Genetic and biochemical characterization of the two glutamine synthetases GSI and GSII of the phosphinothricyl-alanyl-alanine producer, *Streptomyces viridochromogenes* Tü494

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The 1410 bp DNA region (*glnA*) encoding glutamine synthetase I (GSI) from *Streptomyces viridochromogenes* was amplified by PCR, cloned and sequenced. The molecular mass of the deduced GSI protein (469 residues) was determined to be 50 kDa. The DNA region showed 90% nucleotide identity with the *Streptomyces coelicolor* A3(2) *glnA* gene, but no significant nucleotide sequence similarity with the *glnII* (GSII) gene of *S. viridochromogenes*. The chromosomal *glnA* and *glnII* genes of *S. viridochromogenes* were disrupted by site-specific mutagenesis. Neither *glnA* nor *glnII* single mutants required glutamine for growth and both were normal in their sporulation. Measurement of the GS activity in cultures grown with different nitrogen sources revealed that GSI (heat-stable) and GSII (heat-labile) were always expressed together, with GSI as the predominant activity. It could be proposed that GSI, but not GSII is inactivated by adenylation under conditions of nitrogen excess. GSI and GSII activities are inhibited by amino acids and by nucleotides.

Introduction

Glutamine synthetase (GS) (glutamate–ammonia ligase, EC 6.3.1.2) catalyses the conversion of L-glutamic acid to L-glutamine in the presence of ammonia and ATP. Glutamine is a key metabolite in the synthesis of nitrogen-containing compounds such as purines, pyrimidines, amino sugars and various amino acids. Different forms of GS in one organism have been found in *Rhizobiaceae* (Darrow & Knotts, 1977; Fuchs & Keister, 1980), *Frankia* sp. (Edmands *et al.*, 1987) and *Streptomyces* (Behrmann *et al.*, 1990; Kumada *et al.*, 1990), and a third type of GS was detected in the anaerobe *Bacteroides fragilis* (Hill *et al.*, 1989).

There are three different types of GS. GSI are generally found in bacteria and are relatively heat-stable polymeric enzymes consisting of 12 identical subunits, each with 450 to 470 amino acids. GSII, composed of eight smaller

subunits, each with 350 to 420 amino acids, are typical of eukaryotes but are also found in some bacteria, and are heat-labile. GSI, but not GSII, are known to be modified post-translationally by adenylation (Darrow *et al.*, 1981). GSI and GSII have about 15% amino acid identity concentrated in five conserved regions (Janssen *et al.*, 1988; Rawlings *et al.*, 1987). The GS of *B. fragilis* has six subunits, each with 729 amino acids, is not regulated by adenylation and has no sequence similarity to the GSI and GSII enzymes (Hill *et al.*, 1989).

Transcriptional and post-translational regulation of *glnA* have been extensively studied in the *Enterobacteriaceae* (for reviews see Reitzer & Magasanik, 1987; Merrick, 1988).

Rhizobiaceae and actinomycetes contain both a GSI and a GSII activity. They are differently regulated in the well-studied *Rhizobiaceae* (Carlson & Chelm, 1986). In *Bradyrhizobium japonicum* (Adams & Chelm, 1988; Carlson & Chelm, 1986) and *Agrobacterium tumefaciens* (Rossbach *et al.*, 1987, 1988) the *glnA*-homologous gene appears to be expressed constitutively and GSI activity is controlled post-translationally (Darrow & Knotts, 1977; Darrow *et al.*, 1981; Ludwig 1978, 1980a, b). *glnII* expression is, however, regulated in response to the cellular nitrogen status by the Ntr system (Martin *et al.*, 1988).

The metabolic regulation of nitrogen metabolism has been studied in different *Streptomyces* species. GS

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Abbreviations: GS, glutamine synthetase; *glnA*, gene encoding GSI; *glnII*, gene encoding GSII; MCS, multiple cloning site; PTT, phosphinothricyl-alanyl-alanine; SVPDE, snake venom phosphodiesterase.

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activity is dependent on the nitrogen source in *S. cattleya*, *S. clavuligerus* and *S. coelicolor* (Pares & Streicher, 1985; Bascarán *et al.*, 1989; Fisher & Wray, 1989) but not in *S. venezuelae* (Shapiro & Vining, 1983). Transcriptional control of the *glnA* gene of *S. coelicolor* is mediated through an activator protein, *glnR* (Wray *et al.*, 1991) and GSI activity is regulated post-translationally via adenylation in *S. cattleya*, *S. coelicolor*, *S. clavuligerus* (Streicher & Tyler, 1981; Fisher & Wray, 1989; Bascarán *et al.*, 1989). In recent studies, the existence of two GS genes in *S. viridochromogenes* and *S. hygroscopicus* was proved (Behrmann *et al.*, 1990; Kumada *et al.*, 1990). Much about the respective roles of the two *gln* genes and about their expression and regulation remains unclear (Fisher, 1992).

In *S. viridochromogenes*, examination of nitrogen assimilation metabolism is of particular interest, because this strain produces the antibiotic phosphinothricyl-alanyl-alanine (PTT), which contains the GS inhibitor phosphinothricin (Pt) (Bayer *et al.*, 1972) and none of the GS enzymes are Pt-resistant, although overexpression of GSII gives PTT resistance (Behrmann *et al.*, 1990). To analyse the expression and regulation of the two *gln* genes of *S. viridochromogenes* we constructed and analysed *glnA* and *glnII* mutants by gene disruption techniques.

