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Transcriptional analysis of the gene for glutamine synthetase II and two upstream genes in *Streptomyces coelicolor* A3(2)

Received: 12 April 2000 / Accepted: 15 June 2000 / Published online: 21 July 2000
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Abstract The glutamine synthetase II (GSII, encoded by *glnII*) activity detectable in crude extracts from *Streptomyces coelicolor* is low compared to the activity of glutamine synthetase I (GSI, encoded by *glnA*) and to that of GSII from *S. viridochromogenes*. We have identified and sequenced a 3.9-kb *BglII*-*Bam*HI fragment carrying the glutamine synthetase II gene (*glnII*) from *S. coelicolor*. Besides *glnII*, this region contains four ORFs (*orf1*–*orf4*). While homologues of *orf1* and *orf2* were also found in the *glnII* region of the *S. viridochromogenes* chromosome, this was not the case for *orf3* and *orf4*, which encode a putative hydrolase and a transcriptional regulator (Ptr) of the MarR family, respectively. High-resolution S1 nuclease mapping showed that the *S. coelicolor* *glnII* gene is expressed from two overlapping promoters. The first comprises a vegetative promoter sequence and the second contains sequence elements that are recognized by $E\sigma^{31}$. Similar promoter structures were found upstream of the *S. viridochromogenes* *glnII* gene. The involvement of *ptr* in *glnII* regulation was studied by gel retardation assays. Recombinant Ptr interacted with the upstream region of *ptr*, but not with the promoter region of *glnII*. A *ptr* gene replacement mutant (*S. coelicolor* IP) was also constructed. RT-PCR analysis of RNA from wild-type *S. coelicolor* and the IP mutant demonstrated that 0expression of *orf3* depends on Ptr. Thus, the difference in gene organization between *S. coelicolor* and *S. viri-*

dochromogenes is not responsible for the difference in GSII activity.

Key words *Streptomyces coelicolor* · Nitrogen metabolism · Glutamine synthetase

Introduction

In most microorganisms, glutamine synthetase (GS) is especially important in nitrogen metabolism, as it is the only enzyme capable of ammonium assimilation under conditions of nitrogen starvation. So far, four different glutamine synthetase types (GSI, GSII, GSIII and GlnT) have been discovered in bacteria (for review, see Merrick and Edwards 1995). Although all these glutamine synthetases are characterized by conserved sequence domains, they differ in expression pattern, the number of subunits, kinetic properties and by post-translational modification.

In enteric bacteria, only one glutamine synthetase (GSI, encoded by *glnA*) is present, which is regulated at the transcriptional as well as the post-translational level by a complex mechanism involving at least five proteins of the nitrogen regulation (Ntr) system (for reviews, see Stadtman et al. 1980; Reitzer and Magasanik 1987; Merrick and Edwards 1995).

In contrast to enteric bacteria and also to other gram-positive prokaryotes, like bacilli (for review see Fisher 1999), streptomycetes contain at least two distinct GS enzymes (Behrmann et al. 1990; Kumada et al. 1990). GSI (encoded by *glnA*, Wray and Fisher 1988) is composed of 12 identical subunits and is similar to other bacterial GSs of type I. The heat-sensitive GSII (encoded by *glnII*; Hillemann et al. 1993) is an octamer and resembles the eukaryotic GS type.

In *S. coelicolor*, both GSI activity and *glnA* expression are subject to complex control mechanisms. We previously identified a *glnE* gene (encoding an adenyltransferase) from *S. coelicolor* (Fink et al. 1999) which seems to be ubiquitous among *Streptomyces*

Communicated by A. Kondorosi

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species. Analysis of a *glnE* replacement mutant demonstrated that GSI in *S. coelicolor* is inactivated under conditions of high nitrogen supply by the *glnE* gene product, possibly by adenylation. In addition to *glnE*, the identification of *glnB* (Fink et al. 1999) and *glnD* (by the *S. coelicolor* Genome Sequencing Project at the Sanger Centre, Hinxton, UK) provides further evidence that *S. coelicolor* GSI is post-translationally modified by Ntr-like proteins. Fisher and Wray (1989) have shown that *glnA* is monocistronically transcribed from the same promoter during all phases of growth; the sequence of this promoter is compatible with the consensus sequence of *Streptomyces* vegetative promoters in the -10 but not the -35 region. *glnA* expression might be controlled by the positively acting transcription factor GlnR; the corresponding gene was isolated by complementation of a glutamine auxotrophic *S. coelicolor* mutant (Wray and Fisher 1993).

The regulation and function of *Streptomyces* GSII are poorly understood. Sequence data for *glnII* are available only from *S. viridochromogenes* (Behrmann et al. 1990) and *S. hygroscopicus* (Kumada et al. 1990). The C-terminal region of the prokaryotic GSI enzyme subunit, which in *E. coli* carries the site of reversible covalent GSI modification (Tyr-398), is not present in the GSII sequence. Therefore, it is assumed that - like the eukaryotic GS type - GSII from streptomycetes is not modified post-translationally (Behrmann et al. 1990). The GSII in *S. viridochromogenes* is active under various nitrogen conditions and can comprise up to 30% of the total GS activity (Hillemann et al. 1993). In contrast, only 4-8% heat-labile GSII activity was detected in *S. coelicolor* (Nguyen et al. 1994). Because no post-translational modification seems to occur, these

differences in detectable GSII activity may be caused by different levels of *glnII* transcription.

