bacterial strain. B. subtilis 168 trp^- was used throughout this study as the parent strain. For genetic mapping the reference strains prepared by Dedonder et al. (11) were used.

Media, growth and sporulation. Bacteria were cultivated at 30°C or 37°C in Difco Nutrient Broth of Schaeffer (NBS) (12) or in minimal media such as M34 (12) or that of Spizizen (13) supplemented with glutamine when necessary. Thermoresistant spores were counted 18 hrs after the end of growth at 30°C in NBS by plating aliquots previously heated 10 min at 70°C.

Isolation of mutants. Five ml aliquots of a washed spore suspension at 2 x 10^9/ml in 0.1 M $MgSO_4$ were irradiated with a U.V. germicidal lamp in a glass petri dish for 2.5 min with agitation to give a survival level of 0.01%. Irradiated spores were immediately centrifuged and resuspended in 25 ml of NBS medium plus 1 mg/ml glutamine in the dark and shaken at 30°C in the dark for 4 hours to allow adequate germination, outgrowth and expression. They were then centrifuged, washed and resuspended in medium M34 with normal NH_4 but no glutamine at 30°C for 1 hour then subjected to amphicillin at 50 µg/ml overnight (16 hrs) to selectively kill all survivors that could grow in the absence of glutamine. Intact cells were collected on 0.45 µ millipore filters, washed and resuspended in the medium M34 without NH_4 but containing glutamine at 1 mg/ml. After 5-6 hrs incubation at 30°C the culture was plated onto M34 minimal plates containing NH_4 plus 5 µg/ml of glutamine. After 2 days incubation the tiny colonies (supposedly limited by the low glutamine conc.) were picked and tested on minimal media plus NH_4, with and without glutamine. Nineteen of 1000 colonies from a total of 3 irradiation experiments were found to grow with either glutamine or glutamate as the nitrogen source. Four isolates which required glutamine alone as a nitrogen source were retained for further analysis. Mutant C4B which sporulated normally and C4D which was asporogenic under all conditions tested were analyzed in detail and are characterized here.

Genetic mapping. Bacteriophage PBS1 was passed 3 times on each of the glutamine requiring mutants to prepare efficient transducing lysates. These lysates were used to transduce highly motile cultures of various recipients to prototrophy. The auxotrophs were all from the "Kit" of Dedonder et al. (11). Transductants for each marker were picked and tested to determine the frequency of those that simultaneously became Gln^-. Procedures for PBS1 mediated transduction were the same as those used by Dubnau (14) and Hoch et al. (15).

Enzyme assays. GS activity was determined in cells grown at 30°C in NBS, harvested by centrifugation at various times during growth and after T_0 (the end of exponential growth) and stored at -20°C as a frozen pellet. When thawed, the cells were immediately suspended in a 50 mM imidazole-HCl buffer pH 7.1 containing 1mM mercaptoethanol and 1 M NaCl. They were centrifuged, washed once with the same buffer lacking NaCl and resuspended to a density of approximately 60-80 A_{570} for breakage with a Branson B12 Sonifier. Suspensions having an A_{570} of less than 10 were centrifuged for 30 minutes at 20,000 rpm. Enzyme assay was carried out directly with this supernate. The biosynthetic reaction of GS was analyzed at 25°C using the method described by Elmerich (2). The non-physiological or "transfer reaction" was analyzed at ambient temperature (~ 22°C) for 12 min in a final volume of 3 ml according to a modification of method of Deuel and Turner (6). The reaction mixture in 40 mM imidazole-HCl buffer pH 7.1, contained 30 mM l-glutamine, 3 mM

TABLE 1

PHENOTYPES OF B. SUBTILIS 168

AND OF GLUTAMINE REQUIRING MUTANTS OF C4B AND C4D

Strain	Glutamine Synthetase					Sporulation Frequency
	Activity(a)				Antigen(b)	
	Biosynthetic reaction U/mg/ml	%	Transfer reaction U/mg/ml	%		
168	0.035	100	0.0056	100	+++	1
C4B	0.033	94	0.0072	128	+++	1
C4D	0.009	26	0.0012	21	+	10^{-6}

(a): a unit of activity was defined as 1 μ mole of substrate metabolized per minute under the conditions described in Materials and Methods.