Methods

Strains. The wild-type strain *Streptomyces viridochromogenes* Tü494 (Bayer *et al.*, 1972) was obtained from Professor H. Zährner, University of Tübingen, Germany. The GS mutants DH11 and DH22 were derived from this strain. *Escherichia coli* JM83 (Vieira & Messing, 1982) was used in cloning experiments. Single-stranded DNA was isolated from *E. coli* NM522 (Gough & Murray, 1983), after infection with helper phage M13KO7 (Martin, 1987). *E. coli* S17-1 (Simon *et al.*, 1983), which carries the RP4 transfer functions chromosomally integrated, was used as donor in mating experiments between *E. coli* and *S. viridochromogenes*. Heterologous expression of *S. viridochromogenes glnA* and *glnII* genes was investigated in *E. coli* strain YMC11 (Backman *et al.*, 1981), which is a *glnA* deletion mutant.

Culture conditions and genetic manipulations. For protoplasting, *S. viridochromogenes* mycelium was grown in S medium containing 0.75% glycine (Strauch *et al.*, 1987). Transformation of *S. viridochromogenes* was carried out according to the protocol of Hopwood *et al.* (1985). Protoplasts were spread on R5 agar (Hopwood *et al.*, 1985) and selection of thiostrepton-resistant colonies was carried out by over-laying the plates with thiostrepton after 18 h incubation. Thiostrepton was kindly provided by Dr S. J. Lucania (E. J. Squibb & Sons, New York, USA).

Mating experiments with *E. coli* as donor and *S. viridochromogenes* spores as recipient were performed as described by Wohlleben & Pielsticker (1989). Transformation of *E. coli* strains was performed as described by Cohen *et al.* (1972).

S. viridochromogenes was cultivated on MYG agar containing (l⁻¹): 10 g malt extract, 4 g yeast, 4 g glucose and 15 g agar, pH 7.3. Cultivation of *S. viridochromogenes* for enzyme assays was carried out in chemically defined medium containing 1 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 10 g glucose in 50 mM-HEPES

buffer pH 7.6. The medium was supplemented with different nitrogen sources at 20 mM concentration, as indicated.

Preparation, manipulation and sequencing of DNA. Total *Streptomyces* DNA was isolated by the method of Hopwood *et al.* (1985). *E. coli* plasmid DNA was isolated by the method of Holmes & Quigley (1981), isolation of single-stranded phage DNA as described by Hillemann *et al.* (1991).

Manipulations in *E. coli* were performed using standard procedures (Maniatis *et al.*, 1982). Restriction enzymes, T4 DNA ligase, alkaline phosphatase, DNA polymerase I and *Taq* polymerase were obtained from Gibco BRL or Boehringer Mannheim and used according to the suppliers' recommendations. Radioactive deoxynucleotides were purchased from Amersham or DuPont. DNA was sequenced following the protocol of Sanger *et al.* (1977). Both strands were sequenced. Fragments of interest were cloned into the sequencing vector pSVB30 (see Table 1).

Oligodeoxynucleotide synthesis. The chemicals required for the synthesis of DNA were purchased from Applied Biosystems. The oligonucleotides were synthesized with an Applied Biosystems DNA Synthesizer, Model 380B, using the phosphoramidite method (Beaucage & Caruthers, 1981).

PCR. PCR (Mullis *et al.*, 1986) was set up at 4 °C. The 50 µl reaction mixture consisted of 200 nM of each oligonucleotide primer, 200 µM-d(NTP), 2.5 units *Taq* polymerase, 1 µg linearized template DNA in PCR buffer (67 mM-TRIS, 0.17 mM-EDTA, 0.17 mM-BSA ml⁻¹). The reaction mixture was overlaid with mineral oil. After the first denaturation, which lasted for 2.5 min at 94 °C, 31 cycles of amplification (at 94 °C for 90 s, 57 °C for 90 s, and 72 °C for 90–150 s dependent on the expected length of the template) were performed in a thermocycler (Collasius *et al.*, 1989). PCR products were electrophoretically separated in a 0.8% agarose gel, isolated by the glass wool method (Mukhopadhyay & Roth, 1991) and directly employed for cloning.

For the inverse PCR reaction (Ochman *et al.*, 1990), the template DNA was pretreated by digestion with *SalI* and religated at low DNA concentration to promote circularization (Collins & Weissman, 1984). The primer pair 2 and 4 (Fig. 1) was used for the PCR reaction. Primers for PCR are homologous to the end of the core region, but oriented such that chain elongation proceeds across the unknown region of the circle.

The following oligonucleotide primers were used: 5' ATG TTC CAG AAC GCC GAC 3' (primer 1), 5' GAC GGC ATC AAG AAC AAG ATC 3' (primer 2), 3' CTG CCG TAG TTC TTG TTC TAG 5' (primer 3), 3' TAC ATG AAG CTG CAC ACT 5' (primer 4).

