Mutations in the β subunit of the *Bacillus subtilis* RNA polymerase that confer both rifampicin resistance and hypersensitivity to NusG

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Mutations conferring resistance to the antibiotic rifampicin (Rif^r) occur at specific sites within the β subunit of the prokaryotic RNA polymerase. Rif^r mutants of *Escherichia coli* are frequently altered in the elongation and termination of transcription. Rif^r *rpoB* mutations were isolated in *Bacillus subtilis* and their effects on transcription elongation factor NusG and Rho-dependent termination were investigated. RNase protection assay, Northern analysis and the expression of *nusG-lacZ* fusions in cells with an inducible NusG suggested the *B. subtilis* *nusG* gene was autoregulated at the level of transcription. Rif^r mutations that changed residue Q469 to a basic residue (Q469K and Q469R) enhanced autoregulation of *nusG*. A mutant expressing a truncated form of NusG, due to a nonsense mutation within the *nusG* gene, was isolated on the basis of the loss of autoregulation. The mechanism of autoregulation was found to be independent both of transcription termination factor Rho and of the promoter transcribing *nusG*. Autoregulation required sequences within the 5' coding sequence of the *nusG* gene or immediately upstream. This is the first evidence from any bacterium that Rif^r RNA polymerases can display altered transcription regulation by NusG.

Keywords: rifampicin resistance, RNA polymerase, NusG, *Bacillus subtilis*

INTRODUCTION

The core RNA polymerase of the Gram-negative bacterium *Escherichia coli* requires only four subunits (two α and one each of β and β') to elongate the nascent RNA chain (Burgess *et al.*, 1969). A sigma subunit is required for initiation of the RNA polymerase holoenzyme (core polymerase with a sigma factor) at a promoter. The sigma factor is released after formation of a productive elongation complex. The essential core subunits of the bacterial RNA polymerase are highly conserved throughout nature; homologues with structural similarities have been identified in both eukaryal and archaeal RNA polymerases (Archambault & Friesen, 1993; Wang *et al.*, 1998).

Bacterial transcription can be inhibited by a small

number of chemotherapeutic agents, most notably the semi-synthetic antibiotic rifampicin (Wehrli *et al.*, 1968). Rifampicin binds to the RNA polymerase and prevents productive initiation of transcription, but does not inhibit transcription after promoter clearance. The effectiveness of rifampicin varies depending on the sigma factor that is directing the initiation of transcription (Wegrzyn *et al.*, 1998). Rifampicin derivatives are highly effective clinically. However, resistant bacteria arise during treatment due to mutations within the *rpoB* gene encoding the β subunit of the RNA polymerase (Jin & Gross, 1988). Rif^r RNA polymerases have been extensively characterized in *E. coli*; these have altered properties in transcription elongation and/or termination (Jin *et al.*, 1988a, b; Jin & Gross, 1991). In *E. coli*, Rif^r mutations are usually located in the central region of the β subunit polypeptide within clusters I, II and III (Jin & Gross, 1988; Severinov *et al.*, 1993). Rif^r mutations can also occur near the N terminus of the β subunit (Severinov *et al.*, 1994). In *Bacillus subtilis* it is known that Rif^r mutations map to *rpoB* (Boor *et al.*,

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Table 1. Strains of *B. subtilis* used in this work

<i>B. subtilis</i>	Relevant genotype*	Reference/source
<i>B. subtilis</i> 168	<i>trpC2</i>	C. R. Harwood, University of Newcastle, UK
BSNusG1	<i>nusG::pMutin2 [ermE, Φ(<i>nusG-lacZ</i>) pSPAC-<i>nusG</i>]</i>	Ingham <i>et al.</i> (1999)
BSNusG2	<i>nusG::pMutin2 [Φ(<i>nusG-lacZ</i>) <i>nusG::ermE</i>]</i>	Ingham <i>et al.</i> (1999)
BSRif1	Wild-type, 168 with Rif ^r (<i>rpoB</i> Q469K)	This work
BSNusG3	BSNusG1, Rif ^r (<i>rpoB</i> Q469K)	This work
BSNusG4	BSNusG1, Rif ^r (<i>rpoB</i> Q469R)	This work
BSNusG5	BSNusG1, Rif ^r (<i>rpoB</i> H482Y)	This work
BSNusG6	BSNusG2, Rif ^r (<i>rpoB</i> Q469K)	This work
BSNusG7	BSNusG3 with mutated NusG [lysine 155 (AAA) to ochre stop codon (TAA)]	This work
BSRho2	<i>rho::pMutin2 [ermE Φ(<i>rho-lacZ</i>) pSPAC-<i>rho</i>]</i>	Ingham <i>et al.</i> (1999)
BSRho9	BSRho2, Rif ^r (<i>rpoB</i> Q469K)	This work
BSRho10	BSRho2, Rif ^r (<i>rpoB</i> Q469R)	This work
BSRho11	BSRho2, Rif ^r (<i>rpoB</i> H482Y)	This work

* All pMutin2 constructs are integrated into the chromosome by homologous recombination. Φ (*nusG-lacZ*) indicates a transcriptional fusion of the upstream chromosomal region and 5' section of the *nusG* gene to *lacZ*, and pSPAC-*nusG* indicates expression of the complete *nusG* gene from the pSPAC promoter and the native ribosome-binding site (Fig. 1a). Notation for *rho* is analogous.

