Small subunits of RNA polymerase: localization, levels and implications for core enzyme composition

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Bacterial RNA polymerases (RNAPs) contain several small auxiliary subunits known to co-purify with the core α , β and β' subunits. The ω subunit is conserved between Gram-positive and Gram-negative bacteria, while the δ subunit is conserved within, but restricted to, Gram-positive bacteria. Although various functions have been assigned to these subunits via *in vitro* assays, very little is known about their *in vivo* roles. In this work we constructed a pair of vectors to investigate the subcellular localization of the δ and ω subunits in *Bacillus subtilis* with respect to the core RNAP. We found these subunits to be closely associated with RNAP involved in transcribing both mRNA and rRNA operons. Quantification of these subunits revealed δ to be present at equimolar levels with RNAP and ω to be present at around half the level of core RNAP. For comparison, the localization and quantification of RNAP β' and ω subunits in *Escherichia coli* was also investigated. Similar to *B. subtilis*, β' and ω closely associated with the nucleoid and formed subnucleoid regions of high green fluorescent protein intensity, but, unlike ω in *B. subtilis*, ω levels in *E. coli* were close to parity with those of β' . These results indicate that δ is likely to be an integral RNAP subunit in Gram-positives, whereas ω levels differ substantially between Gram-positives and -negatives. The ω subunit may be required for RNAP assembly and subsequently be turned over at different rates or it may play roles in Gram-negative bacteria that are performed by other factors in Gram-positives.

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INTRODUCTION

Bacterial RNA polymerase (RNAP) comprises α_1 , α_2 , β and β' subunits forming the catalytic core. The α subunits act as a scaffold for the β and β' subunits to form the characteristic crab-claw structure (Vassilyev *et al.*, 2002). Sigma (σ) factors facilitate initiation of transcription at the appropriate start points and usually dissociate upon promoter escape (Gross *et al.*, 1998). There are at least two other auxiliary subunits in Gram-positive bacteria, the δ and ω subunits, which co-purify with RNAP, but their exact roles remain elusive (Pero *et al.*, 1975; Gentry & Burgess, 1986; Lampe *et al.*, 1988; Gentry & Burgess, 1990). Of the two, δ has been studied the most in Gram-positives, and *in vitro* experiments suggest it plays a role in transcription specificity, promoter melting, RNAP recycling and even sigma factor switching, although *in vivo*

experiments are yet to confirm any of this (Hyde *et al.*, 1986; Juang & Helmann, 1994; López de Saro *et al.*, 1995, 1999). It is highly conserved throughout the firmicute branch of Gram-positive bacteria and is even present in the minimal genome of *Mycoplasma genitalium*. It consists of two domains, an ordered N-terminal domain of four α helices and an anti-parallel β sheet, and an unstructured C-terminal domain (López de Saro *et al.*, 1999; Motáčková *et al.*, 2010). It has been reported that a δ -null *Bacillus subtilis* strain has a slightly altered colony morphology and elongated cells along with a 30 min lag leading into exponential growth (López de Saro *et al.*, 1999) and this mutation may lead to a block in suppression of sporulation (Gao & Aronson, 2004). More recently, the abundance of δ has been shown to play a role in the virulence of *Streptococcus agalactiae* (Jones *et al.*, 2003; Seepersaud *et al.*, 2006). The authors found that by reducing the expression of δ by transposon mutagenesis, attenuation of virulence was observed, which could be restored by expressing *rpoE* from a low-copy-number plasmid.

Abbreviations: GFP, green fluorescent protein; HTP, high throughput; LIC, ligation independent cloning; RNAP, RNA polymerase; SPA, sequential peptide affinity; TF, transcription foci.

In contrast with the δ subunit, ω is highly conserved between not only Gram-positive and Gram-negative bacteria but also archaea (RpoK) and eukaryotes (Rpb6) (Minakhin *et al.*, 2001). It has been shown to play a role in RNAP maturation, by facilitating the binding of β' to $\alpha_2\beta$ (Ghosh *et al.*, 2001). Early *in vitro* work on *Escherichia coli* ω suggested it could be essential for the stringent response, in which there is a dramatic downregulation of rRNA and an upregulation of biosynthetic operon expression (Igarashi *et al.*, 1989). However, subsequent *in vivo* work showed that *E. coli* lacking ω could still carry out the stringent response, and other proteins, such as DksA, may be able to compensate for the lack of ω (Gentry *et al.*, 1991; Vrentas *et al.*, 2005). The ω knockout strain in *E. coli* has slightly retarded growth compared with the wild-type strain but otherwise appears morphologically the same (Gentry & Burgess, 1989; Mukherjee *et al.*, 1999). Very little is known about the role of ω in Gram-positive organisms such as *B. subtilis*. Indeed, there is speculation that *Bacillus* spp. contain two ω subunits, ω_1 (encoded by *yzkG*) and ω_2 (*yloH*). However, ω_1 is unrelated in sequence to ω /Rpb6 from eubacteria, archaea and eukaryotes, our unpublished data suggest that it does not bind at the same site as ω /Rpb6 subunits bind their cognate RNAPs (Minakhin *et al.*, 2001), and therefore it is unlikely to function as a true ω subunit.