Determination of PTT resistance and PTT production. Measurement of the PTT resistance level was performed on minimal agar containing (NH₄)₂SO₄ (20 mM) as nitrogen source (Hopwood *et al.*, 1985) supplemented with different PTT concentrations. PTT was kindly supplied by Hoechst. The bioassay for determining PTT production was done as described by Alijah *et al.* (1991).

GS assays. GS assays were performed in crude extracts. Before preparation of crude extracts the cells were washed twice with buffer (50 mM-imidazole, pH 7.0, 150 mM-NaCl, 1 mM-MnCl₂, 0.5 mM-DTT). The cells were resuspended in the same buffer and disrupted in a French press at 1.07 × 10⁸ Pa. GS was assayed by the γ-glutamyltransferase method (Bender *et al.*, 1977), modified as described previously (Behrmann *et al.*, 1990). The procedure measures the ability of GS to replace the γ-amino group of glutamine with hydroxylamine in the presence of ADP and AsO₄³⁻. Reactions were started by adding glutamine. Since GSI and GSII have approximately the same pH optima in the transferase assay reaction (data not shown), mixtures containing 50 mM-imidazole buffer (pH 7.0) were used. The activity of the heat-stable GSI was determined after heat treatment of the crude

Table 1. Plasmids

Plasmid	Relevant characteristics and marker gene(s)*	Source or reference
pSVB30	Sequencing vector derived from pUC8 (Vieira & Messing, 1982); mcs bla lacZ'	Arnold & Pühler (1988)
pUC18/19†	Cloning vector; bla lacZ'	Norrande <i>et al.</i> (1983)
pK18::mob	Mobilization vector derived from pK18 (Pridmore, 1987); aphII	Sonnen <i>et al.</i> (1991)
pDH5	Multifunctional vector derived from pUC19 with T ₇ and T ₃ promoters and phage f1 intergenic region for the isolation of single- stranded DNA; mcs bla lacZ' tsr	Hillemann <i>et al.</i> (1991)
pDH18.1	pUC18 containing the glnA gene of <i>S. viridochromogenes</i> expressed from the lacZ promoter; bla	This study
pDH19.2	pUC19 containing the glnII gene of <i>S. viridochromogenes</i> expressed from the lacZ promoter; bla	This study
pDH22	pDH5 carrying an internal fragment of the glnII coding region of <i>S.</i> <i>viridochromogenes</i> ; bla tsr (Fig. 3)	This study
pDH11	pK18::mob carrying a 1.2 kb internal glnA fragment (PCR-generated fragment with the primers 1 and 3) disrupted by the 1.05 kb BclI tsr cassette; aphII tsr (Fig. 3)	This study

* Genes: *aphII* aminoglycoside phosphotransferase gene from Tn5 (selection in *E. coli* and *Streptomyces*); *bla*, β -lactamase gene from *E. coli* (selection in *E. coli*); *lacZ'*, truncated β -galactosidase (*lacZ*) gene; *tsr*, thiostrepton resistance gene from *S. azureus* (selection in *Streptomyces*).

† This pair of plasmids differ in the orientation of their mcs.

extracts at 60 °C for 10 min prior to the enzyme assay (Edmands *et al.*, 1987). The contributions of GSI and GSII in the wild-type were determined by comparing γ -glutamyltransferase activity of crude extracts before and after heat treatment. Protein concentration was determined by the dye-binding protein assay (Bio-Rad).

Modification of GS from exponentially grown cells was performed as described by Braña *et al.* (1986), but examined in crude extracts and not in whole cells. Reactivation of GS in crude extracts was tested in 20 mM-imidazole/HCl pH 7.0, containing 2.5 mM-MnCl₂. The extracts were incubated at 37 °C with snake venom phosphodiesterase (SVPDE, Boehringer Mannheim) at 100 μ g ml⁻¹.

Construction of plasmids pDH18.1 and pDH19.2. The plasmid pDH19.2 carries the *glnII* gene of *S. viridochromogenes* expressed under the control of the *E. coli lacZ* promoter in the plasmid pUC19. Partially *Sau3AI* digested fragments in the 2–4 kb range were isolated from *S. viridochromogenes* total DNA and ligated into the *Bam*HI site of the vector pUC19. The ligation mixture was used for the transformation of the *E. coli glnA* mutant YMC11 (Backman *et al.*, 1981). Two transformants that grew on minimal medium were selected. Further analysis of these transformants showed that they harboured plasmids carrying an identical 1.5 kb insert. Restriction analysis revealed that the cloned fragments of these plasmids represent the *glnII* gene of *S. viridochromogenes* (Behrmann *et al.*, 1990).

To construct the plasmid pDH18.1 which carries the *glnA* gene of *S. viridochromogenes* expressed under the control of the *E. coli lacZ* promoter of the plasmid pUC18, three fragments of *glnA* DNA were cloned in 5'–3' direction into the *Sma*I/*Sal*I-digested *E. coli* vector pUC18: (1) a 0.6 kb blunt-ended/*Pst*I fragment generated by *Pst*I-digestion of the 0.8 kb PCR product (primer pair 1 and 4); (2) a 0.7 kb *Pst*I fragment isolated from the *S. viridochromogenes* chromosomal DNA; and (3) a 0.1 kb *Pst*I/*Sal*I fragment in the 3' region of the *glnA* gene generated by the inverse PCR with the primer pair 4 and 2 (Fig. 1).