1995) but the effect of these mutations on transcription elongation and termination is unknown. Indeed, little is known of the effect of Rif^r mutations on transcription elongation and termination in non-enteric bacteria.

B. subtilis and closely related Gram-positive bacteria differ in several aspects of transcription elongation and termination compared to *E. coli* (Henkin, 2000). *B. subtilis* has a number of distinctive means of regulating Rho-independent termination that are lacking in Gram-negative bacteria, including tryptophan RNA-binding attenuator proteins (TRAP) (Babitzke, 1997), as well as the T-box antitermination system and the S-box regulon (Grundy & Henkin, 1998). In addition, both transcription termination factor Rho and transcription elongation factor NusG are essential in *E. coli* but neither are required for growth by *B. subtilis* (Quirk *et al.*, 1993; Ingham *et al.*, 1999). NusG has also been shown to be inessential in *Staphylococcus aureus* (Xia *et al.*, 1999). NusG appears to be a universal transcription factor. A *nusG* homologue has been found in all eubacteria for which the genome has been sequenced and has been found in the archaea and eukaryotes (Hartzog *et al.*, 1998). In *E. coli*, NusG regulates Rho-dependent termination (Sullivan & Gottesman, 1992) and increases the rate of transcription elongation by decreasing pausing by the RNA polymerase (Burova *et al.*, 1995). The function of NusG in other bacteria is largely unknown and even in *E. coli* it is not clear in detail how NusG regulates the RNA polymerase.

In this work, Rif^r mutants of *B. subtilis* were isolated and characterized with respect to the autogenous regulation of the *rho* and *nusG* genes. Two Rif^r mutants with a highly conserved glutamine residue mutated to a

basic residue (Q469K and Q469R) enhanced autogenous regulation of *nusG*. Q469K and Q469R are the first RNA polymerase mutants identified which show increased sensitivity to regulation by NusG. Auto-regulation of *nusG* was used to isolate a strain expressing a truncated NusG which had lost this regulation. This confirmed that we had identified a genetic system for examining fundamental aspects of transcription elongation/termination in a non-enteric bacterium.

METHODS

Bacterial strains and culture methods. *B. subtilis* 168 strains BSNusG1, BSNusG2 and BSRho2 used in this work are summarized in Table 1 and are shown in detail in Fig. 1. These strains have single copies of the vector pMutin2 integrated into the *nusG* or *rho* genes by homologous recombination, as previously reported (Ingham *et al.*, 1999). Strains were grown in 2TY medium at 37 °C with 1 µg erythromycin ml⁻¹ and 25 µg lincomycin ml⁻¹ to maintain integration of pMutin2. Where *B. subtilis* strains had *nusG* or *rho* genes transcribed from the artificial pSPAC promoter (Fig. 1) induction of NusG or Rho synthesis was by growth for 5 h with 1 mM IPTG.

Isolation and mapping of Rif^r mutants. Between 10⁸ and 10⁹ cells of strain BSNusG1 were spread on L agar plates containing 200 µg rifampicin (BDH) ml⁻¹, 1 µg erythromycin ml⁻¹ and 25 µg lincomycin ml⁻¹ (Sigma) to select for spontaneous Rif^r mutants. Chromosomal DNA was prepared by using a Puregene kit (Flowgen). The region containing Rif^r clusters I–III (nt 1063–1794 of *rpoB*) was amplified from a chromosomal DNA template as a 732 bp PCR product using *Pfu* DNA polymerase (Stratagene). The primers used to amplify Rif^r clusters I–III were: *rpoBF*, 5' CAAGAAGGAG-AACAGGTTATCA, and *rpoBR*, 5' CCCGCGGAAACGA-GCTACGATG. The resulting PCR products were cloned by

Fig. 1. Modification of the *B. subtilis* *nusG* and *rho* genes using the chromosomal integration vector pMutin2 to construct strains BSNusG1 and BSRho2, respectively. (a) Map (not to scale) of the region surrounding the *nusG* gene in strain BSNusG1. Co-ordinates (in nt) are relative to the first nucleotide of the *nusG* start codon (+1 = A of ATG; a negative co-ordinate therefore lies upstream of the *nusG* ORF). BSNusG1 makes a *lacZ* transcription fusion to *nusG*, $\Phi(nusG-lacZ)$, and places a complete copy of *nusG* downstream of the pSPAC promoter; NusG protein is only present in cells that are grown with IPTG. The antisense RNA probes (N1 and N2) used in RNase protection mapping (Figs 4a, 5a) are shown. Strain BSNusG2 was similar to BSNusG1 in terms of $\Phi(nusG-lacZ)$, but induction of the pSPAC promoter only transcribes genes downstream of *nusG* as this gene is disrupted by the integration event. The positions of the 3400 and 4000 nt transcripts, deduced from Northern analysis (Fig. 6), originating from putative promoters upstream of the *nusG* and *secE* genes (respectively), is also shown. (b) Map (not to scale) of the region surrounding the *rho* gene in *B. subtilis* strain BSRho2. Co-ordinates are relative to the first nucleotide of the *rho* start codon. BSRho2 makes a *lacZ* transcription fusion to *rho*, $\Phi(rho-lacZ)$, and has the complete *rho* gene downstream of the pSPAC promoter so that Rho is induced when cells are grown with IPTG. The *rho* promoter is located at nt -293 to -320 and *tywji* is the Rho-independent terminator of the *tywji* ORF upstream of the *rho* promoter (nt -369 to -414).