In this work, we have studied the subcellular dynamics of *B. subtilis* RNAP and the δ and ω subunits *in vivo*. We have shown that both δ and ω are closely associated with RNAP, and appear to be components of both mRNA and rRNA transcription complexes. Strains lacking either δ or ω showed no altered phenotype compared with wild-type and did not affect the ability of the cells to undergo the stringent response. Quantification of each of these subunits suggested that δ is present in most, if not all, mature RNAP complexes in exponentially growing cells. The ω subunit, however, was only half as abundant, suggesting that only around half of the RNAP complexes within the cell contain an ω subunit at any one time. We also created *E. coli* β' -green fluorescent protein (GFP)- and ω -GFP-labelled strains. We found that both of these fusions were closely associated with the DNA and formed subnucleoid regions of high GFP intensity, similar to the transcription foci seen in *B. subtilis*, and those described previously in *E. coli* (Cabrera & Jin, 2003). Quantification of these subunits revealed that around 84% of the cellular pool of RNAP contains an ω subunit, which may be indicative of differences in RNAP assembly/turnover dynamics between *B. subtilis* and *E. coli*.

METHODS

Bacterial strains and growth conditions. All strains and plasmids used in this work are listed in Table 1. Cloning was performed using either *E. coli* DH5 α (Gibco-BRL) using 100 μ g ampicillin ml⁻¹ for selection, or NovaBlue GigaSingles (Novagen) with 100 μ g carbenicillin ml⁻¹. Transformation of *B. subtilis* was carried out as described by Kunst & Rapoport (1995). Cells harbouring pYG1-derived plasmids were selected using nutrient agar plates containing 35 μ g

erythromycin ml⁻¹ and supplemented with 0.5 mM IPTG where appropriate. Cells harbouring pNG621-derived plasmids were selected using nutrient agar plates containing 5 μ g chloramphenicol ml⁻¹ supplemented with 0.5% (w/v) xylose where appropriate.

Fusions of GFPmut3 to β' and ω in *E. coli* were created by using PCR using the λ Red system, as described elsewhere (Datsenko & Wanner, 2000). Briefly, *gfpmut3* was amplified from pYG1 using primers GFP F and GFP R (Table 1), while the *cat* gene was amplified from pKD3 using primers cat F and cat R (Table 1). The subsequent PCR products were digested with *Acc651* and ligated. The ligation mixture was then amplified by PCR using GFP F and cat R and the GFP-Cat construct was purified by using gel electrophoresis. This was used as the template for creating the *rpoC*-GFP and *rpoZ*-GFP fusions by amplifying with the *rpoC* lambdaRed F/*rpoC* lambdaRed R and *rpoZ* lambdaRed F/*rpoZ* lambdaRed R primer combinations (Table 1), respectively. *E. coli* DH5 α harbouring pKD46 (Table 1) was then transformed via electroporation with these PCR products and resultant chloramphenicol-resistant colonies were checked for fluorescence. The chloramphenicol resistance gene was then removed using pCP20 as described previously (Datsenko & Wanner, 2000), to create strains 773 (β' -GFP) and 774 (ω -GFP), respectively (Table 1). Growth kinetics of these strains compared with wild-type DH5 α showed no detectable difference under the conditions used in this work (data not shown).

Plasmid construction. Plasmid pYG1 was derived from pMUTIN-SPA [O. Delumeau, Institut National de la Recherche Agronomique (INRA), France], which had previously been adapted for ligation independent cloning (LIC) to facilitate high throughput (HTP) cloning (pMUTIN-LICSPA). Briefly, the LIC site was introduced by mutagenesis of the *Acc651* and *NcoI* sites immediately upstream of the sequential peptide affinity (SPA) sequence by whole plasmid amplification using the pMUT-LICSPA oligonucleotides (Table 1), thereby generating a unique *Ascl* recognition site enabling linearization of the plasmid for HTP cloning. To produce pYG1, the pMUTIN-LICSPA sequence encoding the SPA tag was excised by restriction enzyme digestion with *Ascl* and *EagI*; GFPmut3 was amplified by PCR from the plasmid pJBA27 (Andersen *et al.*, 1998), with the recognition sequences for *Ascl* and *EagI* appended to the forward (pYG1F) and reverse (pYG1R) primers, respectively (Table 1). The *gfpmut3* PCR product was digested with *Ascl* and *EagI* and ligated into the similarly digested pMUT-LICSPA plasmid prior to transformation. LIC was performed as described by Botella *et al.* (2010) using primers *rpoC* F, *rpoC* R, *rpoE* F, *rpoE* R, *rpoZ* F and *rpoZ* R (Table 1), to create 3' gene fusions to *gfp*. This resulted in full-length gene fusions to *gfp* when integrated into the *B. subtilis* chromosome via single crossover, and a downstream truncated copy of the gene lacking a start codon required for translation.

To create pNG621, the *mCherry* gene was amplified from mCherry-pBAD (Shaner *et al.*, 2004) using the primers mCherry F and mCherry R (Table 1), before the PCR product was digested using *EcoRI* and *SpeI*. The *gfp* gene from pSG1164 was then excised using the same restriction enzymes and replaced with the digested *mCherry* PCR product. Plasmid pNG622 was created by amplifying the 540 bp 3' region of the *rpoC* gene using primers *rpoC* 3057 F and *rpoC* 3597 R (Table 1) and cloning it into pNG621 using *XhoI* and *EcoRI*.

Growth conditions for microscopy and image analysis. Wells of a 96-well microplate (Nunc) containing 100 μ l Luria-Bertani (LB) medium were inoculated with a single colony from a nutrient agar plate. Strains EU38 and EU171 were also supplemented with 0.5 mM IPTG to drive expression of genes downstream of *icd*. Serial dilutions were made and the plate was cultured overnight at 37 °C on a shaking platform. After overnight growth, exponentially growing cells were then used to inoculate fresh LB in a new microplate, which was cultured until an OD₆₀₀ of 0.5 was reached. The stringent response

Table 1. Plasmids, strains and primers used and constructed in this work

bla, ampicillin resistance; *P_{spac}*, IPTG inducible promoter; *P_{xyb}*, xylose inducible promoter; LIC, ligation independent cloning site; *erm*, erythromycin resistance; *cat*, chloramphenicol resistance; *spec*, spectinomycin resistance.