(b): +++ = normal level, + = low level.

$MnCl_2$, 0.4 mM ADP, 20 mM sodium arsenate, 60 mM NH_2OH and 0.4 M K_2SO_4. Water was substituted for NH_2OH in the control. The reaction was arrested as a function of time by withdrawing 0.5 ml aliquots and adding an equal volume of a mixture which contained 3.3% $FeCl_3$ and 2% TCA in 0.25 NHCl. If a precipitate resulted, the tubes were centrifuged at 4000 rpm before reading the absorbance of the supernate at A_{540}.

Proteolytic activity of both the serylprotease and metalloprotease of the culture supernates at T_{24} were assayed with azocoll as substrate as described for intracellular protease analysis by Millet et al. (16). Esterase activity of the same supernate was analyzed by the method of Martin et al. (17) using Z-tyrosine-p-nitrophenyl ester as the substrate.

Since B. subtilis glutamine synthetase crossreacts efficiently with antiserum to purified B. megaterium glutamine synthetase, the general procedure for detecting antigic material in B. subtilis was the same as that previously described for B. megaterium (8).

RESULTS

Two general categories of glutamine requiring mutants have been isolated. One group included those which required specifically glutamine, the other included those which required glutamine or glutamate. Mutants in both categories were asporogenous, oliosporogenous, or sporogenous. Two mutants (C4B, C4D) which required exclusively glutamine were particularly studied. Both mutants were cultivated in minimal medium

TABLE 2

FREQUENCY OF Thy^+ Gln^- AND Ilv^+ Gln^- RECOMBINANTS AFTER TRANSDUCTION OF DEDONDER'S Kit 4 STRAIN BY PBS1 LYSATES OF MUTANTS C4B AND C4D

PBS1 lysate	Primary selection Number	Gln^- transductants Number	Frequency	Secondary marker Number
C4B	Thy^+ (207)	(27)	.130	Ilv^+ (2), Ilv^- (25)
	Ilv^+ (823)	(18)	.021	Thy^+ (16), Thy^- (2)
C4D	Thy^+ (215)	(22)	.102	Ilv^+ (0), Ilv^- (22)
	Ilv^+ (579)	(13)	.022	Thy^+ (10), Thy^- (3)

or NBS containing 1 mg/ml glutamine although the minimal level which facilitated normal growth was 200 µg/ml. Glutamine could not be replaced by 1 mg/ml of asparagine, aspartate, arginine, histidine, glutamate or nitrate.

As shown in Table 1, mutant C4B was apparently unaltered in its GS activity. Normal amounts of functional enzyme appeared to be made and the protein showed good cross reaction with antiserum to purified B. megaterium GS. Contrarily, mutant C4D retained only 20-25% of GS activity of the mother strain and the antigen level was low. The mutants behave oppositely for sporulation, C4B being sporogenous and C4D asporogenous. When mutant C4B was cultivated in NBS supplemented with increased concentrations of glutamine up to 4 mg/ml, or if additional glutamine was added after T_0, the rate of sporulation remained unchanged. From this point of view, mutant C4D seems to be different from mutants isolated by Fisher and Sonenshein (10) whose sporulation depends on the glutamine concentration in the medium. Production after growth of the extracellular serylprotease, metalloprotease and esterase was normal in mutant C4B and was only slightly reduced in the asporogenous mutant C4D. Reversion to glutamine prototrophy occured at the frequency of 1×10^{-5} in mutant C4D. No spontaneous revertants could be obtained with mutant C4B.