blunt-ended ligation into the vector PCRScript (Stratagene) using *E. coli* XL2 Blue (Kan^r) as a host. PCRScript clones were transformed into wild-type (Rif^s) *B. subtilis* 168 with selection for Rif^r by homologous recombination of clusters I–III in a double cross-over event (Boor *et al.*, 1995). PCRScript clones were concluded to contain a Rif^r mutation if they transformed *B. subtilis* 168 to Rif^r at a higher efficiency (> 10-fold) than the same region of *rpoB* derived from a Rif^s strain in at least two independent transformation experiments (Boor *et al.*, 1995). PCRScript vectors containing *rpoB* fragments conferring a Rif^r phenotype were sequenced using an ABI automated sequencer. Transformation of plasmid DNA after being

linearized by restriction digestion was also used to recombine Rif^r mutations into other *B. subtilis* 168 backgrounds (Table 1). Verification that a particular Rif^r mutation had been correctly transferred into a new genetic background was by direct sequencing of PCR products.

Isolation of mutants of *B. subtilis* in which *nusG* is not autoregulated. Strain BSNusG3 was plated on L agar containing 200 µg X-Gal ml⁻¹ with IPTG, erythromycin, lincomycin and rifampicin at the concentrations described previously. Under these growth conditions, induction of NusG with IPTG decreased expression of $\Phi(nusG-lacZ)$ so that colonies of BSNusG3 grown overnight appeared nearly white (allowing for a degree of orange-brown pigmentation due to growth on rifampicin). Over 2000 unmutagenized colonies were visually screened for derepression of $\Phi(nusG-lacZ)$ as indicated by blue colonies despite the presence of IPTG. These mutants were subjected to further analysis by Western blotting for NusG expression and sequencing of the *nusG* gene from PCR products.

Quantification of transcripts by RNase protection assay and Northern analysis. RNA was purified from *B. subtilis* cultures by rapid protoplast formation followed by repeated acid phenol extractions and 2-propanol precipitation (Volker *et al.*, 1994). Radiolabelled antisense transcripts for use in RNase protection experiments were generated by *in vitro* transcription using T7 RNA polymerase from a Maxiscript kit as instructed by the manufacturer (Ambion). NTP concentrations used in transcription reactions were 500 µM unlabelled ATP, CTP and GTP, and 5 µM [α -³²P]UTP [400 Ci mmol⁻¹ (14.8 TBq mmol⁻¹)]. The antisense transcripts used were complementary to RNA transcribed within the *secE-nusG* region (Fig. 1a). The DNA template used for *in vitro* transcription was degraded using RNase-free DNase I as instructed by the supplier (Ambion) and the radiolabelled RNA (> 90% full length) was purified using a spin column-100 (Sigma) before hybridization to *B. subtilis* RNA. RNase protection assays were performed using an HybSpeed RPA kit (Ambion) using 3 µg *B. subtilis* RNA or 3 µg of yeast tRNA as a control. Protected RNAs were resolved on 5% acrylamide, 1× TBE, 8 M urea denaturing gels, sized against Century RNA markers (Ambion). Quantification of protected transcripts was by phosphor-imaging (Bio-Rad).

Northern analysis of transcripts was by the method of Igo & Losick (1986) using RNA prepared as described above. Probes for hybridization to Northern blots were prepared by random-priming (High Prime; Boehringer).

Western immunoblotting. Cells were broken by sonication in lysis buffer containing 20 mM Tris (pH 8.0), 2 mM EDTA and complete protease inhibitor cocktail (Boehringer). Soluble protein was quantified using Bradford reagent (Bio-Rad). Protein samples were resolved by SDS-PAGE (8% acrylamide gels) and blotted to PVDF membrane (ICN). Detection of cross-reacting proteins was by Western blotting using a BM chemiluminescent kit (Boehringer) and a polyclonal rabbit antibody raised against purified *B. subtilis* NusG protein (Ingham *et al.*, 1999).

β -Galactosidase assays. The β -galactosidase activity of *B. subtilis* strains containing *lacZ* fusions was assessed by the hydrolysis of ONPG using standard methods and calculations (Harwood & Cutting, 1990). Strains were grown to an optical density at 600 nm of 0.4–0.6 in 2TY medium. Where a qualitative assessment of *lacZ* expression was required, strains were patched on L agar microtitre plates containing 200 µg X-Gal ml⁻¹ with antibiotics and IPTG as appropriate.