Plasmid/strain/primer	Characteristics or primer sequence (5'→3')	Source
Plasmids		
pKD46	<i>bla</i> , λ Red recombinase expression	Datsenko & Wanner (2000)
pKD3	<i>bla flp-cat-flp</i>	Datsenko & Wanner (2000)
pCP20	<i>bla cat</i> , FLP recombinase expression	Datsenko & Wanner (2000)
pYG1	<i>bla P_{spac}-LIC-gfpmut3-erm</i>	This work
pEU13	<i>bla P_{spac}'-yloH-gfpmut3-erm</i>	This work
pEU16	<i>bla P_{spac}'-rpoE-gfpmut3-erm</i>	This work
pEU21	<i>bla P_{spac}'-rpoC-gfpmut3-erm</i>	This work
pEU22	<i>P_{spac}'-icd-gfpmut3-erm</i>	This work
pSG1164	<i>bla cat P_{xyt}-gfp</i>	Lewis & Marston (1999)
pNG621	<i>mCherry</i> inserted into <i>EcoRI-PstI</i> -cut pSG1164; <i>bla cat P_{xyt} mCherry</i>	This work
pNG622	3' end of <i>rpoC</i> inserted into <i>XhoI-EcoRI</i> -cut pNG621; <i>bla cat P_{xyt}'rpoC mCherry</i>	This work
pETMCSIII	<i>bla P_{spac} 10-6HisTϕ</i>	Neylon <i>et al.</i> (2000)
pNG583	PCR product of <i>gfpmut3</i> inserted into <i>XbaI-Acc651</i> -cut pETMCSIII; <i>bla P_{spac} 10-gfpmut3-6HisTϕ</i>	This work
Strains		
<i>E. coli</i>		
BL21(DE3) pLysS	λ DE3 pLysS, F ⁻ <i>ompT(lon)hsdSB</i> (<i>r_B⁻</i> , <i>m_B⁻</i>)	Studier & Moffatt (1986)
DH5 α	F ⁻ <i>endA1 hsdR17 supE44 thi-1 λ-recA1 gyrA96 relA1 Δ(lacZYA-argF)</i> <i>U169 ϕ80 dlacZ $\Delta$$\mu$15</i>	Gibco-BRL
773	DH5 α , <i>P_{wt}-rpoC-gfp</i>	This work
774	DH5 α , <i>P_{wt}-rpoZ-gfp</i>	This work
<i>B. subtilis</i>		
168trp ⁺	<i>trpC2 chr::trp</i>	Botella <i>et al.</i> (2010)
EU16	168trp ⁺ transformed with pEU13; 168trp ⁺ <i>chr::erm P_{wt} yloH-gfp, P_{spac}'yloH</i>	This work
EU19	168trp ⁺ transformed with pEU16; 168trp ⁺ <i>chr::erm P_{wt} rpoE-gfp, P_{spac}'rpoE</i>	This work
EU38	168trp ⁺ transformed with pEU22; 168trp ⁺ <i>chr::erm P_{wt} icd-gfp, P_{spac}'icd</i>	This work
EU44	168trp ⁺ transformed with pEU21; 168trp ⁺ <i>chr::erm P_{wt} rpoC-gfp, P_{spac}'rpoC</i>	This work
EU128	168trp ⁺ transformed with pNG622; 168trp ⁺ <i>chr::cat P_{wt} rpoC-mCherry, P_{xyt}'rpoC</i>	This work
EU163	EU16 transformed with pNG622; 168trp ⁺ <i>chr::erm P_{wt} yloH-gfp, P_{spac}'yloH</i> <i>chr::cat P_{wt} rpoC-mCherry, P_{xyt}'rpoC</i>	This work
EU166	EU19 transformed with pNG622; 168trp ⁺ <i>chr::erm P_{wt} rpoE-gfp,</i> <i>P_{spac}'rpoE chr::cat P_{wt} rpoC-mCherry, P_{xyt}'rpoC</i>	This work
EU171	EU38 transformed with pNG622; 168trp ⁺ <i>chr::erm P_{wt} icd-gfp,</i> <i>P_{spac}'icd chr::cat P_{wt} rpoC-mCherry, P_{xyt}'rpoC</i>	This work
EU288	168trp ⁺ <i>chr::rpoE':spec':rpoE</i>	E. Bidnenko & P. Noirot, INRA, Jouy en Josas
EU289	168trp ⁺ <i>chr::yloH':spec':yloH</i>	E. Bidnenko & P. Noirot, INRA, Jouy en Josas
EU302	EU288 transformed with pEU21; 168trp ⁺ <i>chr::rpoE':spec':rpoE::chr::erm P_{wt}</i> <i>rpoC-gfp, P_{spac}'rpoC</i>	This work
EU306	EU289 transformed with pEU21; 168trp ⁺ <i>chr::yloH':spec':yloH::chr::erm P_{wt}</i> <i>rpoC-gfp, P_{spac}'rpoC</i>	This work
Primers		
rpoC F	GGGTTCTCTGGCGCGAGCCGTAGAAAGTAATGGTTCGCCAG	
rpoC R	TTGGGCTGGCGCGAGCTTCAACCGGGACCATATCGTC	
rpoE F	GGGTTCTCTGGCGCGAGCCGCATCTTGCTCGGCGTG	
rpoE R	TTGGGCTGGCGCGAGCTTTAATTTCTCTTTCATCATCATAGTC	
rpoZ F	GGGTTCTCTGGCGCGAGCTTAGATCCGTCAATTGATTCTTTAATG	
rpoZ R	TTGGGCTGGCGCGAGCTTCGCGGTCTTCCTTTTCAAACG	
mCherry F	TTTTTTGAATTCATGGTGAGCAAGGGCGAGG	
mCherry R	TTTTTTACTAGTCTACTTGTACAGCTCGTCC	
rpoC 3057 F	AAAATTACTCGAGGCCAAGTACTG	
rpoC 3597 R	AATCAGGAATTCTTCAACCGGGAC	