The genetic locus of both glutamine requiring mutants was determined to be in the thy A region as shown in Table 2. This interpretation derives from the knowledge that the thymine requiring recipient strain QB943 (Kit no. 4) (11), was

TABLE 3

GLUTAMINE SYNTHETASE ACTIVITY AND RATE OF SPORULATION OF REVERTANTS FROM MUTANT C4D SELECTED FOR Gln^+ or Sp^+ CHARACTERS

Strain	Glutamine synthetase activity[(a)]	Sporulation frequency
Sp^+ revertants		
C4D RSA	108	1
C4D RSB	115	1
C4D RSC	93	1
Gln^+ revertants		
C4D RG1	108	1
C4D RG2	106	1
C4D RG3	100	1
C4D RG5	91	10^{-6}
C4D RG10	104	10^{-6}

(a): GS activity was determined by the biosynthetic assay and is expressed as % of the value obtained with strain 168.

pyr D, *ilv* A1, *thy* A1, *thy* B1, *trp* C2 and that among the Thy^+ transductants with PBS1 it is impossible to distinguish *thy* A^+ *thy* B from *thy* A *thy* B^+ since they both are phenotypically identical to the wild type (18). Ten percent of the Thy^+ transductants with PBS1 (C4D) and 13% with PBS1 (C4B) required glutamine, suggesting a linkage between the Gln^- mutation and one of the thymine markers. Since only 2.2% of the Ilv^+ transductants with C4D and 2.1% with C4B were Gln^-, it appears that the *thy* B region is *not* near the Gln^- marker. The data also suggests that the mutations conferring the glutamine requirement on each of these mutants are not identical but relatively close. However, a PBS1 lysate carrying the Gln^- character from C4D was unable to transduce C4B to ptototrophy. Initially the mapping was complicated by the observation that the mutants were 12-15% linked to the *sac* A locus near the

origin of replication. However, subsequent reisolation and testing cultures derived from these transductants revealed that every single clone was unstable ultimately reverting to glutamine prototrophy. Currently we have no explanation for this aberrant expression of the glutamine character which deserves a more thorough analysis.

The relationship between Sp^- and Gln^- phenotypes of C4D was studied by analyzing properties of some transductants and of spontaneous revertants selected either for Sp^+ or Gln^+ phenotypes. Two of the Ilv^+ Thy^+ Gln^- transductants selected for Ilv^+ as primary marker, were analyzed for GS activity and rate of sporulation. As compared to the mother strain QB943 (Kit 4 parent) (11), the two transductants (td 201 and td 202) had about 20% of GS activity and their rate of sporulation was 10^{-5}. Sp^+ revertants were obtained by submitting C4D to repeated cycles of growth and sporulation in NBS medium supplemented with glutamine. After two such cycles the rate of sporulation was equivalent to the wild type. More than 1800 randomly picked clones were tested on plates for glutamine requirement, all of them had recovered glutamine prototrophy. The rate of sporulation of 19 clones, determined in liquid medium, was normal in all cases and GS activity measured in 3 of these revertants was similar to that of strain 168 (Table 3). Gln^+ revertants were obtained by plating cells on M34 agar devoid of glutamine. After reisolation, 40 revertants were studied. On minimal agar, after 4 days of incubation at 30^oC, about a half formed pigmented colonies and a half formed white colonies. All strains deriving from pigmented colonies were sporogenous and all strains deriving from white colonies were asporogenous. As shown in Table 3, GS activity determined in 3 sporogenous and 2 asporogenous revertants was normal. It thus appears from all these results that the Gln and Sp characters can be dissociated only in some Gln^+ revertants.

DISCUSSION

The two glutamine requiring mutants of *B. subtilis* studied here vary significantly in their biochemical and sporulative properties although retaining their absolute requirements for glutamine. Mutant C4B, which contains normal amounts

of GS activity and antigen, is not unlike the B. subtilis mutants reported by Dean et al. (9) or some of the B. megaterium mutants described by Reysset and Aubert (8). In fact, the reason for C4B dependence upon glutamine is unclear. On the other hand, mutant C4D, as well as each of its transductants tested, had about 20% GS activity as measured by the biosynthetic assay, or the transfer assay, and a low antigen level. Note however, that both C4B and C4D required 200 µg/ml glutamine in minimal medium to exhaust 1 mg/ml glucose. This level of growth factor, which is 10-40 times higher than the normal requirement for single auxotrophic characters and which is similar to that observed by glutamine requiring mutants of B. megaterium (2) suggests that in B. subtilis like in B. megaterium, GS plays a major role in ammonia assimilation.