RESULTS

Identification of Rif^r mutations in the *rpoB* gene of *B. subtilis*

Spontaneous Rif^r mutants were selected on plates containing 200 µg rifampicin ml⁻¹ and were obtained at a frequency of 1×10^{-8} , which is three- to tenfold less frequent than comparable selections in *E. coli* (Jin & Gross, 1988). All Rif^r mutants were equally resistant (> 500 µg rifampicin ml⁻¹) when grown at temperatures ranging from 28 to 42 °C on L agar plates. Mutations were mapped to the region of *rpoB* containing Rif^r clusters I–III by transformation and recombination of DNA fragments conferring the resistant phenotype. Mutations were then identified by sequence analysis. In all cases, the mutations conferring the Rif^r phenotype in *B. subtilis* were found in a highly conserved region of the RNA polymerase β subunit that corresponded to Rif^r cluster I (Fig. 2). Despite performing five independent mutant selections, only three different mutations were obtained; these were Q469K (mutation of the CAA codon to AAA), Q469R (CAA to CGA) and H482Y (CAC to TAC). In each case only the observed single-base mutation could have led to these amino acid changes. Additional mutant selections performed after mutagenesis with EMS or NTG did not yield a greater variety of Rif^r mutants (seven mutants mapped and sequenced). The mutation H482Y has previously been isolated as *rfm2103* in *B. subtilis* Marburg (Boor *et al.*, 1995). Mutations identical to Q469R, H482Y and Q469K have also been reported in *E. coli* (Jin & Gross, 1988; Severinov *et al.*, 1993; Yang & Polisky, 1999).

The *nusG* gene is autoregulated and Rif^r mutations Q469K and Q469R increase the sensitivity of a *nusG*–*lacZ* transcription fusion to inhibition by NusG

The Rif^r mutations described above were selected in strain BSNusG1, which contains a Φ (*nusG*–*lacZ*) and in which NusG can be induced by the addition of IPTG to the growth medium (Ingham *et al.*, 1999; Fig. 1a). When BSNusG1 was grown on L agar medium containing X-Gal, induction of NusG with IPTG apparently decreased Φ (*nusG*–*lacZ*) expression. This was confirmed by quantitative β -galactosidase assays (Fig. 3a). When NusG was induced in strain BSNusG1, a twofold decrease in *lacZ* expression was observed. However, in the Rif^r mutants BSNusG3 and BSNusG4 (*rpoB* Q469K and Q469R, respectively) inhibition of *lacZ* expression by NusG was increased to tenfold. In all cases, transformation of cloned fragments (containing the mutation thought to confer Rif^r) into a BSNusG1 background was used to confirm that the mutations identified were responsible for all observed phenotypes. Western blotting of cell extracts indicated that NusG was induced with IPTG to similar levels (e.g. Fig. 4b). Rif^r mutations Q469K and Q469R conferred a phenotype in which expression of Φ (*nusG*–*lacZ*) in strains BSNusG3 and BSNusG4 was unusually sensitive to NusG. NusG hypersensitivity was not a property of all Rif^r strains; BSNusG5 (H482Y) was similar to the Rif^s strain BSNusG1 in that NusG only

Fig. 2. Sequence alignment of the region of the RNA polymerase β subunits of *B. subtilis* (amino acids 461–500) and *E. coli* (amino acids 505–544) containing Rif^r cluster I (underlined). Similar amino acid residues are aligned with a vertical line, identical ones with a dot. Mutations leading to a high level of Rif^r in *B. subtilis* are indicated (boxed) above the alignment; asterisks followed by a number indicate the times a particular mutation was identified from independent mutant selections. Rif^r mutations at corresponding amino acid residues in *E. coli* (Jin & Gross, 1988; Severinov *et al.*, 1993) are shown below the alignment. Allelic designations of *E. coli* mutations are indicated in parentheses where commonly used (e.g. Jin & Gross, 1988).

weakly inhibited *lacZ* expression (Fig. 3a). Growth of strains BSNusG3 and BSNusG4 in the presence of sufficient bicyclomycin to inhibit Rho-dependent termination (Magyar *et al.*, 1996; Ingham *et al.*, 1999) did not alleviate this apparent repression of Φ (*nusG*–*lacZ*) (Fig. 3a). This suggests that NusG was regulating *nusG* transcription by an alternative mechanism to any involvement in Rho-dependent termination.

A plausible alternative explanation for the above observation is that the apparent inhibition of transcription of *nusG* was due to increased expression of one or more genes downstream of *nusG* when pSPAC was induced with IPTG (Fig. 1a). To exclude this possibility, the Rif^r mutation Q469K was transformed into strain BSNusG2 to create strain BSNusG6. BSNusG2 and BSNusG6 make essentially the same *nusG*–*lacZ* fusion as BSNusG1, except that activation of the pSPAC promoter with IPTG only results in transcription of the genes immediately downstream of *nusG*. The addition of IPTG to the growth medium did not result in synthesis of NusG in BSNusG2 or BSNusG6 as the *nusG* gene is disrupted (Ingham *et al.*, 1999). Growth of strains BSNusG2 or BSNusG6 in the presence of 1 mM IPTG did not decrease the expression of *lacZ* (Fig. 3b). This confirms that the down-regulation of expression of the *nusG*–*lacZ* fusion observed in BSNusG1 and BSNusG3 was genuinely dependent on NusG. These data support the hypothesis that the *nusG* gene is autogenously regulated.

