Nitrogen metabolism in *Streptomyces coelicolor* A3(2): modification of glutamine synthetase I by an adenylyltransferase

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An internal adenylyltransferase gene (*glnE*) fragment from *Streptomyces coelicolor* was amplified using heterologous PCR primers derived from consensus motifs. The sequence had significant similarity to bacterial *glnE* genes, and included a motif typical of the C-terminal adenylyltransferase domain of GlnE. *glnE* from *S. coelicolor* lies on the Asel-C fragment of the chromosome and is localized near *glnA* (encoding glutamine synthetase I, GSI) and *glnII* (encoding GSII). To analyse the function of GlnE in *S. coelicolor*, *glnE* (*S. coelicolor* E4) and *glnA* (*S. coelicolor* HT107) gene replacement mutants were constructed. The GSI activity of the *glnE* mutant was not down-regulated after an ammonium shock. However, the GSI activity of the wild-type cells decreased to 60% of the original activity. The *glnA* mutant is not glutamine auxotrophic, but in the γ-glutamyltransferase assay no GSI activity was detected in unshifted and shifted HT107 cells. By snake venom phosphodiesterase treatment the GSI activity in the wild-type can be reconstituted, whereas no alteration is observed in the E4 mutant. Additionally, the loss of short-term GSI regulation in the E4 mutant was accompanied by an increased glutamine:glutamate ratio.

**Keywords:** *Streptomyces coelicolor*, nitrogen metabolism, adenylyltransferase, glutamine synthetase

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**INTRODUCTION**

Current knowledge of bacterial nitrogen regulation (for reviews see Magasanik, 1996; Merrick & Edwards, 1995) points to the evolution in bacteria of different regulatory principles that might be a consequence of various life strategies. Rapidly dividing enteric bacteria possess only one glutamine synthetase (GSI, encoded by *glnA*) which, in addition to being feedback-inhibited by nine different end products of the glutamine metabolism, is regulated by a complex action of the components of the nitrogen regulation (Ntr) system (for reviews see Merrick & Edwards, 1995; Reitzer & Magasanik, 1987; Stadtman et al., 1980). The Ntr system triggers synthesis of glutamine in response to nitrogen avail-

ability, and is composed of at least five proteins, acting at the transcriptional as well as the post-translational level: the two-component system NtrB/NtrC controlling *glnA* expression, the primary nitrogen sensor protein uridylyltransferase (encoded by *glnD*), the signal transmitter protein PII (encoded by *glnB*) and the bifunctional adenylyltransferase GlnE (encoded by *glnE*) which reversibly modifies GSI by adenylylation/deadenylylation of a specific residue (Tyr-398) in the C-terminal region of GSI (Shapiro & Stadtman, 1970). The function of a recently found second PII protein (encoded by *glnK* in *Escherichia coli*) is still under investigation (van Heeswijk et al., 1996; Atkinson & Ninfa, 1998; He et al., 1998). To date, sequence data for the native GlnE are available from three organisms: *E. coli* (van Heeswijk et al., 1993), *Haemophilus influenzae* (Fleischmann et al., 1995) and *Mycoplasma tuberculosis* (Cole et al., 1998). The predicted protein sequence of the *E. coli* GlnE (composed of 945 amino acids) indicates that the N-terminal part is highly similar to its C-terminal part (van Heeswijk et al., 1993). GlnE from *E. coli* is a monomer of 115 kDa (Caban & Ginsburg,
1976). Jaggi et al. (1997) have shown that its antagonistic activities (adenylylation and deadyenylylation) are catalysed by different active sites: the deadyenylylation activity is mediated by the N-terminal part (amino acids 1–423) and the adenylylation activity is located on the C-terminal part of the enzyme (amino acids 425–945).

Like enteric bacteria, Gram-positive bacilli contain one GS (for review see Schreier, 1993). However, Bacillus subtilis possesses no homologues of the ntr genes from enteric bacteria (Kunst et al., 1997), and the GS enzyme is not regulated post-translationally by adenylylation (Schreier et al., 1985). Transcription of glnA is negatively controlled in response to the nitrogen status of the cell by two transcription factors, GlnR (Schreier et al., 1989) and TnrA (Wray et al., 1996).