Construction of plasmids pDH11 and pDH22. pDH11 (Fig. 3) consists of the 1.2 kb *glnA* fragment PCR-generated with the primers 1 and 3 (Fig. 1) cloned into the *Sma*I site of the mobilizable *E. coli* vector pK18::mob and the 1.05 kb *Bcl*I *tsr* cassette (thiostrepton resistance) inserted into the unique *Bgl*III-site of the *glnA* fragment. pDH22 (Fig. 3) consists of pDH5, carrying an internal 300 bp *Bgl*III/*Sau*3AI

fragment of the *glnII* coding region of *S. viridochromogenes* inserted into the single *Bam*HI site of the mcs.

Results and Discussion

Cloning and sequence analysis of the *S. viridochromogenes glnA* coding region

To isolate the *glnA* coding region of *S. viridochromogenes*, the PCR method was applied for amplifying and cloning internal segments of the *glnA* gene (Fig. 1). Oligonucleotide primers 1–4 (see Methods and Fig. 1) were designed according to the known *S. coelicolor glnA*

Fig. 1. Physical map, oligonucleotide primers and amplified PCR fragments of the *S. viridochromogenes glnA* coding region. A restriction map of the 1.4 kb *glnA* coding region from *S. viridochromogenes* is shown above the physical map (a). Shaded areas within the *S. viridochromogenes glnA* coding region indicate the conserved regions I–V which are common in GSI and GSII protein sequences. Arrows beneath the physical map mark the location of the oligonucleotide primers used for PCR (b). The bars at the bottom represent the 800 and 1200 bp PCR-amplified fragments (c) and the 550 bp *Sal*I fragment generated by inverse PCR (d).

M F Q N A D E A K K L I A D E D V K F I D V R F C
 ATGTTCCAGAACGCCGACGAGGCCAAGAAGTTGATCGCGGACGAGGACGTCAAGTTCATCGACGTCCGCTTCTGC 75
 D L P G V M Q H F T I P A S A F D P A E E L A F D
 GACCTGCCGGGCGTGATGCAGCACTTCACGATCCCCGCATCGGCCTTCGACCCGGCCGAGGAGCTGGCCCTCGAG 150
 G S S I R G F Q A I H E S D M A V R A D L S T A R
 GGTTCGTCGATTCGGCGGCTTCGAGGCCATCCAGGATCCGACATGGCGGTCGCGCGGACCTGTCCGACCGCCCGG 225
 I Sali
 V D P F R R D K T V N I N F F I H D P I T G E Q Y
 GTCGACCCGTTCCGCCGTGACAAGACGGTCAACATCAACTTCTTCATCCACGACCCGATCACGGGCGAGCAGTAC 300
 S R D P R N V A K K A E A Y L A S T G I G D T G Y
 TCCCGTGACCCGCGCAACGTGGCGAAGAAGGCCGAGGCCTACCTCGCGTCCGACCGGTATCGGGGACACCGGCTAC 375
 Sali
 F G P E A E F Y V F D S V R L A N S A T E S F Y H
 TTCGGCCCCGAGGCCGAGTTCTACGTCTTCGACTCCGTGCGCTTGGCGAACAGCGCGACCGAGTCCTTCTACCAC 450
 I D S E A G A W N T G A L E N N R G Y K V R Y K G
 ATCGACTCCGAGGCCGGCGCCTGGAACACCGGTGCCCTCGAGAACAACCGTGGTTACAAGGTCCGCTACAAGGGC 525
 G Y F P V P P V D H F A D L R A Q I S L E L D R A
 GGCTACTTCCCGGTCCCGCCGGTTCGACCACTTCGCGGACCTGCGTGCCGAGATCTCCCTGGAGCTGGACCGTGCC 600
 Sali BglII
 G L Q V E R H D H E V G T A G Q A E I N Y K F N T
 GGTCTGCAGGTGGAGCGCCACGACCACGAGGTGGCGGCGCGCGCGGAGATCAACTACAAGTTCAACACG 675
 PstII II
 L L A A A D D L Q L F K Y I V K N V A W Q N G K T
 CTGCTCGCCGCGGCCGACGACCTCCAGCTCTTCAAGTACATCGTGAAGAACGTGGCGTGGCAGAACGGCAAGACC 750
 A T F M P K P I F G D N G S G M H V H Q S L W T G
 GCGACCTTCATGCCGAGCCGATCTTCGGTGACAACGGCTCGGGCATGCACGTCCACCACTCGCTGTGGACCGGC 825
 III
 G Q A L F Y D E A G Y A G L S D T A R Y Y M G G I
 GGCCAGGCGCTCTTCTACGACGAGGCCGGTTACGCGGGCCTGTGCGACACCGCCCGCTACTACATGGGGCGGCATC 900
 L K H A P S L L A F T N P T V N S Y H R L V P G F
 CTC AAGCACGCCCCGTGCTGCTGGCCTTCACCAACCCGACGGTGAACCTCGTACCACCGCCTGGTGCCGGGCTTC 975
 E A P V N L V Y S Q R N R S A A M R I P I T G S N
 GAGGCGCCGGTCAACCTGGTCTACTCGCAGCGCAACCCCTCCGCGGCATCGGTATCCGATCACGGGCTCGAAC 1050
 IV
 P K A K R V E F R A P D S S G N P Y L A F S A L L
 CCGAAGGCCAAGCGCTCGGATTCGCGCGCCCGGACTCTCCGCGACCCGTCGCTGGCCTTCGCGCGCTGCTG 1125
 V
 L A G L D G I K N K I E P A E P I D K D I Y E L A
 CTCGCGGTCTGGACGGCATCAAGAACAAGATCGAGTCCGCGCGCGGATCCGACAGGACCTCGAGAGCTGGCC 1200
 ADENYLYLATION
 P E E H A G V P Q V P T S L P A V L D R L E A D H
 CCCGAGGAGCACGCGGGCGTCCCGCAGGTCCCGACCTCGCTCCCGGCGCTCCTCGACCGTCTGGAGGCCGACCAC 1275
 E F L L A G D V F T P D L I E T W I D Y K R T N E
 GAGTTCCTGCTCGCGGGCGACGTCTTCACGCCGGACCTGATCGAGACGTGGATCGACTACAAGCGCACGAACGAG 1350
 I A P L Q L R P H P Y E Y E Q Y Y D V
 ATCGCCCCGCTGCAGCTCCGCCCCGACCCGTACGAGTACGAGCAGTACTACGACGTGTGA 1410
 PstI