Table 1. cont.

Plasmid/strain/primer	Characteristics or primer sequence (5'→3')	Source
GFP F	TGGCGCGCTCTAGAAATGCGTAAAG	
GFP R	CCCCTCGGTACCTTATTTGTATAG	
Cat F	AAAAAAGGTACCGTGTAGGCTGGAGCTGCTTC	
Cat R	AAAAAATCATGAATGGGAATTAGCCATGGTCC	
rpoC lambdaRed F	CCAGCCTGGCAGAACTGCTGAACGCAGGTCTGGGCGGTTCTGATAACGAGCGC GCCAGCCCAATGCGTAAAGGAGAAGAACT	
rpoC lambdaRed R	ATAAAAAAACC CGCCGAAGCGGGTTTTACGTTATTTGCGGATTAACGATTATG GGAATTAGCCATGGTCC	
rpoZ lambdaRed F	CAGGAAGCCGCTGAATTACAAGCCGTTACCGCTATTGCTGAAGGTCGTCGTCGCG CCAGCCCAATGCGTAAAGGAGAAGAACT	
rpoZ lambdaRed R	TCAGTTGATTGAGGCTTTCAAACAGATACAAGGGCGACCCGCTTTGTGATTATGG GAATTAGCCATGGTCC	
pMUT-LICSPA F	GGTTCCTGGCGCGCCAGCCCAATGGAAAAAGAGAAGATGGAAAAAGAATTTTCATAG CCGTCTCAGCAGCCAACC	
pMUT-LICSPA R	TGGGCTGGCGCGCCAGGAACCCCTCGACTCTAGATCACCTCCTTAAGCTTAATTG TTATCCGCTCACAATTCC	
pYG1 F	TTCCTGGCGCGCCAGCCCAATGCGTAAAGGAGAAGAACTTTTCACTGGAGTTG AATTAG	
pYG1 R	TAATCCGGCCGTCCTCAGGTCATTATTTGTATAGTTCATCCATGCCATGTG TAATCC	

was induced by the addition of arginine hydroxamate to a final concentration of 500 $\mu\text{g ml}^{-1}$ and cells were imaged after 30 min as described previously (Lewis *et al.*, 2000; Davies & Lewis, 2003). To induce collapsing of the nucleoid, chloramphenicol was added to a final concentration of 50 $\mu\text{g ml}^{-1}$ as described previously (Doherty *et al.*, 2006). Image acquisition and processing were performed as detailed by Davies *et al.* (2005).

Cloning, overproduction and purification of GFPmut3. To create a standard curve, GFPmut3 (Cormack *et al.*, 1996) was cloned, overproduced and purified. *gfpmut3* was amplified by PCR from pYG1 using primers GFP F and GFP R and inserted into *Xba*I–*Acc*65I-cut pETMCSIII (Neylon *et al.*, 2000) to give plasmid pNG583 which was then transformed into *E. coli* BL21(DE3) pLysS (Studier & Moffatt, 1986). Overproduction was performed by growing this strain at 37 °C to OD₆₀₀ 0.7 before IPTG was added to a final concentration of 0.1 mM and the culture was grown for a further 5 h at 25 °C. Cells were pelleted, then stored at –80 °C before being processed. Cell pellets were resuspended in 10 ml resuspension buffer (20 mM KH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0 with KOH) and lysed in an Emulsiflex-C5 Homogenizer (four passes at 20 000 kPa; Avestin). Samples were clarified by centrifugation and the supernatant was loaded onto a 1 ml HisTrap column (GE Healthcare) using an ÄKTA FPLC and purified according to the manufacturer's instructions using a 25 mM to 500 mM imidazole gradient over 10 column volumes. Samples were analysed on a 10% SDS–polyacrylamide gel to determine purity, and appropriate samples were pooled and dialysed into storage buffer [20 mM KH₂PO₄, 200 mM NaCl, 10% glycerol (v/v), pH 7.6] and stored at –80 °C. Purity of the sample was assessed by analysing the density plot of a Coomassie-stained SDS–polyacrylamide gel using ImageQuant 5.2 software (GE Healthcare), and total protein content was determined using a Bradford assay (Bio-Rad) and confirmed using a Nanodrop 1000 (Thermo Scientific).