In contrast to other Gram-positive prokaryotes, streptomycetes contain at least two distinct GS enzymes (Behrmann et al., 1990; Kumada et al., 1990): GSI (encoded by glnA; Wray & Fisher, 1988) is composed of 12 identical subunits and is similar to other bacterial GSs; the heat-sensitive GSII (encoded by glnII; Hillemann et al., 1993) is an octamer and resembles eukaryotic GSs. Little is known about the function and regulation of the two GS enzymes in streptomycetes. Wray & Fisher (1993) isolated a regulatory gene (glnR) by complementation of a glutamine auxotrophic Streptomyces coelicolor mutant. GlnR might represent a positive transcription factor for both glnA and glnII (Merrick & Edwards, 1995). A residue corresponding to Tyr-398, the site of reversible covalent GSI modification in E. coli, is not present in the GSII sequence. Therefore, it was assumed that, as expected for the eukaryotic GS type, GSII from streptomycetes cannot be modified post-translationally (Behrmann et al., 1990). In contrast to glnII, the deduced amino acid sequence of the S. coelicolor glnA gene (Wray & Fisher, 1988) contained the conserved tyrosyl residue at position 397. Physiological evidence for post-translational GSI modification was obtained for Streptomyces cattleya (Streicher & Tyler, 1981), S. coelicolor (Fisher & Wray, 1989) and Streptomyces viridochromogenes (Hillemann et al., 1993). Grown on poor nitrogen sources, the GSI activity was followed by 35 cycles of amplification (1 min at 92 °C, 2 min at 60 °C, 2.5 min at 72 °C) in a robocycler (Stratagene). PCR products were separated electrophoretically in a 1% agarose gel, isolated by elution (Qiagen; Qiagen) and cloned directly.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Streptomyces strains used to detect glnE sequences (Fig. 2) were obtained from the ‘Tübingen strain collection’. All other bacterial strains and plasmids used in this study are listed in Table 1.

Streptomyces coelicolor M145 (Hopwood et al., 1985) was used as the S. coelicolor wild-type. Streptomyces were cultivated on R2YE (Thompson et al., 1980), HA (Schwartz et al., 1996) or on HEPES-buffered minimal medium as described by Hillemann et al. (1993). S-medium (Okanishi et al., 1974) or minimal medium (Hopwood, 1967) was used as liquid medium. If necessary, 25 µg kanamycin ml⁻¹, 25 µg thio-estrepton ml⁻¹ or 25 µg hygromycin ml⁻¹ was added to the growth medium.

Escherichia coli was cultivated at 37 °C in LB medium or on LB agar (Miller, 1972). Ampicillin or kanamycin was added at a concentration of 150 or 50 µg ml⁻¹, respectively.

**Molecular cloning.** Plasmid and chromosomal DNA of Streptomyces or E. coli were isolated by established techniques (Hopwood et al., 1985; Sambrook et al., 1989). Procedures for cloning were based on the specific codon usage of streptomycetes (Wright & Bibb, 1992). ORF analysis was carried out as described by Hopwood et al. (1985). The enzymes used in this study were purchased from Boehringer, New England Biolabs or Pharmacia, and used as recommended by the manufacturer. Hybridization experiments were carried out according to Southern (1975).

**PCR.** The oligonucleotide primers used (Table 1) were synthesized by MWG Biotech. For PCR, the reaction mixture used was: 0.2 µg S. coelicolor chromosomal DNA as template, 10 µM of each primer, 10 µl × reaction buffer (with 20 mM MgCl₂), 50% DMSO, 0.2 mM dNTPs, 1.0 µl Tag polymerase (Qiagen). The reaction mixture was overlaid with 60 µl mineral oil. An initial denaturation (2 min, 94 °C) was followed by 35 cycles of amplification (1 min at 92 °C, 2 min at 60 °C, 2.5 min at 72 °C) in a robocycler (Stratagene). PCR products were separated electrophoretically in a 1% agarose gel, isolated by elution (Qiagen; Qiagen) and cloned directly.

**DNA analysis and sequencing.** The PCR-generated glnE fragment cloned in pBluescript II SK(+) (resulting in pDOL15) was resoiolated by digestion with EcoRI/BamHI and purified from a preparative gel. By a Klenow fill-in reaction, blunt ends were created for ligation into the Hincll site of a plBluescript II SK(+) vector. Two constructs with inserts in opposite orientations were identified by restriction analysis and designated pND4 and pND7. They were used to ‘Double-stranded Nested Deletion Kit’ from Pharmacia, according to the manufacturer's recommendations.