The genetic locus of both C4B and C4D mutations in the thy A region of the chromosome is in agreement with the mapping of other B. subtilis glutamine requirers (9,10). According to Dean et al. (9), the GS structural gene would be located in this region. In our case, we have no direct evidence that mutants C4B or C4D are structural mutants, although the instability of the residual GS activity in C4D suggests that this strain might be impaired in its GS structural gene.

In previously studied glutamine requiring mutants of B. megaterium (2,8) it was shown that both Sp^+ or Sp^- phenotypes could be observed. This seems to be also the case in B. subtilis since C4B is sporogenous and C4D asporogenous. Attempts to determine whether or not the Gln^- and Sp^- phenotypes of C4D were the result of a single mutation, by studying properties of Gln^- transductants and Sp^+ or Gln^+ revertants show a dissociation of the two characters only in the case of the Gln^+ revertants. Although the possibility of a double mutation cannot be completely excluded, these results are rather in favor of the existence, in B. subtilis of a relationship between GS and sporulation, as already suggested in B. megaterium (8). Two general hypotheses could account for this relationship, depending whether GS would be involved in sporulation either by its catalytic properties or as a regulatory protein. Further knowledge of characteristics of the mutants described here, and of other GS^- mutants, would appear to be useful in any inter-

pretation of the overall role played by GS in metabolic activities and sporulation of Bacilli.

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REFERENCES

1. Magasanik, B. (1976) Prog. in Nucleic Acid Res. and Mol. Biol.,17,99-115.
2. Elmerich, C. (1972) Eur. J. Biochem., 27,216-224.
3. Tempest, D.W., Meers, J.L., and Brown, C.M. (1973) The Enzymes of Glutamine Metabolism, pp. 167-182, Academic Press.
4. Deuel, T.F., Ginsburg, A., Yeh, J., Shelton, E., and Stadtman, E.R. (1970) J. Biol. Chem., 245,5195-5206.
5. Deuel, T.F., and Stadtman, E.R. (1970) J. Biol. Chem., 245,5206-5213.
6. Deuel, T.F. and Turner, D.C. (1972) J. Biol. Chem., 247,3039-3042.
7. Elmerich, C., and Aubert, J.P. (1975) Spores VI, pp. 385-390, Am. Soc. Micro.
8. Reysset, G., and Aubert, J.P. (1974) Biochem. Biophys. Res. Comm., 65,1237-1241.
9. Dean, D.R., Hoch, J.A., and Aronson, A.I. (1977) J. Bacteriol., 131,981-987.
10. Fisher, S.H., and Sonenshein, A.L., in press. (This issue)
11. Dedonder, R.A., Lepesant, J.A., Lepesant-Kejlarova, J., Billaut, A., Steinmetz, M., and Kunst, F. (1977) Appl. & Envir. Micro., 33, 989-993.
12. Schaeffer, P., Millet, J., and Aubert, J.P. (1965) Proc. Natl. Acad. Sci., U.S.A., 54,704-711.
13. Spizizen, J. (1958) Proc. Natl. Acad. Sci., U.S.A., 44,1072-1078.
14. Dubnau, D. (1971) Methods in Enzymology, Vol. XXI, pp. 430-438, Academic Press.
15. Hoch, J., Barat, M., and Anagnastopoulos, C. (1966) J. Bacteriol., 93,1925-1937.
16. Millet, J., Larribe, M., and Aubert, J.P. (1976) Biochemie., 58, 109-117.
17. Martin, J., Golubow, J., and Axelrod, A.E. (1958) Biochem. Biophys. Acta., 27,430-431.
18. Wilson, M.C., Farmer, J.L., and Rothman, F. (1966) J. Bacteriol., 92,186-196.