NusG inhibits transcription within the *secE*–*nusG* region

RNase protection assays were used to quantify transcription within the *secE*–*nusG* intergenic region (Fig. 1). In strain BSNusG1 grown without NusG, three protected RNAs were detected, including a 230 nt RNA

Fig. 3. β -Galactosidase expression by $\Phi(nusG-lacZ)$ in Rif^r and Rif^s *B. subtilis* 168, showing the effect on *nusG* autoregulation. Results shown are the mean of three replicates $\pm$ SD. (a) Expression of $\Phi(nusG-lacZ)$ in BSNusG1 (Rif^r) and the Rif^r mutants BSNusG3, BSNusG4, BSNusG5, demonstrating the NusG-hypersensitive phenotype of BSNusG3 and BSNusG4. C, control cells grown in the absence of IPTG; I, cells grown with 1 mM IPTG to induce NusG; B, cells grown with 300 μ g bicyclomycin ml⁻¹ to inhibit Rho-dependent termination; IB, cells grown with both IPTG and bicyclomycin. (b) Analysis of *lacZ* fusions demonstrating that the decrease in expression of $\Phi(nusG-lacZ)$ shown in Fig. 3a is dependent on NusG. C, I, B and IB as in (a), except that in BSNusG1 and BSNusG3 IPTG induces synthesis of NusG protein, whilst in BSNusG2 and BSNusG6 activation of the P_SPAC promoter with IPTG does not lead to induction of NusG.

full-length RNA, indicating transcription from *secE* into *nusG* (Fig. 4). Two smaller RNAs (120 and 160 nt) were also detected. It is not clear if these truncated transcripts were generated by different initiation, termination or processing of the 230 nt RNA. When NusG was induced in BSNusG1, the 230 nt RNA decreased in abundance more than tenfold (Fig. 4a, b). The truncated transcripts were reduced around threefold by NusG expression. In the absence of NusG, the pattern of transcription within this region was similar when comparing strains BSNusG1 (Rif^r) and BSNusG3 (Q469K). In strain BSNusG3, induction of NusG to a similar level to BSNusG1 (Fig. 4b) almost completely eliminated all transcripts detected in the *secE-nusG* region, except for

Fig. 4. RNase protection of transcripts in the *secE-nusG* intergenic region showing the effect of NusG in a Rif^r strain (BSNusG1) compared with a Rif^r and NusG-hypersensitive strain (BSNusG3) and Western blotting of NusG from these strains. (a) RNase protection of 3 μ g total RNA from *B. subtilis* (or 3 μ g yeast tRNA as a negative control) with antisense probe N1, protecting transcripts within the *secE-nusG* intergenic region (Fig. 1a). NusG was induced by growth with 1 mM IPTG; RNA was extracted from strains grown with and without NusG induced with IPTG. Lanes: 1, BSNusG1 (-IPTG); 2, BSNusG1 (+IPTG); 3, BSNusG3 (-IPTG); 4, BSNusG3 (+IPTG); 5, tRNA negative control. The 230 nt RNA represents full-length protection of the antisense probe. (b) Chemiluminescent Western blots using 20 μ g of soluble protein from the same strains as in (a), showing the induction of NusG with IPTG. Lanes: 1, BSNusG1 (-IPTG); 2, BSNusG1 (+IPTG); 3, BSNusG3 (-IPTG); 4, BSNusG3 (+IPTG).

Fig. 5. Quantification of transcripts within the *secE* region by RNase protection of RNA from strain BSNusG3. NusG was induced by growth with 1 mM IPTG; RNA was extracted from strains grown with and without NusG induced with IPTG. Western blotting for NusG in this strain is also shown. (a) RNase protection using probe N2 at the 5' end of the *secE* gene (Fig. 1a). Lanes: 1, 3 μ g yeast tRNA; 2, 3 μ g RNA from strain BSNusG3 (-IPTG); 3, 3 μ g RNA from strain BSNusG3 (+IPTG). (b) Western blots using NusG antiserum against 20 μ g samples of soluble protein from the same cultures used to prepare the RNA used in (a). Lanes: 1, no extract; 2, BSNusG3 (-IPTG); 3, BSNusG3 (+IPTG).

a minor amount of the 120 nt RNA (Fig. 4a). RNase protection supported the results obtained from the analysis of $\Phi(nusG-lacZ)$ expression (Fig. 3a). The Q469K mutation in the RNA polymerase confers hypersensitivity to NusG in that NusG becomes a more potent inhibitor of *nusG* transcription in this genetic background.