The DNA sequence of the cloned 1.5 kb PCR fragment was determined by a standard protocol modified for double-stranded DNA, using the AutoRead Sequencing Kit (Pharmacia) for sequencing on an ALF Sequencer (Pharmacia). Nucleic acid sequences were analysed using the software packages Staden (Staden & McLaughlin, 1982), BLAST (Gish & States, 1993; Altschul et al., 1990), FASTA (Pearson & Lipman, 1988) and CLUSTAL W (Thompson et al., 1994). ORF analysis was based on the specific codon usage of streptomycetes (Wright & Bibb, 1992).

**Construction of the S. coelicolor mutant E4 and HT107.** For the construction of a glnE replacement plasmid, a kanamycin-resistance cassette (aphII) was ligated as a SmaI–Hincll fragment (from pUC19aphII) into BstEII-restricted and
### Table 1. Bacterial strains, plasmids, vectors and oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant phenotype/characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli XL-1 Blue</td>
<td>recA1 bsdR17 relA1 lac[F lacP::ZM15 Tn10(Tet')]</td>
<td>Bullock et al. (1987)</td>
</tr>
<tr>
<td>E. coli ET12567</td>
<td>F' dam 13;: Tn9 dcm-6 bsdM bsdR lacY1</td>
<td>MacNeil et al. (1992)</td>
</tr>
<tr>
<td>S. coelicolor M145</td>
<td>S. coelicolor A3(2) free of plasmids</td>
<td>Hopwood et al. (1985)</td>
</tr>
<tr>
<td>S. coelicolor E4</td>
<td>Mutant strain of S. coelicolor A3(2) with insertionally inactivated glnE gene; aphII</td>
<td>This work</td>
</tr>
<tr>
<td>S. coelicolor HT107</td>
<td>Mutant strain of S. coelicolor A3(2) with insertionally inactivated glnA gene; byg</td>
<td>This work</td>
</tr>
</tbody>
</table>

### Vectors and plasmids

<table>
<thead>
<tr>
<th>Plasmid or vector</th>
<th>Relevant phenotype/characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18/19</td>
<td>bla, lacZ'-x-complementation system</td>
<td>Vieira &amp; Messing (1982)</td>
</tr>
<tr>
<td>pK18/19</td>
<td>pUC18/19 derivative; aphII, lacZ'-x-complementation system</td>
<td>Pridmore (1987)</td>
</tr>
<tr>
<td>pUC19aphII</td>
<td>pUC19 derivative with aphII ligated in SmaI and HindIII vector; bla aphII</td>
<td>C. Bormann (Tübingen)</td>
</tr>
<tr>
<td>pBluescript II SK(+)</td>
<td>bla; f1 (+) origin</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pWHM3</td>
<td>bla tsr; E. coli origin, Streptomyces origin</td>
<td>Vara et al. (1989)</td>
</tr>
<tr>
<td>pDOL15</td>
<td>pBluescript II SK(+) carrying a 1.5 kb internal glnE fragment (PCR-generated fragment with primers P1/P3 from total S. coelicolor DNA as template); bla</td>
<td>This work</td>
</tr>
<tr>
<td>pDOF4</td>
<td>pDOL15 restricted by BstEII and then disrupted by a 1.3 kb SmaI–HindIII aphII cassette; bla aphII</td>
<td>This work</td>
</tr>
<tr>
<td>pDOL4</td>
<td>pWHM3 containing the disrupted glnE fragment from pDOF4; bla aphII tsr</td>
<td>This work</td>
</tr>
<tr>
<td>pND4</td>
<td>glnE fragment isolated as 1.5 kb EcoRI–BamHI fragment from pDOL15, blunt-ended by Klenow fill-in and ligated into the HindIII site of pBluescript II SK(+); bla</td>
<td>This work</td>
</tr>
<tr>
<td>pND7</td>
<td>pND4 which carries the glnE fragment in opposite direction</td>
<td>This work</td>
</tr>
<tr>
<td>pSF205</td>
<td>pUC18 derivative carrying glnA from S. coelicolor; bla</td>
<td>S. H. Fisher (Boston)</td>
</tr>
<tr>
<td>pJ963</td>
<td>pJ2922 derivative; byg</td>
<td>D. Lydiate (Norwich)</td>
</tr>
<tr>
<td>pDFG1</td>
<td>pWHM3 derivative with glnA (1562 bp SmaI fragment) from pSF205; bla tsr</td>
<td>This work</td>
</tr>
<tr>
<td>pDFH107</td>
<td>pDFH1 derivative with glnA inactivated by insertion of bygB as 1.7 kb BglII fragment from pJ963; bla tsr byg</td>
<td>This work</td>
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### Primers