As a first step towards understanding *glnII* transcriptional control, we have identified and characterized the *glnII* gene from *S. coelicolor* and analysed the role of its 5' upstream region.

Materials and methods

Strains, plasmids and media

Strains and plasmids used in this study are listed in Table 1. All strains were cultivated as described previously (Fink et al. 1999), unless stated otherwise. *S. coelicolor* M145 (Hopwood et al. 1985) was used as the *S. coelicolor* wild-type strain.

Molecular cloning, Southern hybridization, DNA sequencing and analysis

For all recombinant DNA techniques and analyses the standard procedures published previously (Fink et al. 1999) were used.

PCR

The oligonucleotide primers used in this study (Table 2) were synthesized by MWG Biotech (Munich) or Sigma Genosys (Cambridge). For PCR, the following reaction mixture was used: 0.2 µg of *S. coelicolor* chromosomal DNA as template, 1.0 µM of each primer, 10 µl of 10 × reaction buffer (with 20 mM MgCl₂), 5.0% DMSO, 0.2 mM dNTPs, 2.5 U of polymerase (Boehringer). After the first denaturation (2 min, 94 °C), 30 amplification cycles (1 min denaturation at 92 °C, 2 min annealing at 60 °C, 0.5 min elongation at 72 °C for *ptr* amplification, or 1.5 min elongation at 72 °C for *ptr-aphII* amplification) were performed in a thermocycler (Pharmacia). PCR products were electrophoretically separated in an 1% agarose gel, isolated by gel elution (QIAquick, Qiagen) and directly employed for cloning.

Table 1 Bacterial strains, plasmids and vectors used in this study

Strains/plasmids	Relevant phenotype/characteristics	Reference/source
Strains		
<i>E. coli</i> XL1-Blue	<i>recA1 hsdR17 relA1 lac[F' lacI^qZM15 Tn10 (Tet^r)]</i>	Bullock et al. (1987)
<i>E. coli</i> ET12567	<i>F⁻ dam⁻13::Tn9 dcm-6 hsdM hsdR lacY1</i>	MacNeil et al. (1992)
<i>E. coli</i> JM109	<i>F' traD36 lacI^qΔ(lacZ)M15 proA⁺ B⁺ e14⁻ (McrA⁻) Δ(lac-proAB) thi gyrA96 (Nal^r) endA1 hsdR17(r_k⁻m_k⁺) relA1 supE44 recA1</i>	Yanisch-Perron et al. (1985)
<i>E. coli</i> Ptr	<i>E. coli</i> JM109 carrying pANK48; <i>bla</i>	This work
<i>S. coelicolor</i> M145	<i>S. coelicolor</i> A3(2) free of plasmids	Hopwood et al. (1985)
<i>S. coelicolor</i> IP	Mutant of <i>S. coelicolor</i> A3(2) with insertionally inactivated <i>ptr</i> gene; <i>aphII</i>	This work
Plasmids		
pUC18/19	<i>bla</i> , <i>lacZ'</i> α-complementation system	Vieira and Messing (1982)
pK18/19	pUC18/19 derivative; <i>aphII</i>	Pridmore (1987)
pUC19aphII	pUC19 derivative with <i>aphII</i> ; <i>bla</i>	C. Bormann (Tübingen)
pWHM3	<i>E. coli</i> origin, <i>Streptomyces</i> origin; <i>bla</i> , <i>tsr</i>	Vara et al. (1989)
pJOE2702	pBR322-derived vector with <i>rha</i> _p expression cassette	Volf et al. (1996)
pSV32	<i>S. coelicolor</i> A3(2) 3.2-kb genomic <i>Bgl</i> II fragment in pUC19	This work
pSV27	<i>S. coelicolor</i> A3(2) 2.7-kb genomic <i>Bam</i> HI- <i>Sac</i> I fragment in pUC19	This work
pDF1618	1.6-kb <i>Sph</i> I- <i>Bam</i> HI fragment from pSV32 containing the <i>glnII</i> gene and 600 bp of the upstream region in pUC18	This work
pANK29	PCR-generated <i>ptr</i> inserted into the <i>Nde</i> I and <i>Bam</i> HI sites of pUC18; <i>bla</i>	This work
pANK48	PCR-generated <i>ptr</i> inserted into the <i>Nde</i> I and <i>Bam</i> HI sites of pJOE2702; <i>bla</i>	This work
pANK43	pANK29 derivative with <i>ptr</i> inactivated by insertion of <i>aphII</i> ; <i>bla</i> , <i>aphII</i>	This work
pANK90	pANK43 derivative with <i>ptr</i> inactivated by insertion of <i>aphII</i> in pWHM3; <i>bla</i> , <i>aphII</i> , <i>tsr</i>	This work