Fig. 6. Confirmation by Northern analysis that *nusG* is autogenously regulated. Ten microgram total RNA samples purified from strain BSNusG3 were resolved on a 1.1% agarose gel, blotted onto nylon membrane (Hybond N; Amersham) and then probed using randomly primed PCR fragments. Lanes: 1 and 3, RNA from BSNusG3 grown without IPTG; 2 and 4, RNA from BSNusG3 grown with IPTG. Lanes 1 and 2 were probed for the *rho* gene using a PCR fragment comprising this ORF as a control for equal RNA loading. Lanes 3 and 4 were probed for the *secE-nusG* intergenic region to detect transcripts fused to the *lacZ* reporter gene (Fig. 1).

Fig. 7. Analysis of the properties of the truncated NusG protein in strain BSNusG7 by Western blotting, showing the unusual mobility and the high level of expression of NusG in strain BSNusG7. Twenty microgrammes of soluble protein was loaded in all lanes except lane 4 (4 µg). Lanes: 1, wild-type *B. subtilis*; 2, BSNusG3 (+IPTG); 3, BSNusG3 (−IPTG); 4, BSNusG7 (+IPTG, 4 µg); 5, BSNusG7 (+IPTG, 20 µg); 6, BSNusG7 (−IPTG); 7, BSNusG3 (+IPTG).

To understand the transcription regulation in more detail, an antisense RNA corresponding to the first 81 nt of the *secE* ORF (probe N2; Fig. 1a) was used to protect RNA purified from strain BSNusG3. Transcription of the 5' region of the *secE* gene was the same in the presence or absence of NusG (Fig. 5a, b). Autoregulation of *nusG* was therefore not due to regulation of any promoter(s) co-transcribing *secE* and *nusG*. This suggests that the site(s) of NusG regulation were within either the *secE* gene, the *secE-nusG* intergenic region or the 5' region of the *nusG* gene.

Northern analysis of transcription was used to confirm autoregulation and clarify the pattern of transcription within the *secE-nusG* region (Fig. 6). Probing for mRNAs transcribed from the *secE-nusG* into the *lacZ* reporter gene in strain BSNusG3 detected two transcripts of 3400 and 4000 nt which were no longer detected

when NusG was induced with IPTG. In contrast, *rho* transcription was not significantly regulated by NusG (Fig. 7). The size of these mRNAs (including the *lacZ* fusion) suggests that *nusG* is transcribed from at least two promoters, one upstream of *secE* and one apparently within the *secE-nusG* intergenic region (Fig. 1). Neither transcript was detected when NusG was induced with IPTG, which is consistent with the RNase protection data (Fig. 4a).

Isolation of mutant BSNusG7 expressing a truncated NusG polypeptide

Strain BSNusG3 was used to isolate a mutant (BSNusG7) in which NusG could still be induced by growth with IPTG but in which NusG no longer repressed $\Phi(nusG-lacZ)$. In total, 27 apparently derepressed (blue on X-Gal with IPTG) mutants were obtained out of 2000 colonies screened and BSNusG7 was chosen for further analysis. Western blotting showed that BSNusG7 over-expressed NusG by fivefold when compared to BSNusG3 or wild-type *B. subtilis* (Fig. 7). NusG in BSNusG7 appeared truncated by approximately 3 kDa when judged by SDS-PAGE. The *nusG* gene from BSNusG7 and the pSPAC promoter region was amplified by PCR using *Pfu* DNA polymerase and sequenced. A single point mutation, lysine 155 of NusG to an ochre stop codon (AAA to TAA), was found, which is consistent with the truncation of NusG from 20 to 17 kDa. Quantitative β -galactosidase assays confirmed that expression of the 17 kDa NusG did not repress $\Phi(nusG-lacZ)$ (data not shown). The loss of 23 amino acid residues from the C terminus of NusG therefore resulted in a polypeptide which was no longer capable of autoregulation of transcription. In both BSNusG3 and BSNusG7, NusG was expressed from functional and unmutated pSPAC promoters. There was only 31 bp upstream of the NusG start codon present in these constructs downstream of pSPAC. This suggests that autoregulation of *nusG* required very little sequence upstream of the *nusG* gene to function and was not promoter-specific as NusG still limited its own expression from the artificial promoter pSPAC.

Taken together with RNase protection experiments (Fig. 4a), these data suggest that autoregulation of transcription was mediated by sequences within the region −31 to +272 relative to the start codon of the *nusG* gene (the latter co-ordinate within the *nusG* gene was deduced from the 3' limit of the fusion of *nusG* to *lacZ*). In this work, we have excluded regulation of the initiation of transcription and Rho-dependent termination as possible explanations. Rho-independent termination and/or the regulation of mRNA stability are therefore the most likely candidates. A search of RNA secondary structure in this region using the GCG program mFold (University of Wisconsin) did not reveal any likely Rho-independent terminators (data not shown). Therefore, if NusG is acting as a Rho-independent termination factor, the mode of action is an unusual one. Termination of transcription within the −31 to +272 region does not explain the down-

Fig. 8. The effect of *Rif^r* mutations on autoregulation of the *rho* gene. Expression of $\Phi(\rho\text{--}lacZ)$ was assayed in strain BSRho2 (*Rif^r*) and *Rif^r* mutants BSRho9, BSRho10 and BSRho11. C, control cells grown in the absence of IPTG; I, cells grown with 1 mM IPTG to induce Rho; B, cells grown with 300 μ g bicyclomycin ml^{-1} to inhibit Rho-dependent termination; IB, cells grown with both IPTG and bicyclomycin.

regulation of all of the transcripts (observed by RNase protection and Northern analysis) of the region -229 to $+4$ (Figs 4a, 6) unless decreased stability and/or processing of terminated transcripts is also a factor. Therefore, NusG may act (directly or indirectly) within the -31 to $+272$ region with a knock-on effect on the abundance of upstream transcripts within the *secE*–*nusG* intergenic region, but not further upstream within the 5' sequence of the *secE* gene.