Determining the number of cells ml⁻¹. Overnight exponentially growing cultures of *B. subtilis* 168trp⁺ and *E. coli* DH5 α (Table 1) were used to inoculate 35 ml LB in 500 ml non-baffled shake flasks,

which were grown at 37 °C until an OD₆₀₀ of 0.5 was reached. Cells were then harvested by centrifuging 1 ml aliquots at 13 000 *g* for 5 min. The resultant cell pellets were then resuspended in 500 μl ice-cold 70% (v/v) ethanol and incubated on ice for 30 min to make septa visible (Wu *et al.*, 1995), pelleted by centrifugation as described above, and resuspended in 1 ml TES [20 mM Tris/HCl (pH 7.5), 5 mM EDTA, 100 mM NaCl]. Cells were counted in triplicate in a Helber Bacteria chamber with Thoma ruling (Hawksley) according to the manufacturer's instructions. Cell counts indicated that there were 1.84×10^8 ($\pm 1.81 \times 10^7$) cells ml⁻¹ at an OD₆₀₀ of 0.5 for *B. subtilis* and 2.27×10^8 ($\pm 1.46 \times 10^7$) cells ml⁻¹ at an OD₆₀₀ of 0.5 for *E. coli*.

Absolute quantification of GFP fusion proteins using 96-well microplates. Strains EU16, EU19 and EU44, along with *B. subtilis* 168trp⁺ were cultured in LB to an OD₆₀₀ of 0.5 in 500 ml non-baffled shake flasks as detailed above; cell pellets from 1 ml culture were snap-frozen in liquid nitrogen and stored at –80 °C. Pellets were then resuspended in 400 μl TES, 100 μl of lysis cocktail [TES, 10 mg lysozyme ml⁻¹ (Sigma), 0.25% (v/v) Triton X-100] was added and these were gently mixed. A 100 μl volume of lysis mixture was added to four wells in a 96-well optical bottom black plate (Nunc) for each strain. *B. subtilis* 168trp⁺ lysates were added to additional wells in order to measure the natural (background) autofluorescence. Background fluorescence was subtracted from the total fluorescence of each of the GFP-labelled strains. A standard curve was created by adding increasing amounts of purified GFPmut3 to *B. subtilis* 168trp⁺ cell lysate to ensure the fluorescence of the standard curve samples mimicked that of GFP in the labelled strains as closely as possible (i.e. pH, salinity). The plate was then placed in a FLUORostar Optima (BMG LabTech) and incubated with shaking at 37 °C for 10 min to ensure complete cell lysis, which was checked microscopically. GFP levels were then measured using appropriate filters (485/10 nm excitation, 520/10 nm emission) and analysed in Microsoft Excel. Pellets from three different cultures were quantified for each strain. Four technical replicates were performed on each pellet. Quantification of β' and ω in *E. coli* was performed as detailed above, using strains 773 and 774, along with DH5 α (Table 1) to correct for autofluorescence and to generate the standard curve.

RESULTS

Subcellular localization of RNAP subunits

The subcellular localization of RNAP using a fluorescently tagged β' subunit is well established (Lewis *et al.*, 2000; Davies *et al.*, 2005; Doherty *et al.*, 2006). To investigate the subcellular dynamics of the δ and ω subunits with respect to the RNAP core (defined as $\alpha_2\beta\beta'$, and using fluorescently labelled β' subunit), a pair of vectors were constructed to allow co-localization of two proteins within live *B. subtilis* cells using GFP and mCherry. These two fluorescent proteins were chosen due to their rapid maturation times, bright signals and the extent of separation of the excitation and emission spectra between the proteins, which makes them ideal for co-localization experiments. Furthermore, the use of the red-shifted mCherry protein allows the blue DNA stain DAPI to be used, permitting co-localization of tagged proteins with the nucleoid.

Plasmid pYG1 (Fig. 1a) contains a LIC site for HTP cloning and, once integrated into the *B. subtilis* chromosome via single crossover, results in a 3' fusion of *gfp* to the gene of interest, driven by the wild-type promoter. To prevent transcription polarity within operons, an IPTG-inducible promoter (P_{spac}) has been engineered into the vector. Plasmid pNG621 is derived from the heavily utilized pSG1164 vector (Lewis & Marston, 1999) and is also designed to integrate into the chromosome via single crossover to yield a 3' fusion to the gene of interest expressed via its native promoter. In this vector, a xylose-inducible promoter (P_{xyl}) prevents transcriptional polarity in downstream genes. Due to the different vector backbones, antibiotic resistance, auxiliary promoters and the spectral properties of the fluorescent proteins used, this vector pair is ideal for efficient co-localization studies in *B. subtilis*.

At high growth rates β' -tagged RNAP localizes exclusively to the nucleoid, suggesting that the vast majority of β' is present as a part of the mature RNAP, and assembly of RNAP is rapid (Lewis *et al.*, 2000). Furthermore, subnucleoid regions of higher GFP intensities, termed transcription foci (TF), were also present, which were subsequently shown to comprise several of the highly expressed ribosomal rRNA operons (Lewis *et al.*, 2000; Davies & Lewis, 2003).

To determine if the δ and ω subunits co-localize with the core RNAP, strains containing a β' -mCherry fusion with either δ -GFP (EU166) or ω -GFP (EU163) were created. As shown in Fig. 1(d and e), β' localization was restricted to the nucleoid, and also forms TF under these conditions (arrows). Fig. 1(f and g) suggests that both the δ and ω subunits are closely associated with β' , which localize throughout the nucleoid and also form the distinctive foci that are observed at high growth rates. Overlays of the mCherry and GFP images show that the foci formed from GFP-tagged δ and ω (Fig. 1h and i, respectively) co-localize with TF from β' . This is graphically illustrated by the

linescans shown in Fig. 1(j and k) and suggests that δ and ω occupy the same subcellular domains as mature RNAP.