Table 2 Oligonucleotides used in this study

Use	Primer	Strand	Position in <i>glnII</i> region	Sequence (5'→3') ^a
S1 mapping	SI01	+	3811–3838	CAGACCGGCAGGGCGTCCAGAC
	SI02	–	2651–2672	<i>GATGGATCGATCGCGTGAGCCGCGCCACAACGAG</i>
<i>ptr</i> expression	ptru	+	2158–2186	<i>AAAAAAACATATGCCGGAAGAGATGGCAGAGCCGGAAGAG</i>
	ptrl	–	2652–2681	<i>AAAGGATCCTTACCCGGGCTCGTTGTGGCGCGGCTCACG</i>
Gel retardation assays	ptrglnIIu	+	2735–2752	<i>AAGAATTCCACGGCAGTAGACGCGGCACC</i>
	ptrglnIIl	–	2805–2826	<i>AAAAAGCTTGAAGGTCACGGGGCCACATCC</i>
	orf3ptru	+	2045–2066	<i>AAGAATTCCGAGCGGTGCCCCCTGTTTCGC</i>
	orf3ptrl	–	2136–2156	<i>AAAAAGCTTTTCCGGCTCTTCCGCCTCTGC</i>
RT-PCR	orf31	+	1409–1438	TGACCGGCCTGCGCTCGGGGAAGCCGACGG
	orf32	–	1909–1938	CGGTGCGACGCACGTCCACCTGACCGGCC
	ptr1	+	2157–2186	GTGCCGGAAGAGATGGCAGAGCCGGAAGAG
	ptr2	–	2652–2681	TTACCCGGGCTCGTTGTGGCGCGGCTCACG
	glnII1	+	2817–2839	GTGACCTTCAAGGCCGAGTACAT
	glnII2	–	3822–3845	CTGGCCGGCCTTCTCCAGCGC
	glnA1	+		GGCGAGCAGTACTCCCGCGACC
	glnA2	–		GTACACCAGGTTACCCGGCGCCTC

^a Nucleotides that deviate from the original gene sequence are printed in *italics*

Heterologous expression of *ptr*

Using the primers ptru/ptrl (Table 2), the *ptr* gene was amplified as a 529-bp *NdeI*-*Bam*HI fragment from total *S. coelicolor* DNA. After digestion with *NdeI* and *Bam*HI, the *ptr* gene was ligated into the *NdeI*/*Bam*HI-digested expression vector pJOE2702 (Vofft et al. 1996). The resulting recombinant plasmid (pANK48) was used to transform *E. coli* JM109 (resulting in *E. coli* Ptr). *E. coli* Ptr was grown in LB and expression was induced at an OD₆₀₀ value of 0.2 after addition of L-rhamnose to a final concentration of 0.2%.

Crude extracts were prepared and heat treated at 70 °C for 15 min before proteins were fractionated by SDS-PAGE (Laemmli 1970).

Gel retardation assays

For gel-shift assays of Ptr binding to double-stranded oligonucleotides, *E. coli* Ptr cells were harvested, washed twice in potassium phosphate buffer (pH 7.0) and disrupted by two consecutive passages through a French press (American Instruments Corporation) at 1000 psi (6900 kPa) to yield cell extracts. PCR-generated DNA fragments (see Table 2 for primer sequences) were purified using the QIAquick PCR Purification kit (Qiagen), labelled with [α -³²P]dATP (1480 kBq) by Klenow fill-in, and free dNTPs were removed with the QIAquick Nucleotide Removal kit (Qiagen). Labelled DNA fragments and cell extracts were incubated at room temperature for 10 min as described by Garabedian et al. (1993). Protein-DNA-complexes and free DNA were resolved on non-denaturing 6% polyacrylamide-TBE gels (Novex). Electrophoresis was carried out at 30 mA and 4 °C.

Construction of the *S. coelicolor ptr* mutant (IP)

For the construction of a *ptr* replacement-plasmid, a kanamycin resistance cassette (*aphII*) was ligated as a *SmaI*-*HincII* fragment (from pUC19aphII) into *HincII*-digested pANK29 (Table 1). From the resulting plasmid, pANK43, a 1870-bp *NdeI*-*Bam*HI fragment containing the disrupted *ptr* fragment was ligated into *EcoRI*/*Bam*HI-cleaved pWHM3 (Vara et al. 1989) following a Klenow fill-in reaction of the *NdeI* and *EcoRI* sites. The resulting plasmid was designated pANK90 and used as replacement plasmid for the construction of the *S. coelicolor ptr* mutant (IP), as described previously (Fink et al. 1999).

Recovery and breakage of cells, crude extract preparation and glutamine synthetase assay

Harvesting and disruption of cells, crude extract preparation, GS activity measurement and protein determination were done as described previously (Fink et al. 1999).

RT-PCR analysis

For RT-PCR analysis wild-type *S. coelicolor* and the *S. coelicolor* IP mutant cells were grown in YEME (Hopwood et al. 1985). Total RNA was isolated from log-phase cultures with the RNeasy kit from Qiagen according to the manufacturer's instructions. The reverse transcription reaction was carried out with the RT-PCR Kit from Sigma following the recommendations of the manufacturer.

High-resolution S1 nuclease mapping

Wild-type *S. coelicolor* RNA was isolated from mycelium grown on plates of nitrogen-limited Evans medium (N-Evans; modified from Evans et al. 1970), overlaid with cellophane discs prior to spore inoculation.