Effect of *Rif^r* mutants on autoregulation of the *rho* gene

Despite being inessential (Quirk *et al.*, 1993), the *B. subtilis rho* gene is known to be autoregulated by a similar attenuation mechanism to *rho* in *E. coli*. Autoregulation of *rho* in *B. subtilis* occurs at the level of transcription, requires sequences downstream of the *rho* promoter and is mediated by Rho-dependent termination within the mRNA leader and coding region of the *rho* gene (Ingham *et al.*, 1999). Given that *Rif^r* mutants in *E. coli* usually antiterminate or facilitate Rho-dependent termination (Jin *et al.*, 1988b), it was logical to test the effects of *B. subtilis* *Rif^r* mutations on the expression of a *lacZ* transcription fusion to *rho* in *B. subtilis*. The *Rif^r* mutants Q469K, Q469R and H482Y were transformed into strain BSRho2 (Fig. 1b) to create strains BSRho9, BSRho10 and BSRho11, respectively. In each strain Rho was induced with IPTG and Rho-dependent termination was assessed by a *lacZ* fusion to the *rho* gene. In all cases, induction of Rho from pSPAC (to a similar level in all strains as judged by Western blotting; data not shown) decreased expression of $\Phi(\rho\text{--}lacZ)$. However, the effect of Rho was twofold greater in BSRho9 and BSRho10 and was twofold less in BSRho11 when compared to the *Rif^s* strain with the same *lacZ* fusion (Fig. 8). Therefore, the Q469K and

Q469R mutations enhanced autogenous regulation of *rho* and the H482Y was inhibitory. The observed changes in *lacZ* expression due to Rho induction were alleviated by bicyclomycin (Fig. 8), confirming that these were dependent on Rho. As autoregulation of Rho is independent of NusG (Ingham *et al.*, 1999), this phenotype appears to be distinct from *nusG* hypersensitivity.

DISCUSSION

X-ray crystallography (e.g. Zhang *et al.*, 1999) and related structural models of the RNA polymerase during elongation (Korzheva *et al.*, 2000) and rifampicin cross-linking studies (Severinov *et al.*, 1995) all suggest that the transcript within the RNA–DNA heteroduplex and the RNA polymerase active site pass extremely close to the rifampicin-binding pocket of the β subunit. Binding of a rifampicin molecule to this region in a wild-type RNA polymerase appears to be sufficient to block elongation beyond 3 nt (Korzheva *et al.*, 2000). It is therefore perhaps unsurprising that defects in elongation and termination are common to most *Rif^r* mutants (Jin *et al.*, 1988a, b). The mutations found in *B. subtilis* which conferred a high level of *Rif^r* were also found within this region (Fig. 2). The *rpoB* mutations Q469K and Q469R enhanced the action of Rho in *B. subtilis* whilst H482Y was inhibitory. This is consistent with the effect that *rpoB* mutations at the equivalent residues in *E. coli* have on Rho-dependent termination (Jin *et al.*, 1988b), suggesting that interactions between Rho and the RNA polymerase are similar in both bacteria. Given that the *E. coli* RNA polymerase can be terminated by the *B. subtilis* Rho (Ingham *et al.*, 1999), this is logical.

Unusual features of NusG function in *B. subtilis* allowed us to screen for *Rif^r* RNA polymerase mutants that were also altered with respect to regulation by NusG; the *nusG* gene is both inessential for viability and appears autoregulated. Transcription of *nusG* was dependent on NusG (Figs 4a, 6) but transcription of *secE* was not (Fig. 5a), although the mRNA co-transcribing *secE* and *nusG* is regulated in some fashion, possibly by termination immediately downstream of *secE*. Transcription regulation within the *secE*–*nusG* region in *B. subtilis* appears more complex than reported in other bacteria, despite the arrangement of genes around *nusG* being highly conserved within the eubacteria (Downing *et al.*, 1990; Joeng *et al.*, 1993; Puttikhunt *et al.*, 1995). The upstream *secE* gene encodes a secretory protein that is essential in both *E. coli* and *B. subtilis* (Joeng *et al.*, 1993). In *E. coli*, *secE* and *nusG* are invariably co-transcribed (Downing *et al.*, 1990). In *Streptomyces coelicolor* both genes appear essential but have their own promoters (Puttikhunt *et al.*, 1995). In *E. coli*, the intergenic region between *secE* and *nusG* is only 1 bp (Downing *et al.*, 1990); in *B. subtilis* this region is 180 bp and may contain additional regulatory elements. Sequences sufficient to mediate autoregulation were located within the 5' region of *nusG* (nt -31 to $+272$). NusG appears to act in this system after the initiation of transcription, which is consistent with its known properties as a transcription elongation and anti-