<table>
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<th>Primer</th>
<th>Relevant phenotype/characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Lower primer used to amplify an internal S. coelicolor glnE fragment corresponding to aa 752–759 in E. coli GlnE, 5'-AAGGATCCCTC/GC/GG/GCT/CG/GC/AG/AG/T/T/GC/GCGT/3'</td>
<td>This work</td>
</tr>
<tr>
<td>P3</td>
<td>Upper primer used to amplify an internal S. coelicolor glnE fragment corresponding to aa 327–335 in E. coli GlnE, 5'-AAGATCCGTATCCGC/GC/GT/AG/GAGCT/GC/CT/GC/CAGCT/3'</td>
<td>This work</td>
</tr>
<tr>
<td>P5</td>
<td>Upper primer used to amplify an internal S. coelicolor glnE fragment corresponding to aa 245–254 in E. coli GlnE, 5'-AAGGATTCAGACGG/CG/TGGG/AG/AG/AAG/AG/AG/AG/AG/3'</td>
<td>This work</td>
</tr>
</tbody>
</table>

Klenow-filled-in pDOL15. From the resulting plasmid pDOF4, a 3000 bp EcoRI–SpeI fragment containing the disrupted glnE fragment was ligated into EcoRI/XhoI-restricted pWHM3 (Vara et al., 1989), which can be used as a suicide vector in many Streptomyces species (Bruntner & Bormann, 1998). The resulting replacement plasmid was designated pDOL4. For the construction of a glnA replacement plasmid, a 1562 bp SmaI fragment from pSF205 carrying the complete glnA gene was ligated into EcoRI-restricted and Klenow-filled-in pWHM3 (pDFG1). glnA was inactivated by a hygromycin-resistance cassette (bygB) which was ligated as a 1.7 kb BglII fragment (from pJ963) into the single BglII site of pDFG1. This plasmid was designated pDFH107. The S. coelicolor glnE and glnA single knock-out mutants were constructed using the same mutagenesis protocol: the replacement plasmid (pDOL4 or pDFH107) was used to transform E. coli ET12567 and reisolated. After alkaline denaturation (Oh & Chater, 1997), 1 μg plasmid DNA was introduced into S. coelicolor protoplasts via PEG-mediated transformation (Hopwood et al., 1985). Several thiostrepton-resistant colonies were obtained on R2YE media which had been overlaid with 3 ml NB soft agar (Shirahama et al., 1981) containing 25 μg thiostrepton ml⁻¹. They were allowed to undergo two rounds of sporulation in the absence of antibiotic and the resulting spores were replica-plated on HA media containing 25 μg kanamycin ml⁻¹ (for selection of glnE).
mutants) or 25 μg hygromycin ml⁻¹ (for selection of glnA mutants).

**Cell harvesting, breakage and crude extract preparation.** In general, *S. coelicolor* was grown for 2 d. Mycelium was homogenized, harvested by centrifugation, washed twice with disruption buffer (50 mM imidazole, pH 7.0; 150 mM NaCl; 1 mM MnCl₂; 0.5 mM DTT) and resuspended in the same buffer. *S. coelicolor* cells were broken by two consecutive passages of the mycelium through a French press (American Instruments) at 1000 p.s.i. (6900 kPa). Cell debris and membrane fractions were separated from the soluble fraction by centrifugation (20 min, 13000 g) and the supernatant was used as crude extract. Total protein was determined according to Smith et al. (1985).

**GS assay.** GS was assayed by the γ-glutamyltransferase method adapted from Shapiro & Stadtman (1970). The activity of the heat-stable GSI was determined after heat treatment of crude mycelium extracts at 60 °C for 10 min prior to the enzyme assay (Edmands et al., 1987). Modification of GSI was examined as described by Braña et al. (1986). However, crude extracts were used instead of whole cells. Reactivation of GS in crude extracts was monitored in 20 mM imidazole/HCl, pH 7.0, containing 2.5 mM MnCl₂ at 37 °C. The extracts were incubated at 37 °C for 20 min with SVPDE (Boehringer) at 100 μg ml⁻¹.