To assess the close association of δ and ω with RNAP *in vivo* in more detail, the nucleoids in these strains were collapsed using high concentrations of chloramphenicol (Woldringh *et al.*, 1995). This unambiguously permits resolution between nucleoid-associated proteins and those free to diffuse throughout the cytoplasm. We have previously shown using this method that GreA-GFP is closely associated with RNAP (Doherty *et al.*, 2006). As a control, a dual-labelled strain containing β' -mCherry and a GFP fusion to isocitrate dehydrogenase was used. Isocitrate dehydrogenase is a citric acid cycle protein and has no known DNA or RNAP binding ability. As seen in the DAPI-stained images (Fig. 2a–c), chloramphenicol treatment caused nucleoid collapse. Furthermore, the corresponding β' -mCherry images also show a similar pattern of localization (Fig. 2d–f), which is indicative of the close association of RNAP with the nucleoid. Both the δ - and ω -GFP images (Fig. 2g and h, respectively) also show a similar pattern of localization. Linescans taken through the image overlays of the DAPI, mCherry and GFP images graphically show that both δ and ω are closely associated with the nucleoid (Fig. 2m and n). The δ and ω subunits do not have any known DNA binding ability, which would suggest the vast majority of these subunits are associating with mature RNAP, with very little free to diffuse throughout the cytoplasm. In contrast with δ and ω , isocitrate dehydrogenase-GFP did not colocalize with the nucleoid, and was free to diffuse unhindered throughout the cytoplasm (Fig. 2i, l and o).

To investigate whether the localization patterns of β' and ω are conserved across Gram-positive and Gram-negative bacteria, GFP fusions to these subunits were created in *E. coli*. These strains were created using the λ Red system and resulted in GFP fusions to β' (773) and ω (774) driven by the wild-type promoter. The subcellular localization patterns of these strains showed a close association with the nucleoid, and formed the characteristic TF-like regions of high GFP intensity similar to those observed in *B. subtilis*, as indicated by arrows in Fig. 3(c and d) and reported previously for a β' -GFPuv RNAP fusion in *E. coli* (Cabrera & Jin, 2003). To investigate the close association of β' and ω in more detail, chloramphenicol-induced collapsing of the nucleoid was performed (Fig. 4). As observed in *B. subtilis*, the vast majority of these subunits are in close association with the DNA, with very little free to diffuse throughout the cytoplasm.

Regions of high GFP intensity for *B. subtilis* δ and ω are TF

To confirm that the foci of high GFP intensity seen in the δ and ω localization patterns were representative of rRNA synthesis, the stringent response was induced. The stringent response is a mechanism that allows bacteria to adapt to harsh conditions, resulting in the downregulation

Fig. 1. Plasmid maps of pYG1 and pNG621, and the subcellular localization of core RNAP subunit β' with respect to the δ and ω subunits in *B. subtilis*. (a) Plasmid maps of pYG1 and pNG621. Abbreviations are given in the legend to Table 1. MCS, multiple cloning site as for pSG1164 (Lewis & Marston, 1999), without the *Pst*I site. Plasmid maps not to scale. (b and c) DAPI-stained DNA overlaid with the phase-contrast images from strains EU166 and EU163, respectively. A schematic cartoon of a single cell adjacent to cell units is shown. Under these conditions cells typically contain two nucleoids. (d and e) β' -mCherry from strains EU166 and EU163, respectively. (f) δ -GFP. (g) ω -GFP. (h and i) Image overlays of DAPI, mCherry and GFP images, respectively (phase-contrast excluded). (j, k) Linescan taken through cells in (h) and (i). Arrows indicate TF. Bar, 3 μ m.

of rRNA synthesis and the upregulation of several biosynthetic operons (Cashel *et al.*, 1996). The addition of serine or arginine hydroxamate is commonly used to induce the stringent response and results in increased levels of ppGpp in a RelA-dependent manner, which reduces the level of cellular GTP available for initiation of rRNA operons (Krásný & Gourse, 2004). It has previously been shown that TF rapidly disappear upon induction of the stringent response using serine or arginine hydroxamate in

both *B. subtilis* and *E. coli*, indicative of a downregulation of rRNA operons under these conditions (Lewis *et al.*, 2000; Cabrera & Jin, 2003; Davies *et al.*, 2005; Doherty *et al.*, 2006). Fig. 5 shows the results of inducing the stringent response in strains EU166 (β' -mCherry; δ -GFP) and EU163 (β' -mCherry; ω -GFP). As previously shown for β' -GFP (Lewis *et al.*, 2000), TF from β' -mCherry rapidly disappear upon induction of the stringent response (Fig. 5a and b). Likewise, the foci previously observed for δ and ω

Fig. 2. RNAP subunits δ and ω are tightly associated with core RNAP in *B. subtilis*. (a–c) DAPI-stained collapsed nucleoids of strains EU166, EU163 and EU171, respectively. (d–f) β -mCherry from strains EU166, EU163 and EU171, respectively. (g) δ -GFP. (h) ω -GFP. (i) Isocitrate dehydrogenase-GFP. (j–l) Image overlays of DAPI, mCherry and GFP images, respectively. (m–o) Linescan taken through cells in (j), (k) and (l), respectively. Bar, 3 μ m.

also disappeared when the stringent response was induced (Fig. 5c and d, respectively). The linescans taken through the image overlays of the DAPI, mCherry and GFP signals for these strains graphically show the absence of peaks that are associated with TF (compare Fig. 5i and j with Fig. 1j and k). These cytological experiments suggest that the ω and δ subunits are present as components of RNAP involved in all classes of RNA synthesis, unlike a transcription factor such as NusB, which is required for rRNA synthesis and only localizes to TF, or GreA, which has no known role in rRNA transcription regulation and localizes to nucleoids, but does not form TF (Doherty *et al.*, 2006).