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the *glnA* coding region from *S. viridochromogenes*. Restriction sites shown in Fig. 1 are underlined. The five regions (I–V) of high amino acid homology between GS enzymes and the putative adenylation region are highlighted by stippling. The tyrosyl residue which may be subject to adenylation is boxed.

Table 2. GS activity of different *E. coli* and *S. viridochromogenes* strains

Strain*	GS activity† [nmol γ -GH min ⁻¹ (mg protein) ⁻¹]		Reduction of GS activity after heat treatment (%)
	Without heat treatment	With heat treatment (10 min, 60 °C)	
<i>E. coli</i>			
YMC11 (pUC19)	0	0	—
YMC11 (pDH18.1) (<i>glnA</i>)	1381	1340	3
YMC11 (pDH19.2) (<i>glnII</i>)	1220	15	99
<i>S. viridochromogenes</i>			
Wild-type	943	778	17.5
DH22 (<i>glnII</i> mutant)	838	822	2
DH11 (<i>glnA</i> mutant)	249	6	97.5

* *S. viridochromogenes* and its mutants were grown in minimal medium supplemented with 20 mM-asparagine as nitrogen source as indicated in Methods. *E. coli* cells were grown in penassay medium (antibiotic medium III; Oxoid).

† GS activity was measured by the γ -glutamyltransferase assay. The GS activity values are means of five assays, SEM was not more than 7%.

DNA sequence (Wray & Fisher, 1988). Primer 1 corresponds precisely to the 5' end of the *S. coelicolor* *glnA* coding sequence. Primers 1 and 4 produced a 800 bp DNA fragment, primers 1 and 3 a 1200 bp fragment. Furthermore inverse PCR (Ochman *et al.*, 1990) with primers 4 and 2 produced a 550 bp *SalI* fragment, which included the putative 3' terminus of the *glnA* coding region.

The amplified fragments of 800, 1200 and 550 bp (Fig. 1) were cloned by blunt-end ligation into the unique *SmaI*-site of the sequencing vector pSVB30. This, together with an internal 700 bp *PstI* fragment (Fig. 1) cloned from the *S. viridochromogenes* chromosomal DNA represented the complete coding region of the *S. viridochromogenes* *glnA* gene except for the 18 bp oligonucleotide primer of the 5' region. The 1410 bp DNA sequence (the entire coding region) of the *glnA* gene and the deduced sequence of the 469 amino acid GSI enzyme are shown in Fig. 2. The sequences of the 700 bp chromosomal *PstI* fragment and the DNA sequence derived from the PCR-amplified fragments were identical, indicating that the PCR amplifications, at least in this region, worked with high fidelity.

The coding region has a G + C content of 67%, and a codon usage typical for *Streptomyces* genes with 63, 40 and 97% G + C in the first, second and third codon position (Wright & Bibb, 1992).

The amino acid sequence of the putative *S. viridochromogenes* GSI protein showed 91% identity with the putative *S. coelicolor* A3(2) GSI protein sequence. Interestingly, *S. viridochromogenes* (and *S. coelicolor*) GSI seemed more closely related to those of Gram-negative bacteria such as *E. coli*, *Azospirillum brasilense* and *Azotobacter vinelandii* (51, 54 and 53% identity,

respectively) than to those of Gram-positive bacteria like *Bacillus* species or *Clostridium acetobutylicum* (40–44% identity). Only 14% identity was found with the *S. viridochromogenes* GSII protein sequence.

To our knowledge, this is the first example where two GS genes from the same organism have been sequenced. Kumada *et al.* (1993) propose that the two genes are products of a gene duplication which occurred very early in the evolution of life, before the emergence of eukaryotes.