**Determination of glutamate and glutamine concentrations.** For measuring the glutamate and glutamine pools in *S. coelicolor* cell extracts, amino acids were derivatized with o-phthalaldehyde (OPA) (Lindroth & Mopper, 1979). Derivatives were loaded on a reverse-phase column (46 × 125 mm; Shandon Hypersil ODS) with 4 μl OPA + 2 μl sample + 4 μl OPA, and separated by HPLC using a linear gradient of elution buffer A (12.5 mM sodium phosphate buffer, pH 7.3; 0.5% tetrahydrofuran) and elution buffer B (20% elution buffer A, 40% methanol, 40% acetonitrile). Components were detected by their UV absorbance at 340 nm (Liquid chromatograph: HP 1090M, built-in diode array detector, autosampler, thermostatbility controlled column compartment; detection: Pascal Workstation HP 79994B).

**RESULTS AND DISCUSSION**

**S. coelicolor contains a glnE homologue**

To examine whether a GS regulation system similar to that of the Enterobacteriaceae is present in *S. coelicolor*, we set out to identify a glnE homologous gene. The sequences of all four adenyllytransferase enzymes available from databases were aligned. The overall identity, including nine conserved regions, was approximately 30%. From these regions consensus sequences were determined and degenerate oligonucleotides were designed based on codon usage in *S. coelicolor* (Wright & Bibb, 1992), and applied in PCR experiments.

From all primers tested, only the combinations P1/P5 and P1/P3 (see Table 1 and Fig. 3a) resulted in fragments of the expected size of 1500 bp and 1300 bp, respectively.

The putative 1500 bp glnE fragment was isolated and cloned in pBluescript II SK(+) utilizing EcoRI and BamHI sites fused to the primers. The resulting plasmid was designated pDOL15 (Table 1) and the 1.5-kb insert was sequenced and analysed. It has a G+C content of 75.6 mol%, which is high even for streptomycetes (typical G+C content 72 mol%; Wright & Bibb, 1992). Only one reading frame gave a region with a high coding probability for streptomycete DNA.

Comparison of the deduced amino acid sequence with protein sequences in the Protein Information Resource database yielded significant similarities to enzymes belonging to the group of nucleotidyltransferases, especially to GlnE proteins (Table 2). A significant similarity (39%) was found to the sequence of the *E. coli* GlnE, which is the covalent modifier of GSI (van Heeswijk et al., 1993). Similarities to GlnE proteins are distributed over the whole 1500 bp fragment, which furthermore contained the conserved amino acid sequence (Fig. 1) assumed to be the functional adenyllylation motif in the *E. coli* enzyme (Holm & Sander, 1995; Jaggi et al., 1997). Identification of a glnE gene from *S. coelicolor* is the first example of an Ntr-like component in a Gram-positive, sporulating bacterium.

**glnE maps between glnA and glnII in S. coelicolor and seems to be ubiquitous among Streptomyces**

In hybridization experiments to locate glnE on the *S. coelicolor* chromosome, the glnE fragment and the *S. coelicolor* glnA and glnII genes as well as a glnB fragment (A. Engels, unpublished) were used as probes. Genomic Southern blots indicated that all of these genes are physically linked (data not shown). This was confirmed by mapping glnE to the *S. coelicolor* cosmids 3H12 (Redenbach et al., 1996), which contains glnA as well as glnII. Both glnE and glnB are located on a 12 kb sub-

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**Table 2. Identities and similarities of the partial GlnE sequence derived from the** *S. coelicolor* **glnE fragment to other GlnE proteins**

<table>
<thead>
<tr>
<th>GlnE (aa)</th>
<th>Sequence matching partial <em>S. coelicolor</em> GlnE fragment</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> (994)</td>
<td>251–750</td>
<td>46</td>
<td>60</td>
<td>Cole et al. (1998)</td>
</tr>
<tr>
<td><em>E. coli</em> (946)</td>
<td>284–759</td>
<td>23</td>
<td>39</td>
<td>van Heeswijk et al. (1993)</td>
</tr>
<tr>
<td><em>H. influenzae</em> (981)</td>
<td>293–816</td>
<td>19</td>
<td>38</td>
<td>Fleischmann et al. (1995)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (partial, 506)</td>
<td>1–506</td>
<td>13</td>
<td>24</td>
<td>U63816</td>
</tr>
</tbody>
</table>
Adenylyltransferase from \textit{Streptomyces coelicolor}