Role of δ and ω in the stringent response

To determine if either δ or ω was required for the stringent response, δ - and ω -null strains containing the β' -GFP fusion were used (EU302 and EU306, respectively; Table 1). Although ω was required for the stringent response *in vitro* using *E. coli* proteins, it has subsequently been found to be

dispensable *in vivo* (Igarashi *et al.*, 1989; Gentry *et al.*, 1991). Other proteins that interact with ppGpp, such as DksA, have been suggested to play a role in the redundancy of ω during the stringent response (Vrentas *et al.*, 2005). However, most Gram-positive bacteria, including *B. subtilis*, lack a homologous DksA, which may render ω indispensable for the stringent response. Furthermore, *Streptomyces kasugaensis* strains lacking ω were unable to utilize ppGpp during antibiotic production (Kojima *et al.*, 2002). Unlike results reported in *E. coli*, for ω knockout strains (Gentry & Burgess, 1989; Mukherjee *et al.*, 1999), there was no obvious phenotype or change in growth rate with respect to wild-type in this knockout strain, as determined by analysis of growth rate in various media, cell morphology and cell dimensions. Likewise, the δ knockout strain also appeared to grow at wild-type rates with no apparent increased lag time or morphological changes as has been previously reported in *B. subtilis* (López de Saro *et al.*, 1999; data not shown). As seen in Fig. 6, the lack of either δ or ω does not have any effect on the localization of β' in exponentially growing cells

Fig. 3. Subcellular localization of *E. coli* RNAP β' and ω subunits. (a and b) DAPI-stained images of strains 773 (β' -GFP) and 774 (ω -GFP), respectively, and (c and d) the corresponding GFP images. (e and f) Image overlays of the respective DAPI and GFP images, with the DAPI signal pseudocoloured red for easier signal discrimination. Arrows indicate TF. Bar, 2 μ m.

Fig. 4. Subcellular localization of *E. coli* RNAP β' and ω subunits after chloramphenicol-induced nucleoid collapse. (a and b) DAPI-stained images of strains 773 (β' -GFP) and 774 (ω -GFP), respectively, and (c and d) the corresponding GFP images. (e and f) Image overlays of the respective DAPI and GFP images, with the DAPI signal pseudocoloured red for easier signal discrimination. Bar, 2 μ m.

(compare Fig. 6b and c with Fig 6a). Also, the lack of these subunits appears to have no effect on the ability of the cell to undergo the stringent response, as can be seen in Fig. 6(d–f). This would suggest that any role ω plays in the stringent response is redundant.

Quantification of β' , ω and δ

Although it appears from the localization patterns that the majority of β' , ω and δ subunits are present as a part of the mature RNAP complex, the stoichiometry of these complexes has yet to be comprehensively determined. Indeed, previous studies have estimated that δ could be five times more abundant than RNAP in exponentially growing *B. subtilis* (López de Saro *et al.*, 1999). We have previously shown that as long as a GFP fusion protein is under the control of its respective wild-type promoter, and the fusion protein is functional, accurate cellular protein levels can be determined by measuring GFP levels, when compared with both quantitative Western blots and protein levels determined by mass spectrometry using AQUA-labelled peptides [Davies *et al.*, 2005; Doherty *et al.*, 2006; D. Becher and J. Muntel (Ernst-Moritz-Arndt-University Greifswald, Germany), personal communication]. In this work we determined the cellular abundance of the β' , δ and ω subunits in *B. subtilis* at an OD₆₀₀ of 0.5 in cells cultured in LB. The results (Fig. 7) show a cellular abundance of $21\,700 \pm 580$ molecules per cell for β' , $23\,020 \pm 900$ molecules per cell for δ and $11\,375 \pm 590$ molecules per cell for ω . As shown in Fig. 7(a), δ has a 1:1 ratio with RNAP, while ω is only half as abundant. Furthermore, micrographs show no detectable heterogeneity in fluorescence between cells in the culture, suggesting that this ratio is indicative of single-cell values (see Figs 1 and 2). Fig. 7(b) shows heat-ramped images of representative cells imaged under identical conditions, with all the backgrounds equalized, and corroborates the cellular numbers from quantification using the microplate reader. From this, it appears that every mature RNAP in *B. subtilis* contains a δ subunit, but only about half contain an ω subunit.

Although still less abundant than β' , a much larger proportion of mature RNAP contain an ω subunit in *E. coli*. In stark contrast to the results in *B. subtilis*, in *E. coli* there are $18\,980 \pm 860$ molecules of β' compared with $15\,885 \pm 1330$ molecules of ω in *E. coli* (Fig. 7a), suggesting that around 85 % of the cellular pool of RNAP contain an ω subunit.

DISCUSSION

In this work, we have presented a pair of vectors that are ideal for co-localizing two different proteins within a single bacterial cell. Furthermore, the spectral properties of GFPmut3 and mCherry allow for the DNA stain DAPI to be used to allow the two proteins to be co-localized with respect to the nucleoid. Minimal homology in plasmid backbones, different antibiotic resistance and auxiliary

Fig. 5. Effect of the stringent response on the localization of RNAP subunits β' , δ and ω . (a and b) DAPI-stained nucleoid from strains EU166 and EU163, respectively, 30 min after induction of the stringent response, and (c and d) the corresponding β' -mCherry images at the same time point. (e and f) The corresponding δ -GFP and ω -GFP images. (g) Image overlay of (a), (c) and (e). (h) Image overlay of (b), (d) and (f). (i and j) The respective line scans taken through cells in (g) and (h). Bar, 3 μm .