A 1.6-kb *SphI*-*Bam*HI fragment containing the *glnII* gene and 600 bp of the upstream region were cloned into pUC18 (pDF1618). An internal *glnII* primer (SI01, Table 2) and an internal *orf4* oligonucleotide with a 12-bp non-homologous tail at its 5' end (SI02, Table 2) were designed. The 5' end of the SI01 primer was labelled using [γ -³²P]ATP (3000 Ci/mmol). A 282-bp PCR fragment was generated with these primers and used as a probe. 40 μ g of RNA and 25 fmol of the labelled probe were vacuum dried and resuspended in 20 μ l of NaTCA buffer. After a 10-min denaturation at 70 °C, hybridization was allowed to proceed for a minimum of 3 h at 45 °C. For high-resolution analysis, sequencing ladders were generated with the TaqTrack Sequencing System from Promega. Both the direct radiolabelling and the incorporation protocols were used.

Nucleotide sequence accession number

The nucleotide sequence of the 3.9-kb *S. coelicolor* region containing *glnII* has been deposited in the EMBL database under Accession No. Y13833.

Results and discussion

Analysis of *glnII* and its upstream region in the *S. coelicolor* genome

Two *S. coelicolor* DNA fragments carrying *glnII* were isolated from cosmids constructed by Redenbach et al. (1996) after Southern hybridization with a *glnII* probe from *S. viridochromogenes* (data not shown): a 3.2-kb *Bgl*III fragment from cosmid C118 and a 2.7-kb *Bam*HI-*Sac*I fragment from cosmid 10B7. Each fragment was ligated into pUC18, generating pSV27 and pSV32 (Table 1). A common *Sac*I-*Bgl*III fragment (2.1 kb, Fig. 1) was identified by restriction analysis. The nucleotide sequence of the 3.9-kb *glnII* region was determined on both strands. Within this region, one incomplete and four complete ORFs with an overall GC content of about 72.4 mol%, typical for *Streptomyces* genes (Wright and Bibb 1992), were identified (*orf1* to *orf4* and *glnII*, Fig. 1).

Nucleotides (nt) 1–356 of *orf1* show high coding probability up to a TAG stop codon. The start codon of this *orf1* is missing, but comparison of the deduced amino acid sequence with proteins from the databases showed similarity (29% identity, 44% similarity) to ArsC from *E. coli* (Chen et al. 1986), which is assumed to be an arsenate reductase. In addition, *orf1* is similar to a gene of unknown function from *S. viridochromogenes* (*orf1*, 86% identity), which also is located in the region upstream of *glnII* (Behrmann et al. 1990). As deduced from the conserved regions in the alignment with ArsC, only five codons are missing from the *orf1* sequence.

orf2 is located downstream of *orf1* and in the same orientation (ATG at position 475–477, TGA at position 1170–1172), and was found to be very similar to an *orf* of unknown function in an equivalent position in *S. viridochromogenes* (85% identity; Behrmann et al. 1990). As deduced from the hydrophobicity plot of the predicted product of *orf2* (231 aa), the N-terminus of the

hypothetical protein contains a hydrophobic region (aa 13–31) and shows all the characteristics of a typical *Streptomyces* signal sequence (aa 1–31; Engels and Koller 1992). Therefore, *orf2* may code for a secretory protein.

In contrast to the case in *S. viridochromogenes*, two additional ORFs are found in the region upstream of *glnII* on the *S. coelicolor* chromosome. The *orf3* is in the opposite orientation to *orf1* and *orf2* and extends from nt 2045 to nt 1173. The amino acid sequence deduced from *orf3* is related to proteins of the α - β -folded family of hydrolases from *Pseudomonas putida* (24.6% identity to XylF; Diaz and Timmis 1995).

The predicted product of *orf4* (nt 2157–2681) is assumed to be a regulatory protein (putative transcriptional regulator, Ptr). Ptr shows similarities to regulators of the MarR family (26.6% identity to PecS from *Erwinia chrysanthemi*; Reverchon et al. 1994) and contains a potential helix-turn-helix motif (aa 21–79).

The deduced sequence of the final ORF (GTG at nt 2817–2819, TGA at nt 3846–3848) exhibits very high similarity (93% identity) to the *S. viridochromogenes* GSII sequence (Behrmann et al. 1990) and was named *glnII*. Regions showing significant similarities to GS proteins are distributed over the whole sequence of 343 aa [75% to *S. hygroscopicus* (Kumada et al. 1990); 60% to *Frankia alni* (Rochefort and Benson 1990); 36% to GSI from *S. coelicolor* (Wray and Fisher 1988)], including all five regions conserved in GS proteins (I, G⁵¹–R⁹³; II, A¹⁷²–P¹⁸⁶; III, T²⁰⁶–K²²⁸; IV, D²⁸⁹–P³⁰⁰; V, I³¹¹–R³²⁷; Rawlings et al. 1987; Janssen et al. 1988).

The *glnII* upstream region from nt 2674 to nt 2816 is also highly conserved (76% identity) within the two species *S. coelicolor* and *S. viridochromogenes* (Fig. 2). The putative promoter elements proposed for *S. coelicolor* (see below) were also present in *S. viridochromogenes*.