\textbf{Fig. 1.} Multiple alignment of the putative adenylylation domains from different GlnE proteins. Black shading depicts identical amino acid residues in all five sequences, dark grey shading depicts identical residues in four sequences and light grey shading depicts identical residues in three of the five sequences. Strain abbreviations: \textit{S.,} \textit{S. coelicolor;} \textit{M., Mycobacterium tuberculosis;} \textit{E., E. coli;} \textit{H., Haemophilus influenzae;} \textit{P., Pseudomonas aeruginosa.} Numbers give the positions of the first and last residues of the partial sequences. The aligned sequence motif is assumed to be the functional adenylyltransferase domain in GlnE from \textit{E. coli} (Holm & Sander, 1995; Jaggi et al., 1997).

\textbf{Fig. 2.} Southern hybridization of the \textit{glnE} probe with genomic DNA from different \textit{Streptomyces} strains. The lanes contained \textit{BamH}I-digested genomic DNA from \textit{S. coelicolor A3(2) (1), S. lavendulae Tu4301 (2), S. hisutus Tu2001 (3), S. albogriseus Tu4101 (4), S. violaceoniger Tu3801 (5), S. ramulosus Tu3401 (6), S. prasinus Tu3001 (7), S. violaceoruber Tu2201 (8), S. tendae Tu2101 (9), S. diastatochromogenes Tu2001 (10), S. michiganensis Tu1400 (11), S. echinatus Tu1201 (12), S. fradiae Tu1101 (13), S. griseoflavus Tu901 (14), S. griseus Tu600 (15), S. olivaceus Tu501 (16), S. phaeochromogenes Tu301 (17) and S. antibioticus Tu201 (18), M, DNA molecular mass marker VII, DIG-labelled (Boehringer), with fragment sizes (bp) of 8000, 7100, 6000, 4800, 3500, 2700, 1900, 1850, 1500, 1400, 1150, 1000, 680, 490 and 370.

fragment of the \textit{AseI-C} fragment and are flanked by \textit{glnA} and \textit{glnII}. This situation differs from that in \textit{E. coli} (van Heeswijk et al., 1993), where \textit{glnE} is physically separated from the GS gene, but is similar to that in \textit{M. tuberculosis} (Cole et al., 1998), where \textit{glnA}, \textit{glnE} and \textit{glnII} are adjacent. The co-localization of \textit{glnE} and \textit{glnB} in \textit{S. coelicolor} resembles the organization in \textit{Mycobacterium leprae} (MycDB, http://www.sanger.ac.uk, cosmid B32), where \textit{glnE} and \textit{glnB} homologues are downstream from \textit{glnII} on the same cosmid.

Further hybridization studies determined whether the presence of \textit{glnE} is a unique feature of \textit{S. coelicolor}. Using the \textit{glnE} fragment from \textit{S. coelicolor} as a probe, the genomes of 18 \textit{Streptomyces} strains were examined for the presence of hybridizing fragments. The strong signals obtained in all strains (Fig. 2) indicated that \textit{glnE} is ubiquitous in \textit{Streptomyces}.

\textbf{Inactivation of the S. coelicolor glnE gene}

To construct a \textit{glnE} mutant of \textit{S. coelicolor} the replacement plasmid pDOL4 (Table 1) was introduced into \textit{S. coelicolor}. Resistance to kanamycin, conferred by the cassette’s \textit{aphII} gene, was used to select transformants in which integration had occurred via a single cross-over.

Putative gene replacement mutants were then screened for by selecting disruption mutants that had lost the ability to grow on thiostrepton-containing medium. Since thiostrepton resistance is mediated by the vector, its loss indicates a second cross-over event. Of 117 kanamycin-resistant colonies obtained by transformation of \textit{S. coelicolor} with pDOL4 and two subsequent rounds of sporulation, 29 colonies were thiostrepton-sensitive. This implies a frequency of about 25\% for the double cross-over event.