All five regions which are conserved in GSI and GSII proteins (Janssen *et al.*, 1988; Rawlings *et al.*, 1987) are also present in the putative GSI protein of *S. viridochromogenes* (stippled regions in Fig. 2). Regions I, II and V were proposed to form the active site which is formed between adjacent subunits (Almassy *et al.*, 1986). Region III may be involved in nucleotide binding (Tischer *et al.*, 1986). Amino acids 385–397 match perfectly the consensus sequence of the GSI adenylation motif in the PROSITE Dictionary [A. Bairoch, University of Geneva; accessed by programme MOTIFS of the UWGCG sequence analysis package (Devereux *et al.*, 1984)]. The tyrosyl residue 398 of *glnA* of *Salmonella typhimurium* is adenylylated under conditions of abundant glutamine (Heinrikson & Kingdon, 1971), but some GS contain a tyrosyl residue in the corresponding region without being adenylylated (Tronick *et al.*, 1973; Fisher *et al.*, 1981). Therefore, the function of the tyrosyl residue 397 of the *S. viridochromogenes* GS needs to be tested experimentally.

The adenylation motif and, of course, the high similarity to the *S. coelicolor* A3(2) GSI enzyme support the claim that the cloned DNA sequence encodes a GSI-type enzyme.

Different heat stability of *S. viridochromogenes* GSI and GSII expressed in *E. coli*

It is known that GSI and GSII activities can be distinguished by their thermostabilities (Darrow & Knotts, 1977). The *S. viridochromogenes* GS were tested for heat-stable and heat-labile GS transferase activities after expression in *E. coli*. The *S. viridochromogenes* *glnA* and *glnII* genes were cloned into the *E. coli* vectors pUC18 and pUC19 to give pDH18.1 and pDH19.2, respectively, and introduced by transformation into the *E. coli* *glnA* mutant YMC11. Both plasmids complemented the glutamine auxotrophy of strain YMC11. Heat-stable GSI activity was detected in crude extracts of YMC11 (pDH18.1), and heat-labile GSII activity in YMC11 (pDH19.2) (Table 2). These experiments also proved that the cloned *glnA* and *glnII* genes of *S. viridochromogenes* were functional and that the *glnII* (GSII) gene of *S. viridochromogenes* is able to replace the natural *glnA* (GSI) gene of *E. coli*.

Construction of a *glnA* mutant of *S. viridochromogenes* by gene disruption

To disrupt the *glnA* gene of *S. viridochromogenes*, pDH11 (Fig. 3) carrying an internal DNA fragment from the *glnA* gene was constructed. pDH11 was introduced by transformation into *E. coli* S17-1 and delivered by conjugation to *S. viridochromogenes*. pDH11 DNA (100 µg) isolated from *E. coli* JM83 was introduced by PEG-mediated transformation into *S. viridochromogenes* protoplasts. Conjugation and transformation gave each a single thiostrepton- and kanamycin-resistant colony, which presumably contained pDH11 integrated into the chromosomal *glnA* gene. This was confirmed for both clones by Southern hybridization experiments using the 1.2 kb PCR fragment (Fig. 1) cloned into pUC19 as probe. Both strains showed the same hybridization pattern (not shown), indicating that pDH11 had integrated via a single crossover in the 5' region of the *glnA* gene. One descendant, *S. viridochromogenes* DH11, derived from the conjugation experiment, was propagated on MYG agar without antibiotics twice, 500 colonies were tested and all of them had retained the thiostrepton and kanamycin resistance markers, indicating stable integration of the entire pDH11 (no second crossover event occurred).

Construction of a *glnII* mutant of *S. viridochromogenes* by gene disruption

For the generation of a *glnII* mutant, plasmid pDH22 (Fig. 3) was constructed. An internal 300 bp *Bgl*III/*Sau*3AI fragment of the *glnII* coding region

Fig. 3. Restriction maps of the plasmids used for the disruption of the *glnA* and the *glnII* genes of *S. viridochromogenes*. pDH11 was derived from the *E. coli* vector pK18::mob with the origin of transfer (*oriT*) of plasmid RP4. It carries a 1.2 kb internal *glnA* fragment (*glnA*), disrupted by the thiostrepton resistance cassette. pDH22 was derived from the *E. coli* vector pDH5 which carries the phage f1 intergenic region for the generation of single-stranded DNA. pDH22 contains a 300 bp internal *glnII* gene fragment (*glnII*). pDH11 and pDH22 contain the pUC (ColE1) replication function (*oriV*). Only restriction sites relevant for construction and cloning are given. For gene designations and further descriptions, see Table 1.

(Behrmann *et al.*, 1990) was cloned into the unique *Bam*HI-site of the vector pDH5 (Table 1), which can be isolated in single- and double-stranded form. About 100 thiostrepton-resistant *S. viridochromogenes* transformants were obtained for 1 µg single-stranded DNA. Southern analysis of six transformants confirmed that in each case one complete plasmid had integrated into the *glnII* gene by homologous recombination (not shown). One of these transformants, *S. viridochromogenes* DH22, with the integrated plasmid pDH22 was chosen for further studies. The stability of the integration was tested by determination of marker loss after two cycles of sporulation on MYG medium without antibiotics. No

loss of thiostrepton resistance occurred in 200 colonies tested. This indicated stable integration of pDH22 into the chromosome of *S. viridochromogenes* DH22.