termination factor (Sullivan & Gottesman, 1992; Li *et al.*, 1993). The most likely mechanism of action is that NusG enhances Rho-independent termination within the *nusG* gene but that these terminated mRNAs are unstable or processed, explaining the apparent regulation of transcription within the upstream *secE–nusG* intergenic region. We have, however, been unable to locate a classical Rho-independent terminator within *nusG* or the *secE–nusG* region. If regulation is due to termination, it is at an unusual site, not an RNA stem-loop with poly(U) tail. Alternatively, we cannot exclude the possibility that NusG may be indirectly autoregulated via another factor. However, Rho was not such an intermediary despite targeting Rho-dependent termination being a major function of the *E. coli* NusG (Sullivan & Gottesman, 1992; Li *et al.*, 1993; Burns & Richardson, 1995).

With the exception of the enteric bacteria, no good genetic systems exist for identifying and characterizing general transcription elongation factors. In part this is due to the lack of analogues of coliphage termination and antitermination systems. λ N-mediated antitermination has proved valuable in identifying enteric Nus factors (Sullivan *et al.*, 1992) and HK022 Nun termination has been used to isolate *nusG* mutations (Burova *et al.*, 1999). Our attempts to isolate a *B. subtilis* phage which is sensitive in its lytic cycle to NusG have failed. However, we have shown in this work that a mutation in *nusG* can be obtained on the basis of the loss of autoregulation. We have therefore identified an approach that may lead to the discovery of novel components of the Gram-positive transcription elongation and termination complex.

In strains of *B. subtilis* with the *Rif^r* mutations Q469K or Q469R, autoregulation of *nusG* was enhanced and transcription of this gene became more sensitive to NusG. In *E. coli*, four *Rif^r* mutations (Severinov *et al.*, 1993) have been found at the equivalent glutamine residue (Q513) in the β subunit of the RNA polymerase. The best characterized of these is *rpoB8* (Q513P). The RNA polymerase with the allele *rpoB8* exhibits increased Rho-dependent and Rho-independent termination, altered transcription elongation and an increased K_m for purine nucleotides (Jin & Gross, 1991). In addition, both *rpoB8* and *rpoB101* (Q513L) have altered properties with respect to interaction with the *nusA1* allele (Jin *et al.*, 1988a). Interestingly, the *nusG4* mutation in *E. coli* also suppresses the *nusA1* allele to restore λ N-mediated antitermination (Sullivan *et al.*, 1992). Taken together with our data, this suggests that the conserved glutamine Q513/Q469 is a critical residue in the regulation of the RNA polymerase by both NusA and NusG. This is despite these factors having generally opposite but non-competitive effects on the RNA polymerase in elongation and Rho-dependent termination (Burns *et al.*, 1998). Basic mutations at this residue may cause the RNA polymerase to adopt a conformation that is more sensitive to NusG, possibly mediated via altered contacts with DNA or RNA. Current structural models suggest that direct contacts between NusG and

the RNA polymerase at this residue are a less likely explanation (Korzheva *et al.*, 2000). Targeted mutagenesis of *Rif^r* cluster I in *E. coli* has yielded the *rpoB* mutation Q513R (Severinov *et al.*, 1993). It is not known how Q513R affects transcription elongation and termination, but this work suggests that the mutants containing Q513R and Q513K in *E. coli* are well worth studying with respect to regulation by NusG. Q513K has been found to affect the copy number of ColE1 plasmids in *E. coli* but it is not known if NusG is involved in this phenotype (Yang & Polisky, 1999). Despite the genetics of Nus factors and *Rif^r* RNA polymerases being well developed in *E. coli*, there have been no reports of RNA polymerases unusually sensitive to NusG. Knowledge of the genetics of NusG–RNA polymerase interactions is limited. It is possible that NusG hypersensitive mutants have not been isolated in *E. coli* because they are lethal, given that the *E. coli* NusG is essential for growth. An intriguing alternative is that NusG hypersensitive mutants have already been isolated but have not been recognized as such. Purified RNA polymerases from *B. subtilis* or *E. coli* with altered sensitivity to NusG should prove useful for future *in vitro* studies on transcription elongation.

Conclusions

- The *B. subtilis* NusG protein regulates *nusG* transcription, apparently by a novel Rho- and promoter-independent mechanism. Sequences required for autoregulation are located within the 5' region of *nusG* or immediately upstream. Autoregulation can be used to isolate mutations within *nusG*, providing the first approach to studying the genetics of this universal transcription elongation factor outside the enteric bacteria.
- *Rif^r* mutations in the β subunit of the RNA polymerase that alter Q513 to a basic residue render *nusG* autoregulation hypersensitive to NusG. This is the first evidence that *Rif^r* RNA polymerases can be enhanced in their sensitivity to NusG.

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