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Glutamine Synthetase Subunit Mixing and Regulation in *Bacillus subtilis* Partial Diploids

A. GARDNER,[1] J. ODEBRALSKI,[2] S. ZAHLER,[2] R. Z. KORMAN,[2] AND A. I. ARONSON[1]*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907,[1] and Section of Genetics and Development, Cornell University, Ithaca, New York 14853[2]

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A specialized transducing phage, SPβ *c2 dglnA2*, of *Bacillus subtilis* was used to construct partial diploids with various glutamine auxotrophs. The overproduction of manganese-stimulated glutamine synthetase no longer occurred in the diploids. The kinetics of heat inactivation of the enzyme extracted from two diploids suggests that there was subunit mixing.

Many glutamine synthetase (GS) mutants of *B. subtilis* overproduce manganese-stimulated activity and antigen (3, 4). These mutants carry mutations that lie in or very close to the structural gene, *glnA*, implying either autogenous regulation or possibly a closely linked regulatory gene. To explore this regulatory mechanism further, we have utilized an SPβ specialized transducing phage (5, 6) to construct partial diploids containing two copies of the *glnA* gene to examine these strains for regulation of GS and for subunit mixing of the dodecameric enzyme.

Strain CU2037 (Table 1) carries a heat-inducible, integration-deficient SPβ *c2 int5* prophage inserted between the *thyA* and *glnA* genes. This strain was heat induced, and the lysate was used to transduce strain CU1769 to Gln^+. A lysate from a transductant was used to transduce strain CU1770 to Gln^+. The resulting strain, CU2103, carries both SPβ *c2* and the defective transducing phage SPβ *c2* d*glnA2*. It also has two copies of the *glnA* gene, $glnA^+$ and *glnA100*, and gives rise to a high-frequency of transduction lysates when it is induced.

Other diploid strains were constructed by mixing equal volumes of SPβ lysates from strain CU2103 and cultures of either of two *glnA* mutants, DRD2 or DRD4, grown to 10^9 cells per ml in Antibiotic Medium No. 3. The cells were washed and plated on selective media. Two isolates from each transduction experiment were chosen for further study (Table 1). To demonstrate the presence of the mutant allele in these transductants, DNA prepared from the diploids was used to transform a closely linked marker, *thyA* (Table 2). The $thyA^+$ and mutant *glnA* genes were cotransformed at approximately the same frequency as that found in the parent, DRD2.

Crude extracts of the diploid strains were prepared, and GS was assayed as previously described (reference 3 and Table 3). The diploids contained the same or slightly lower levels of the Mg^{2+}-stimulated activity as that found in the wild type. In the case of the diploids formed from DRD2, the Mn^{2+}-stimulated activity is about the same as the wild type and well below that of the parental strain. GS antigen content is also reduced in diploid AG2 relative to DRD2 (Fig. 1). In diploids formed from strain DRD4,

TABLE 1. Strains used in this study

Strain	Genotype
CU1769	*metB5 glnA100 attSPβ*
CU1770	*metB5 glnA100* (SPβ *c2*)
CU2037[a]	*trpC2 ilvA3 citD2* (*zee*::SPβ *c2 int5*)[b]
CU2103	*metB5 glnA100* (SPβ *c2*) (SPβ *c2* d*glnA2*)
JH641	*trpC2 phe-1 ilv-1* (SPβ)
DRD2	*trpC2 phe-1 ilv-1 glnA100* (SPβ)
DRD4	*trpC2 phe-1 glnA103* (SPβ)
AG2	*trp C2 phe-1 ilv-1 glnA100* (SPβ) (SPβ *c2* d*glnA2*)
AG3	*trp C2 phe-1 ilv-1 glnA100* (SPβ) (SPβ *c2* d*glnA2*)
AG8	*trpC2 phe-1 glnA103* (SPβ) (SPβ *c2* d*glnA2*)
AG9	*trpC2 phe-1 glnA103* (SPβ) (SPβ *c2* d*glnA2*)

[a] Methods of isolation of strain CU2037 are given in S. A. Zahler, *In* D. Dubnau, (ed.), Molecular biology of the bacilli, Academic Press, Inc., New York, in press.

[b] The nomenclature for the position of the prophage is from Davis et al. (2).

TABLE 2. Presence of mutant *glnA* alleles in diploids detected by cotransformation of *thyA* and *glnA100* or *glnA103*

Donor strain	% Cotransfer[a]
DRD2	14
AG2	11
AG3	11
AG8	20
AG9	13

[a] Transformation was done according to Anagnostopoulos and Spizizen (1). The recipient in all cases was a *thyA thyB* strain, and selection was for thymidine prototrophy at 45°C in the presence of glutamine. *thyA*$^+$ transformants were screened for glutamine auxotrophy, and the percent of *thyA-glnA100* or *glnA103* cotransfer was calculated. Numbers given are averages of at least three transformation experiments.

the Mn^{2+}-stimulated activity is somewhat higher than the activity in the parental strain and is elevated two- to threefold over the wild type as is the GS antigen (Fig. 1). The activity in these diploids may be higher because of a gene dosage effect or greater stability of the enzyme (see Fig. 2).