Mapping of the *glnII* transcriptional start site

In order to localize the *glnII* promoter, the transcription start site of *glnII* was determined by high-resolution S1 nuclease mapping. A 282-bp PCR fragment (positions 2651–3838 of the sequenced *glnII* region; for details, see Materials and methods and Table 2) was generated for use as a probe with 166 bp complementary to the upstream and potential promoter region of *glnII*. This probe was annealed to total RNA isolated from *S. coelicolor*, cultivated under N limitation (Fig. 3). Two transcripts were determined, a major and a minor one, initiating at an adenine and a guanine 78 bp and 74 bp upstream of the *glnII* translational start site, respectively (Fig. 2). In *S. coelicolor*, several σ factors have been identified which are involved in the complex regulation of mycelial growth and differentiation (Westpheling et al. 1985; Buttner 1989; Kang et al. 1997). Among the *Streptomyces* promoters characterized, the –10 promoter region of the major *glnII* transcript (CACGCT) has its

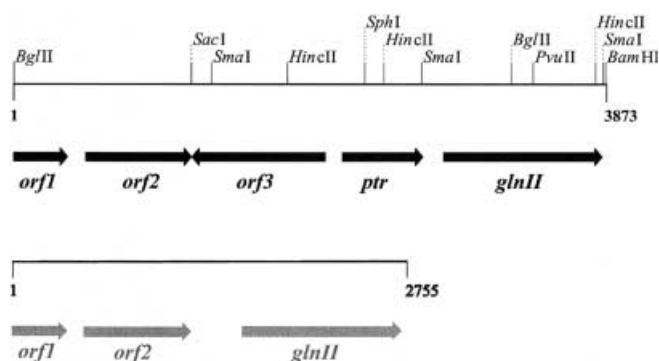


Fig. 1 Schematic representation of the sequenced 3868-bp region of *S. coelicolor* DNA. The identified ORFs and their transcriptional orientation are indicated by arrows. *ptr* is also referred to as *orf4* in the text. For comparison, the *glnII* region of *S. viridochromogenes* (Behrmann et al. 1990) is shown below the *S. coelicolor* scheme

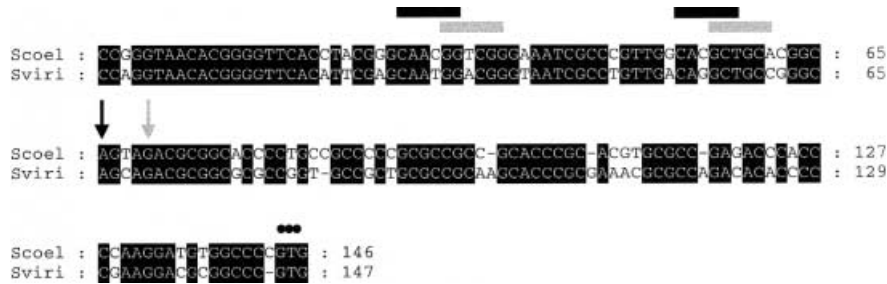


Fig. 2 Alignment of the DNA sequences upstream of *glnII* in *S. coelicolor* (1–146) and *S. viridochromogenes* (1–147). The translational start codon of *glnII* (filled circles) is marked. The two transcriptional start sites of *S. coelicolor glnII* determined by high-resolution S1 nuclease mapping are indicated by a black and a grey arrow, respectively. The corresponding –10 and –35 regions (see text) are marked at the top

closest equivalent (three out of six) in the consensus element proposed for *Streptomyces* vegetative promoters, TTGaca-18 bp-tAGgaT (Strohl 1992; Bourn and Babb 1995), while the –10 and –35 promoter regions of the minor transcript (GGTCGG -17 bp-GCTGCA) are similar to the sequence elements recognized by $E\sigma^{31}$ (GGGcag-N_{17–18}-GttgcN). Interestingly, the p2 promoter of the *S. coelicolor glnR* gene, which encodes a putative transcriptional regulator of *glnA*, also contains a putative $E\sigma^{31}$ recognition element (Kang et al. 1997). However, $E\sigma^{31}$ binds not only to the promoters of *glnR* but also to those of *actIII* and *hrdD* (Kang et al. 1997), which are not involved in nitrogen metabolism. Therefore, the specific function of $E\sigma^{31}$ in nitrogen regulation has to be elucidated in more detail.

Our experimental data clearly show that the *glnII* gene in *S. coelicolor* is transcribed. Moreover, earlier