To characterize the genotype of the mutants (Fig. 3a), chromosomal DNA from four potential gene replacement mutants was analysed by PCR and Southern hybridization experiments. All mutants possessed the genotype shown in Fig. 3(b, c) for the \textit{S. coelicolor glnE} mutant E4 (E4). The fragments amplified in PCR experiments from E4 with primers P1 and P5 were about 2-8 kb in size, whereas a 1-5 kb fragment was obtained with the wild-type (Fig. 3b). The 1-3 kb size difference corresponds to the size of the inserted kanamycin-resistance cassette. Southern blot analysis with the 1-5 kb \textit{glnE} fragment (Fig. 3c) and the 1-3 kb \textit{aphII} resistance cassette (data not shown) as a probe confirmed this result and indicated that only one copy of the antibiotic-resistance cassette is present in the E4 mutant.
Inactivation of the *S. coelicolor* *glnA* gene

From enteric bacteria it is known that GSI (encoded by *glnA*) is the target of the adenylyltransferase. For the investigation of GlnE function it was, therefore, of interest to have an *S. coelicolor* *glnA* mutant. Such a mutant was constructed in the same way as described for the *glnE* mutant (for details see Methods). From 200 kanamycin-resistant colonies obtained, 15 colonies (7.5%) were thiostrepton-sensitive.

The occurrence of a gene replacement event in the *S. coelicolor* *glnA* mutant HT107 was verified by Southern hybridization (data not shown) using a *glnA* probe (1.8 kb *SmaI* fragment from pSF205). As indicated in Fig. 4, a single hybridization signal was observed with *SmaI*-digested wild-type DNA. In contrast, two signals (2.35 and 0.9 kb) were obtained with genomic DNA of HT107. This pattern was expected since the inserted *hyg* cassette carries additional *SmaI* sites. This confirmed that the native *glnA* in the *S. coelicolor* HT107 mutant was disrupted by insertion of the hygromycin-resistance cassette.

**The *glnE* and the *glnA* mutants are prototrophic and not defective in growth**

The *S. coelicolor* E4 and HT107 mutants are glutamine prototrophic, so growth in liquid HA, S or minimal medium with asparagine, aspartate, glutamine, glutamate, histidine, serine, nitrate or ammonia as sole nitrogen source was not impaired. In addition, no defects in differentiation or sporulation on agar (minimal medium containing one of the nitrogen sources described) were observed.

*Salmonella typhimurium* *glnE* mutants have a typical Ntr minus phenotype characterized by reduced growth on poor nitrogen sources (Kustu et al., 1984). *B. subtilis* *glnA* mutants with low residual GS activity require a high glutamine concentration to sporulate (Fisher & Sonenshein, 1977). The observation that neither the *S.
coelicolor E4 nor the HT107 mutant suffered from growth defects may reflect the phenomenon of differently regulated GS enzymes in S. coelicolor, which complement each other under various culture conditions.

The \textit{glnE} mutant has lost the ability for covalent GSI modification by adenylylation

In enteric bacteria, adenylylation of GSI occurs when the cell senses excess nitrogen, i.e. when NH$_4^+$ is available after growth under nitrogen-limited conditions. Therefore, \textit{S. coelicolor} wild-type and E4 mutant cells, growing in minimal medium with asparagine as a sole and poor nitrogen source, were subjected to an ammonium shock (see legend of Fig. 5), and the heat-stable GSI activity in crude mycelium extracts was determined.

The GSI activity of \textit{S. coelicolor} wild-type dropped following the ammonium upshift (Fig. 5). In the first 2 min after the shift, GSI activity decreased 40\%. This reduced activity persisted for a further 8 min. However, 20 min after the shift the GSI activity was partially restored, suggesting that the short-term effect induced by ammonium can be balanced by alternative regulating mechanisms. At any time, the reduction of the GS activity was almost completely abolished by SVPDE treatment.

In contrast to the wild-type, no GSI activity was observed in the \textit{glnA} mutant HT107.

In the E4 mutant, the ammonium upshift resulted in a slight increase in GSI activity and was not affected by SVPDE treatment (Fig. 5). At 2, 5 and 10 min after the shift, GSI activity was about 115\% of the pre-shift value. After 20 min a further slight increase was observed.

Adenylylation of GSI after administering an ammonium shock to cells growing in a nitrogen-limited environment has been detected in a number of Gram-negative prokaryotes (Merrick & Edwards, 1995). In Gram-positive streptomycetes, GSI modification by adenylylation (Streicher & Tyler, 1981; Bascaran et al., 1989; Fisher & Wray, 1989; Hillemann et al., 1993) as well as by ribosylation (Penyige et al., 1994) had been proposed. The results obtained with the E4 mutant demonstrate for the first time that in \textit{S. coelicolor} a GlnE-mediated GSI modification takes place. Moreover, the results obtained after SVPDE treatment confirm that GSI is most likely modified by adenylylation in \textit{S. coelicolor}, since only adenylylation and uridylylation (the latter has never been reported for any GS enzyme), but not ribosylation or phosphorylation, are sensitive to the action of SVPDE.