To test for subunit mixing, the heat stability of GS in crude extracts of diploids AG8 and AG9 was measured since the parental strain, DRD4, contains heat-labile activity (reference 4 and Fig. 2). The activity in crude extracts of DRD4 was rapidly inactivated at 70°C, in contrast to the activity from the wild type. There was a bimodal inactivation curve for a mixture of mutant and wild-type extracts, whereas the activity from crude extracts of the diploid decreased slowly over a 15-min period. The rate of inactivation of extracts from the diploid was

TABLE 3. Specific activities of GS from various strains

Strain	Sp act[a]		
	Mg^{2+}-stimulated	Mn^{2+}-stimulated	Mg^{2+}/Mn^{2+} ratio
JH641	8.4	7.4	1.13
DRD2	4.0	56	0.07
AG2	5.0	7.3	0.69
AG3	5.7	9.2	0.62
DRD4[b]	2.7–5.1	8.4–12.7	
AG8	7.2	22	0.33
AG9	6.5	18	0.36

[a] Strains were grown in minimal medium supplemented with 0.2% glutamine. Specific activity is expressed as nanomoles of glutamyl hydroxamate formed per minute per milligram of protein.

[b] There is some variability in the activity found in cell extracts of this strain and thus in the ratio. This variability may be due to the instability of the GS in this mutant (see Fig. 2).

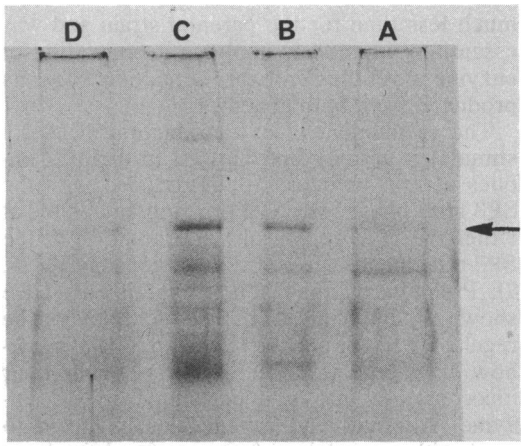

FIG. 1. Fractionation of GS immunoprecipitates by electrophoresis on a 5 to 15% sodium dodecyl sulfate slab gel (4). Extracts were prepared, and portions with equivalent amounts of protein were incubated with varying concentrations of GS antibody as previously described (4). The immunoprecipitates were suspended in 8 M urea–0.05 M dithioerythritol–0.1% sodium dodecyl sulfate, pH 9.5, placed in a boiling water bath for 2 min, and electrophoresed at 35 V for 16 h. One set of stained bands is shown. The arrow indicates the position of migration of *B. subtilis* GS monomer. A, AG2; B, AG8; C, DRD2; D, JH641.

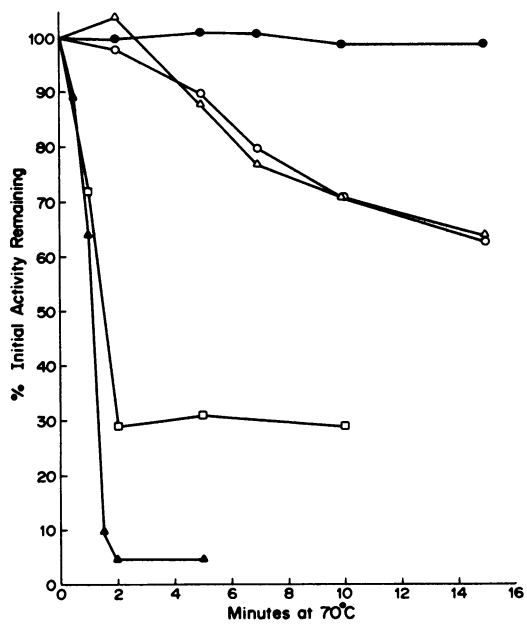

FIG. 2. Heat inactivation of GS. Crude extracts were placed at 70°C, and the samples were removed to an ice bath at the indicated times for assay. Symbols: ●, wild-type JH641; ▲, mutant DRD4; ○, diploid AG8; △, diploid AG9; □, mixture of JH641 and DRD4.

much less than for the parental strain and was essentially unimodal, implying a population of enzyme molecules probably containing subunits produced from both genomes.

The curtailment of overproduction of Mn^{2+}-stimulated activity and antigen in diploids implies a *trans*-acting factor introduced with the SPβ *glnA* phage. The SPβ probably integrated at either the *glnA* region of the genome or at the SPβ attachment site near the *trp* gene cluster (6, 7). Preliminary transduction experiments have shown the latter location in some diploids. The regulatory factor may be GS subunits that somehow exert regulatory action per se or via their incorporation into a dodecameric hybrid enzyme. Alternatively, a gene closely linked to *glnA* with a regulatory function may have been incorporated into the specialized transducing phage and introduced into the diploids. A further analysis of the *glnA* region should elucidate the nature of this regulatory factor.

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