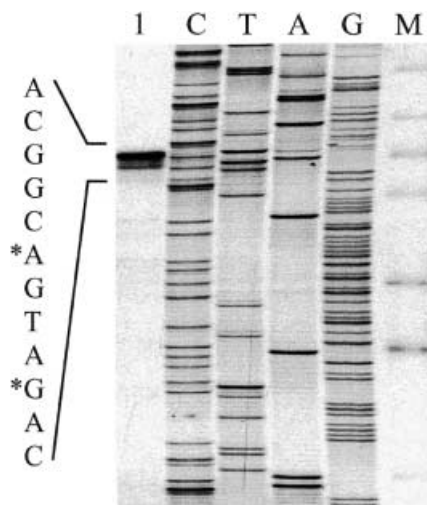


Fig. 3 Transcription of *glnII* in *S. coelicolor* cultures grown on N-Evans medium. Lane 1, S1 protection fragment; lanes C, T, A and G, DNA sequence ladder; M, marker (α ³²P-end labelled *HpaII* digest of pBR322 was used; fragment sizes: 217, 201, 190, 180, 160 and 147 bp) are shown. The asterisks indicate most probable transcription start sites in the coding strand

characterization of a single knock-out *glnA* mutant revealed this strain to be prototrophic for glutamine (Fink et al. 1999). Thus, besides GSI, another functional GS enzyme must exist in *S. coelicolor*. The *S. coelicolor glnII* gene is highly similar (93% identity) to the *glnII* gene from *S. viridochromogenes* (Behrmann et al. 1990). According to Hillemann et al. (1993), the *S. viridochromogenes* GSII can provide up to one-third of total GS activity, suggesting a major role in glutamine synthesis under certain conditions. Taken together, these data strongly indicate that GSII is a functional enzyme in *S. coelicolor*.

Ptr represents a DNA-binding protein

It is well known that transcriptional GS regulators are organized in an operon with *glnA* in gram-negative bacteria (Pahel et al. 1982) as well as in gram positive bacteria (Schreier et al. 1989; Varmanen et al. 2000). Therefore, we speculated that the *ptr* gene, which ends 135 nt upstream of *glnII*, might encode a transcriptional regulator of *S. coelicolor glnII*. This was investigated by performing gel retardation assays.

Using the rhamnose-inducible, positively regulated expression cassette of pJOE2702 (Volf et al. 1996), *ptr* was expressed in *E. coli*. Cell-free extracts with the recombinant *Ptr* protein were assayed for interaction with synthetic DNA fragments, one spanning the intergenic region between *ptr* and *glnII* (Fig. 4, probe B), the other corresponding to the intergenic region between *orf3* and *ptr* (Fig. 4, probe A). The results clearly showed that *Ptr* does not bind to the region upstream of *glnII* (Fig. 4, lane 9 and lane 10), but causes a shift when incubated with its own upstream region (Fig. 4, lane 4 and lane 5). The divergent arrangement of *ptr* and *orf3* raises the possibility that the product of *ptr* either acts to regulate its own transcription and/or controls the expression of *orf3*. Based on these data, co-transcription of *ptr* and *glnII* cannot be conclusively excluded. However, S1 nuclease experiments probing the *glnII* upstream region revealed no *ptr-glnII* readthrough (data not shown).

Inactivation of the *S. coelicolor ptr* gene

For further characterization of *ptr*, a *S. coelicolor* mutant with an insertionally inactivated *ptr* gene was constructed using the replacement-plasmid pANK90

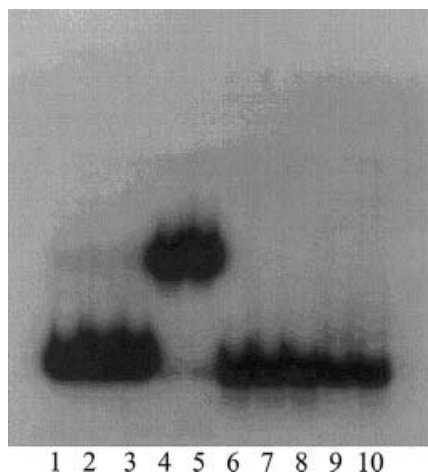


Fig. 4 Gel retardation analysis of specific DNA-binding by Ptr. Ptr interacts with the upstream region of *ptr* (probe A) but not with the upstream region of *glnII* (probe B) as shown by non-denaturing PAGE of DNA-protein mixtures. Lane 1, probe A alone (200 ng DNA); 2, probe A + *E. coli* Ptr crude extract, uninduced (6.5 µg protein); 3, probe A + *E. coli* Ptr crude extract, uninduced (13 µg protein); 4, probe A + *E. coli* Ptr crude extract, induced (6.5 µg protein); 5, probe A + *E. coli* Ptr crude extract, induced (13 µg protein); 6, probe B alone (200 ng DNA); 7, probe B + *E. coli* Ptr crude extract, uninduced (6.5 µg protein); 8, probe B + *E. coli* Ptr crude extract, uninduced (13 µg protein); 9, probe B + *E. coli* Ptr crude extract, induced (6.5 µg protein); 10, probe B + *E. coli* Ptr crude extract, induced (13 µg protein)

(Table 1; for details, see Materials and methods) and the mutagenesis protocol described previously (Fink et al. 1999). The gene replacement event was verified by PCR and Southern hybridization. The fragments amplified from mutant genomic DNA in PCR experiments with primers Ptru and Ptrl were about 1.8 kb in size, whereas a 0.5-kb fragment was obtained when wild-type genomic DNA was used as a template (Fig. 5b). The size difference of 1.3 kb corresponds to the size of the inserted kanamycin resistance cassette. The additional PCR products, observed in both PCR experiments were regarded as artifacts, since the Southern analysis with the *ptr* gene from pANK29 also confirmed the replacement event. As indicated in Fig. 5c, a single hybridization signal was observed in *BglII*-digested wild-type DNA. In contrast, two signals were obtained with genomic DNA from the mutant. This pattern was expected since the inserted *aphII* cassette carries an additional *BglII* site, and confirmed that the native *ptr* in the *S. coelicolor* IP mutant was disrupted by insertion of the kanamycin resistance cassette. Moreover, Southern analysis with the 1.3-kb *aphII* resistance cassette as a probe (data not shown) indicated that only one copy of the antibiotic resistance cassette was present in *S. coelicolor* IP.