Heat-stable (GSI) and heat-labile (GSII) activities in S. viridochromogenes DH11 and DH22

Crude extracts were prepared from exponentially growing wild-type *S. viridochromogenes* DH11 and DH22 cells, and GS transferase activities were determined before and after heat treatment (Table 2). The wild-type strain contained both a heat-stable (82%) and a heat-labile (18%) activity. In DH22 only heat-stable (GSI) activity could be found, confirming functional disruption of the *glnII* gene. In DH11 all GS activity was heat-labile (GSII), confirming the functional disruption of the *glnA* gene.

Growth, sporulation and PTT-production of the S. viridochromogenes gln mutant strains on different media

The two GS in *S. viridochromogenes* might be required for the assimilation (utilization) of different nitrogen sources. Therefore, wild-type and *gln* mutants were tested for growth on minimal plates supplemented with a variety of nitrogen sources (ammonia, nitrate, glutamine, glutamic acid, asparagine, aspartic acid, alanine, glycine, serine). Neither *S. viridochromogenes* DH11 nor DH22 required glutamine for growth, suggesting that sufficient glutamine could be provided by either GS alone.

Both strains grew at about the same rate as the wild-type strain on most nitrogen sources tested. A few minimal differences in growth behaviour could, however, be noticed. For example, on glutamic acid, the *glnA* mutant grew more slowly than the wild-type and the *glnII* mutant. Similar observations were made with *gln* mutants in rhizobia and agrobacteria, where GSI and GSII-deficient strains were glutamine prototrophs and grew on minimal medium supplemented with a variety of nitrogen sources (Rossbach *et al.*, 1988; de Bruijn *et al.*, 1989). Double mutants were, however, auxotrophic (Rossbach *et al.*, 1988; de Bruijn *et al.*, 1989).

Disruption of the *S. viridochromogenes glnII* or *glnA* gene did not result in a general sporulation deficiency. On most nitrogen sources, *gln* mutants could hardly be distinguished from the wild-type with respect to development of aerial mycelia or sporulation. It is interesting to note that aerial mycelium of DH22 developed earlier than that of the wild-type or DH11 strains if alanine was the sole nitrogen source. Sporulation of the *glnA* mutant was delayed and diminished with the branched-chain amino acids isoleucine and leucine as sole nitrogen sources.

Fig. 4. GS activities in wild-type *S. viridochromogenes* and GS mutant strains. Cells growing exponentially in complex medium were transferred at time 0 to media containing asparagine (a), ammonia (b) or no nitrogen source (c). Every 2 d, 2 ml samples were taken. GSI activity was measured in crude extracts of the *glnII* mutant (●), GSII activity in the *glnA* mutant (■) and combined GSI/GSII activity in the wild-type (▲). Heat-stable GS activity of the wild-type (△) was determined after heat treatment of crude extracts for 10 min at 60 °C. The specific activities are expressed as nmol γ -glutamyl hydroxamate (γ -GH) formed min⁻¹ (mg protein)⁻¹. SEM of three measurements was not more than 10%.

Both DH11 and DH22 produced PTT but the amount depended on the nitrogen source. On some nitrogen sources, PTT production of the *gln* mutants and the wild-type was approximately the same (asparagine, valine, aspartic acid, proline, threonine, tyrosine, (NH₄)₂SO₄, NH₄Cl). PTT production of the *glnII* mutant was reduced 66% on glutamic acid, 50% on glycine and 75% on arginine. The *glnA* mutant produced less PTT on glycine and alanine (50% reduction), isoleucine and leucine

Fig. 5. Test for *in vivo* covalent modification of the GSI and GSII in *S. viridochromogenes*. *S. viridochromogenes glnII* mutant (a, DH22) and *glnA* mutant (b, DH11) cells were grown in 100 ml minimal medium supplemented with asparagine and assayed for GS transferase activity by preparing crude extracts of 2 ml samples at time 0. At 5 min the cultures were divided into two equal parts. One was ammonia-shocked by addition of NH_4Cl to 20 mM final concentration (arrow). Sampling and measurement of GS transferase activity were continued. Filled symbols, ammonia-shocked; open symbols, untreated controls. GS activity at time 0 was set to 100%. One-hundred per cent GSI activity (*glnII* mutant culture, a) corresponds to $840 \text{ nmol } \gamma\text{-GH min}^{-1} (\text{mg protein})^{-1}$, 100% GSII activity (*glnA* mutant culture, b) corresponds to $240 \text{ nmol } \gamma\text{-GH min}^{-1} (\text{mg protein})^{-1}$. The effect of SVPDE on GS transferase activity in crude extracts of ammonia-shocked cells of the *glnII* mutant (a, ii) and *glnA* mutant (b, ii) was measured. Ammonia-shocked cultures were incubated with SVPDE (filled symbols) or without SVPDE (open symbols). SEM of three measurements was not more than 10%.

(90% reduction), and no PTT was detected on glutamic acid and glutamine (> 99% reduction).

One can postulate that the failure to isolate a *S. coelicolor glnA* mutant by selection of a Gln^- phenotype (Fisher, 1992) was due to the expression of the GSII. The fact that *glnR* mutants of *S. coelicolor* could be isolated by this procedure (Wray *et al.*, 1991) suggests that *glnR* in this strain might control both the *glnA* and *glnII* genes.