Fig. 6. The RNAP δ and ω subunits are not essential for the stringent response. (a–c) Images taken at $t=0$ of strains EU44 (wild-type with β' -GFP), EU302 (δ knockout with β' -GFP) and EU306 (ω -knockout, with β' -GFP), respectively. (d–f) Images taken at $t=30$ min after induction of the stringent response for strains EU44, EU302 and EU306, respectively. Bar, 3 μm .

Fig. 7. Quantification of RNAP subunits β' , δ and ω in *B. subtilis* and β' and ω in *E. coli*. (a) Absolute protein quantification and (b) normalized representative micrographs heat-ramped to show relative GFP intensity within the cells.

promoters make these vectors simple and effective for creating dual-labelled strains. Because these vectors integrate via single-crossover, the expression of the gene fusion is driven by the wild-type promoter, which allows accurate subcellular localization patterns to be observed, and also provides an easy method for quantifying protein levels using the readily measurable fluorescence. Furthermore, these two plasmids would be ideal for looking into protein-protein interactions using fluorescence resonance energy transfer (Albertazzi *et al.*, 2009).

Using these vectors, we investigated the subcellular dynamics and absolute protein levels of the δ and ω subunits with respect to RNAP core in *B. subtilis*. We have shown that the δ and ω localization pattern is indistinguishable from that of mature RNAP (as represented by the β' -GFP fusion), suggesting that these two auxiliary subunits are part of transcription complexes involved in all classes of transcription (Fig. 1). Tight association of these subunits with mature RNAP was demonstrated by collapsing the nucleoids of exponentially growing dual labelled strains, which showed no detectable fluorescence in the cytoplasm (Fig. 2). Further evidence of the involvement of these subunits in the transcription of rRNA operons was demonstrated by the disassembly of TF upon induction of the stringent response (Fig. 5).

Although these subunits are a part of complexes that can undergo the stringent response, they are not essential for it, as shown by the rapid disassembly of TF in both δ - and ω -null strains (Fig. 6). It has been speculated that there is a degree of redundancy in the stringent response pathway in *E. coli*, and knockout of one pathway can be compensated for by another. The DksA protein in *E. coli* has been shown to interact with the small alarmone ppGpp, which is responsible for the stringent response and has been a candidate for stringent control mechanisms in *E. coli* (Vrentas *et al.*, 2005). Although

B. subtilis does not contain a DksA homologue, there may be unrelated proteins that account for the redundancy in ω 's role in the stringent response. Alternatively, the stringent response in Gram-positive bacteria may be indirect as a result of decreased concentrations of GTP, and may not require a co-factor such as DksA (Krásný & Gourse, 2004).

Quantification suggests that δ and core RNAP are at equal stoichiometric levels. This, coupled with the cytological data showing a close association of the cellular pool of δ and that of mature RNAP, would argue that δ is an integral RNAP subunit in *B. subtilis*. However, deleting δ resulted in no adverse effects on growth rate or phenotype, and did not affect the localization of β' . The δ subunit could be involved in sigma factor switching (López de Saro *et al.*, 1999), which may make it important during the transition to stationary phase and stress responses such as competence. However, we observed no adverse effect on any phase of growth, and the δ -null strain was readily transformed with chromosomal DNA.

In contrast with δ , ω levels were only about half those of β' in *B. subtilis*, suggesting that, at most, only half of the RNAP molecules within the cell contain an ω subunit at any one time. This ratio was constant in cells grown in rich or poor media (data not shown), suggesting it is not growth-rate-specific. Although ω was also present at lower levels in *E. coli*, the ratio of ω to β' was much higher than seen in *B. subtilis* (0.85 versus 0.48). The increased ratio of ω : β' in *E. coli* compared with *B. subtilis* was not due to excess ω within the cell, as shown by the close association of ω with β' in Figs 3 and 4, suggesting that the vast majority of ω is present with mature RNAP in *E. coli*.

There have been several roles attributed to ω from both Gram-positive and Gram-negative bacteria, predominantly via *in vitro* assays. Only mild phenotypes can be observed when ω is knocked out, suggesting a level of redundancy in

the role(s) of ω *in vivo*. Recently, purified RNAP from *Xanthomonas campestris* was characterized, revealing a strong correlation between the presence of ω and both the expression level and activity of the resultant enzyme when overproduced from a plasmid in *E. coli* (Cheng *et al.*, 2010). Mukherjee *et al.* (1999) found that small amounts of the chaperone protein GroEL co-purified with RNAP from wild-type *E. coli* cells. The level of GroEL drastically increased when the purification was performed on ω -null cells. The authors found that either ω or GroEL was needed to reconstitute active RNAP, and without at least one of them, RNAP had severely compromised activity. It is feasible that GroEL may be able to chaperone β' to the $\alpha_2\beta$ complex during RNAP maturation when ω is limiting, and this may explain why the cell requires less ω than other core subunits in *E. coli*. It is tempting to speculate that GroEL or other proteins play a bigger role in RNAP maturation in *B. subtilis*, which could explain why the ω : β' ratio is much lower, although this has yet to be investigated. It is also possible that ω associates with β' only during the initial stage of RNAP maturation and holoenzyme formation, and that it is subsequently turned over once mature RNAP has formed. If this were the case, it suggests that RNAP maturation and ω utilization differ in *B. subtilis* compared with *E. coli*. One thing is clear, the study of the RNAP small auxiliary subunits is in its infancy, and much more work needs to be performed on the roles these subunits play *in vivo*.

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