No defects in differentiation or sporulation of *S. coelicolor* IP on agar (minimal medium containing one of the nitrogen sources described) were observed. The mutant is prototrophic and grows in liquid HA, S or minimal medium with asparagine, aspartate, glutamine, glutamate, histidine, serine, nitrate or ammonia as sole

nitrogen source. In addition, the *ptr* mutant cells expressed about the same GSII (and GSI) activities as the wild type when grown in media containing different nitrogen sources (data not shown). If Ptr were involved in the transcriptional control of *glnII*, changes in GSII activities would be expected in a *ptr* mutant. Since Ptr is obviously not a regulator of *glnII*, the arrangement of *ptr* (Fig. 1) and the bandshift data suggest a role in the regulation of *orf3*. The sequence data suggest that the three adjacent genes (*orf1*, *orf2* and *orf3*) upstream from *ptr* may be involved in an arsenate resistance mechanism. The components of such systems are known to be clustered but the number of genes involved varies (Silver 1996; Rensing et al. 1999; Butcher et al. 2000). Consequently, the IP mutant phenotype was studied in the presence of arsenate. However, both the wild-type and IP mutant cells showed similar levels of resistance to this heavy metal. In both cases, 50% growth inhibition was observed in the presence of 20 mM arsenate.

Ptr is a repressor of *orf3* expression

The possibility that *ptr* is involved in regulation of *orf3* was investigated by RT-PCR analysis. RNA was isolated from wild-type *S. coelicolor* and from the *S. coelicolor* IP mutant, and primer pairs deduced from the sequences of *orf3*, *ptr*, *glnII*, and *glnA* (Table 2) were used. In control experiments without reverse transcriptase no PCR products were formed, confirming that the products were derived from RNA and not from contaminating DNA. As shown in Fig. 6, RT-PCR products of the expected sizes were obtained from wild-type *S. coelicolor* RNA for *glnA* (689 bp), *ptr* (524 bp) and *glnII* (1028 bp). No evidence for an *orf3* transcript was found.

In contrast, RT-PCR with RNA from the *S. coelicolor ptr* mutant generated in addition to *glnA* and *glnII* cDNA signals a strong signal for *orf3* (529 bp), suggesting derepression of the gene under these conditions. Thus, Ptr is a repressor of *orf3* transcription, and is neither involved in the regulation of *glnII* nor in the expression of *glnA*.

Concluding remarks

We have shown that *S. coelicolor* contains a GSII-encoding gene homologous to the *glnII* from *S. viridochromogenes*. However, genes in the *glnII* region of the chromosome are differently organized in the two *Streptomyces* strains. We have demonstrated that the *S. coelicolor ptr* and *orf3* genes, which are not present in *S. viridochromogenes*, are not involved in nitrogen metabolism.

Moreover, we demonstrated that the *S. coelicolor glnII* gene is expressed from two different promoters. This result confirms that *glnII* is not a silent gene, ruling out doubts about the existence of a functional *glnII* gene in *Streptomyces*.

Fig. 5a-c Confirmation of the genotype of the *S. coelicolor* IP mutant. **a** Physical maps of *ptr* from wild-type *S. coelicolor* (WT) and the mutant IP, in which the *ptr* gene is inactivated by insertion of an *aphII* cassette (for details see Materials and methods and Fig. 1). **b** Analysis of genomic DNA by PCR. The primer combination Ptru and Ptrl was used with genomic DNA isolated from wild-type *S. coelicolor* (WT) and the IP mutant (IP). M, DNA marker X (Boehringer), with fragments of 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517, 506, 396, 344, 298, 220, 201, 154, 134 and 75 bp. **c** Analysis by Southern hybridization. Genomic DNA from the wild-type *S. coelicolor* (WT) and the *ptr* mutant (IP) was digested with *SmaI* (lanes 1) or *BglII* (lanes 2) and hybridized with a digoxigenin (DIG)-labelled *ptr* probe. M, DNA marker VII, DIG-labelled (Boehringer), with fragments of 8000, 7100, 6000, 4800, 3500, 2700, 1900, 1850, 1500, 1400, 1150, 1000, 680, 490 and 370 bp