GS activities of wild-type *S. viridochromogenes* and *gln* mutants in liquid cultures with different nitrogen sources

GS transferase activity of wild-type *S. viridochromogenes* and *glnA* and *glnII* mutants DH11 and DH22 was measured in crude extracts over a period of 12 d. Cells grown on asparagine, $(\text{NH}_4)_2\text{SO}_4$ or without added nitrogen source were tested (Fig. 4). The wild-type had the highest total GS activity on all media ($\blacktriangle$). The GSII activity of the *glnA* mutant ($\blacksquare$) was lower than the GSI activity present in the *glnII* mutant strain ($\bullet$).

The heat-stable GSI activity of the wild-type strain ($\triangle$) was always dominant over the GSII activity (difference between $\blacktriangle$ and $\triangle$) irrespective of the type of the nitrogen source in the medium and the ratio between the two activities remained roughly constant. This indicated that there is no differential regulation of the expression of GSI and GSII in response to different

nitrogen sources. This is a marked difference to the situation in the *Rhizobiaceae* and in *Frankia* sp. strain Cpl1, where GSI alone is present during growth on NH_4^+ or N_2 , and GSII is only expressed under conditions of nitrogen starvation (Edmands *et al.*, 1987).

Determination of the covalent modification of GSI and GSII

Post-translational regulation of GS by the covalent attachment of adenylyl groups to prevent metabolic imbalances during sudden changes in ammonia availability is well known (Ginsburg & Stadtman, 1973). GSI and GSII activities in crude extracts from ammonia-shocked cells of *S. viridochromogenes glnII* and *glnA* mutants were examined (Fig. 5). Within 15 min after ammonia treatment of the cells GSI activity decreased to 55% (Fig. 5a, i), but could be re-activated by SVPDE (Fig. 5a, ii), which is known to cleave the AMP moiety of adenylylated *E. coli* GS (Stadtman *et al.*, 1970; Magasanik, 1982). It can be concluded from these results that the *S. viridochromogenes* GSI is partially inactivated after ammonia treatment, probably by adenylylation as has been shown for *S. cattleya*, *S. coelicolor* A3(2) and *S. clavuligerus* (Streicher & Tyler, 1981; Fisher & Wray, 1989; Bascarán *et al.*, 1989), and for the *Frankia* sp. strain Cpl1 (Edmands *et al.*, 1987).

Table 3. Feedback inhibition of GSI and GSII of *S. viridochromogenes*

Test metabolite	Percentage inhibition of GSI*		Percentage inhibition of GSII*	
	1 mM	10 mM	1 mM	10 mM
Alanine	28	81	9	28
Glycine	10	48	3	25
Serine	8	37	5	23
Histidine	2	5	< 1	< 1
Tryptophan	< 1	< 1	< 1	< 1
CTP	17	> 99	48	> 99
UTP	12	> 99	43	> 99
AMP	< 1	< 1	< 1	< 1

*GS activity was measured by the γ -glutamyltransferase assay. Determination of GSI activity was performed with crude extracts of the *glnII* mutant; GSII activity was determined with crude extracts of the *glnA* mutant. The absolute values of GSI and GSII transferase activity are as stated in Fig. 5. GS activity was determined using the means of three assays, SEM was not more than 5%.

The level of adenylylation found in GSI of *S. viridochromogenes* cells grown continuously with ammonia, glutamate or asparagine as nitrogen sources was only about 10% (not shown). This was comparable to *S. cattleya*, where the highest level of adenylylation was 16% (Paress & Streicher, 1985).

GSII activity in crude extracts of DH11 (*glnA*⁻) decreased slowly after addition of ammonia. No reactivation was observed after SVPDE treatment. This result suggested that GSII is not regulated by adenylylation, but it does not exclude the possibility of another type of post-translational regulation as has been suggested for *Rhizobium leguminosarum* GSII (Manco *et al.*, 1992).

Feedback inhibition of GSI and GSII

Several metabolites were tested for their ability to inhibit *S. viridochromogenes* GSI and GSII in crude extracts (Table 3). 10 mM-concentrations of CTP and UTP, but not AMP, inhibited the transferase activities of GSI and GSII very strongly. Alanine, glycine and serine, but not histidine and tryptophan, also at 10 mM concentration gave 23–80% inhibition. With all inhibitors, the GSI activity was more severely reduced than the GSII activity, reflecting the lower pK_i values of GSII in comparison to GSI (data not shown).

These inhibitory patterns are similar to those observed with other GS enzymes (Hubbard & Stadtman, 1967; Bhandari *et al.*, 1983; Tsai & Benson, 1989). Feedback inhibition is of great importance, particularly for organisms with low or no post-translational modification as is the case for some *Bacillus* species (Hubbard & Stadtman, 1967; Ojha & Kantengwa, 1989).

Conclusions

The coding region (*glnA*) from *S. viridochromogenes* has been amplified by the PCR method, cloned and sequenced. Both the *glnA* and the *glnII* mutants were glutamine prototrophs, could sporulate and produce PTT. GSI and GSII were expressed under all conditions tested, the GSI activity was always dominant. No differential expression of the two GS in response to the nitrogen source or differentiation was observed. It will be interesting to find out whether the expression of two GS is common in streptomycetes.

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