The results for the \textit{glnA} mutant HT107 demonstrate that the heat-stable GS activity measured under the conditions used is due to GlnA alone. There is evidence for a putative third GS gene in \textit{S. coelicolor} (localized on cosmid SCI35; accession no. 3581868) from the \textit{S. coelicolor} genome project (Sanger Institute, Cambridge). This GS shows more similarity to the type I (36 and 37\% similarity to \textit{glnA} of \textit{E. coli} and \textit{S. coelicolor}, respectively) than to the type II GS proteins (20\% similarity to \textit{glnII} of \textit{S. viridochromogenes}). If this putative additional GS gene is functional in \textit{S. coelicolor}, the activity is not detectable under our growth and assay conditions.

The decrease in GSI activity (about 40\%) in ammonium-shocked \textit{S. coelicolor} cells is rather low compared to the almost complete inactivation observed for \textit{E. coli} or cyanobacteria (Stadtman et al., 1980; Merida et al., 1991). This may be due to the tendency of \textit{S. coelicolor} to grow in mycelial clusters and not as finely dispersed cells. The NH$_4^+$ shock may be much weaker for \textit{Streptomycyes} cells growing at the centre of a mycelial cluster. As a consequence, the GSI value detected after the shift would be the mean activity of modified GSI from cells growing at the periphery and of unmodified GSI from cells growing at the centre of a cluster. Another reasonable explanation for the incomplete GSI inactivation in \textit{S. coelicolor} after NH$_4^+$ shock is that \textit{glnE} expression or GlnE activity in \textit{S. coelicolor} is just lower than in enteric bacteria.

**Imbalance in the glutamine:glutamate ratio of the E4 mutant**

It has been postulated that adenylylation of the GSI enzyme in enteric bacteria is a short-term protection of the intracellular glutamate pool under conditions of sudden ammonium excess (Kustu et al., 1984). To test this hypothesis for \textit{S. coelicolor}, the internal glutamate and glutamine concentrations before and after an
ammonium upshift were measured in extracts from both wild-type and glnE mutant cells.

In wild-type cells, the glutamate and glutamine concentrations increased in the first 10 min after the upshift and declined thereafter (Fig. 6). In contrast, the glutamate pool of the E4 mutant decreased in the first 5 min after the ammonium upshift and then increased, reaching approximately 71% of the original value after 20 min. A similar course was observed for the glutamine pool. However, the final glutamine concentration after 20 min was 143% of the original value.

In the experiments described above, the glutamine:glutamate ratio of the wild-type was at the beginning about 3, rising up to about 0.4 after the ammonium shock and falling back to 0.33 after 20 min. In the mutant, however, the glutamine:glutamate ratio increased from 0.34 to 0.67 after 20 min. Therefore, it seems that an imbalance in the glutamine:glutamate ratio is compensated for less effectively in the E4 mutant than in S. coelicolor wild-type cells.

In similar experiments with glnE mutants from Sal. typhimurium (Kistu et al., 1984), the glutamate pool decreased to 10% of the pre-shift value and the glutamine pool increased to 180 times the pre-shift value within 30 min after an ammonium upshift. The much weaker effects in the S. coelicolor glnE mutant may be the result of additional regulatory mechanisms that counterbalance the glutamine:glutamate ratio in S. coelicolor.

Concluding remarks

The glnE mutant described here is the first mutant of an Ntr-like gene in a Gram-positive bacterium. Characterization of the E4 mutant demonstrated that GSI in S. coelicolor is affected by the glnE gene product, possibly by adenylylation (suggested by the effect of SVPDE treatment).

Although a further GSI-like gene was discovered during the S. coelicolor genome sequencing project at the Sanger Institute (Cambridge), the properties of the glnA mutant HT107 show that only GlnA represents the heat-stable GS activity. It cannot be excluded, however, that under certain conditions further GS enzymes are active in streptomycetes. It will, therefore, be interesting to isolate and investigate additional compounds of nitrogen metabolism and its regulatory cascade. Elucidation of this network may provide a basic understanding of the complex mechanisms controlling nitrogen metabolism and, in particular, of its involvement in secondary metabolism.

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