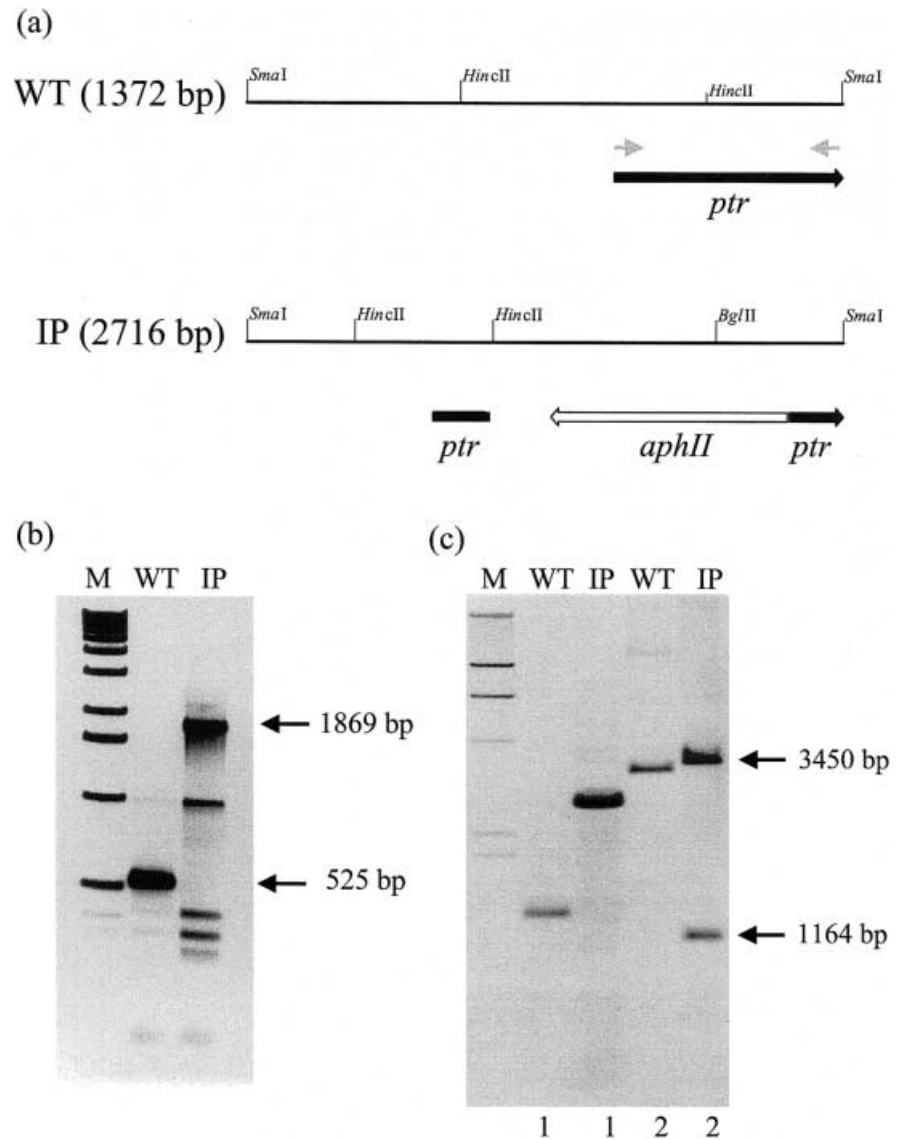
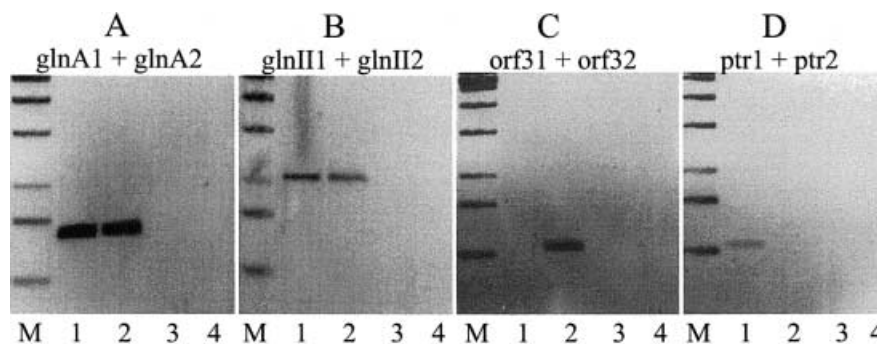


Fig. 6A-D RT-PCR analysis of RNA from wild-type *S. coelicolor* (WT) and the *ptr* mutant (IP). PCR was carried out with *glnA*-specific primers (A), *glnII*-specific primers (B), *orf3*-specific primers (C) and *ptr*-specific primers (D). Lane M, DNA marker (GeneRulerTM 1-kb ladder, Fermentas); the following fragment sizes (bp) are shown: 2500, 2000, 1500, 1000, 750, 500 bp. In lanes 1, wild-type RNA was used as the template for RT-PCR; lane 2, RNA from the IP mutant; lane 3, control, as lane 1 but without reverse transcriptase; lane 4, control, as lane 2 but without reverse transcriptase



Acknowledgements *S. coelicolor* cosmids were kindly provided by M. Redenbach. We are grateful to J. Altenbuchner for the kind gift of plasmid pJOE2702. We wish to thank A. Latus for excellent technical assistance. N.W. acknowledges the receipt of a fellowship from the Landesgraduiertenförderung des Landes Baden-Württemberg. D.F. is grateful for a fellowship from the Deutscher Akademischer Austausch Dienst and for a scholarship from the Studienstiftung des deutschen Volkes. This work was supported by the European Union (BIO4-CT95-0198) and by the Bundesministerium für Bildung und Wissenschaft, Forschung und Technologie (BEO/22 0310814).

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