A simple plasmid-based system that allows rapid generation of tightly controlled gene expression in *Staphylococcus aureus*

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We have established a plasmid-based system that enables tightly controlled gene expression and the generation of GFP fusion proteins in *Staphylococcus aureus* simply and rapidly. This system takes advantage of an *Escherichia coli*–*S. aureus* shuttle vector that contains the replication region of the *S. aureus* theta-mode multiresistance plasmid pSK41, and is therefore a stable low-copy-number plasmid in the latter organism. This vector also contains a multiple cloning site downstream of the IPTG-inducible *Pspac* promoter for insertion of the gene of interest.

Production of encoded proteins can be stringently regulated in an IPTG-dependent manner by introducing a pE194-based plasmid, pGL485, carrying a constitutively expressed *lacI* gene.

Using GFP fusions to two essential proteins of *S. aureus*, FtsZ and NusA, we showed that our plasmid allowed tightly controlled gene expression and accurate localization of fusion proteins with no detrimental effect on cells at low inducer concentrations. At higher IPTG concentrations, we obtained sixfold overproduction of protein compared with wild-type levels, with FtsZ–GFP-expressing cells showing lysis and delocalized fluorescence, while NusA–GFP showed only delocalized fluorescence. These results show that our system is capable of titratable induction of gene expression for localization or overexpression studies.

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INTRODUCTION

Many cellular processes in bacteria, such as cell division and DNA replication, require tightly regulated protein levels in the cell for their proper function (Harry *et al.*, 2006). Many proteins have also been found to interact with each other in a particular stoichiometry, and severe phenotypes are often observed if this stoichiometry is perturbed. Phenotypes that are associated with protein overproduction can often lead to useful insights about protein interactions and/or protein function. For example, in *Escherichia coli*, overproduction of the cell division protein FtsZ is toxic to cells. However, overproduction of the cell division protein FtsA counteracts

this toxic effect (Dai & Lutkenhaus, 1992; Dewar *et al.*, 1992). This led to the suggestion that these proteins interact with each other in a particular stoichiometry in the cell. The severe toxicity associated with FtsZ overproduction also highlights the importance of gene expression systems that allow tightly regulated overproduction of proteins when induced, while causing minimal effects on cell viability in the absence of inducer.

Staphylococcus aureus is a Gram-positive pathogen and is a significant cause of a number of diseases ranging from minor skin infections to life-threatening bacteraemia (García-Lara *et al.*, 2005). The ability of *S. aureus* to develop resistance to current clinically approved antibacterials makes the treatment of these infections increasingly difficult. Despite the clinical significance of *S. aureus*, several factors have hampered molecular analysis of this Gram-positive pathogen. With the exception of strain RN4220, *S. aureus* contains several restriction–modification systems that prevent genetic

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; IFM, immunofluorescence microscopy; MCS, multiple cloning site; RC, rolling circle.

A supplementary figure, showing the construction of plasmid pLOW-GFP, is available with the online version of this paper.

manipulation using recombinant DNA isolated from *E. coli* (Kreiswirth *et al.*, 1983; Veiga & Pinho, 2009). However, strain RN4220 is heavily mutagenized, and is not representative of pathogenic *S. aureus* strains (Lindsay, 2008). Construction of genomically integrated constructs of 'non-RN4220' *S. aureus* strains often requires a two-step process involving phage transduction and screening for the loss of a resistance marker (Lindsay, 2008), which can be time-consuming.

A number of regulated systems which allow the tight control of gene expression and thus protein production have been described for *Bacillus subtilis* and *E. coli* (Cha & Stewart, 1997; Muchová *et al.*, 2002). However, relatively few systems are available for controlled overexpression of genes in *S. aureus*. In general, these systems either utilize an ectopically integrated genomic copy of the gene, expressed under the control of a regulatable promoter (Puyang *et al.*, 2003), or express the gene of interest on a high-copy-number plasmid (Zhang *et al.*, 2000). The integration of DNA constructs into the genome, however, can be time-consuming, and when a high-copy plasmid system is used there is the risk of overproducing proteins, which could then lead to cell death.

In this report, we present a quick and easy plasmid-based system that allows the overexpression of genes placed under an IPTG-inducible promoter in natural *S. aureus* isolates. This system uses a low-copy-number plasmid, called pLOW, which contains an IPTG-inducible promoter, *Pspac*, to allow regulated gene expression. The system also includes a second high-copy-number plasmid (pGL485) expressing *lacI*, the gene encoding the lac repressor protein, to fully repress the *Pspac* promoter. We show using a β -galactosidase assay and quantitative immunoblotting that this plasmid-based system enables tight repression as well as titratable induction of genes placed under the control of the *Pspac* promoter of plasmid pLOW. We also show that this system, at low inducer concentrations, can be used to accurately localize two proteins (FtsZ and NusA) involved in different cellular processes, when fused with a GFP tag, as well as to examine

the phenotypic effects on *S. aureus* cells of overproduction of both proteins when high inducer concentrations are used.

METHODS

Bacterial strains and culture conditions. *E. coli* strain DH5 α (Sambrook & Russell, 2001) and *S. aureus* strains SH1000 (Horsburgh *et al.*, 2002) and RN4220 (Kreiswirth *et al.*, 1983) were employed in this study. *E. coli* strains were cultured at 37 °C in Luria–Bertani (LB) broth or on LB agar containing, where appropriate, 100 μ g ampicillin (Ap) ml⁻¹. *S. aureus* strains were cultured at 37 °C in brain heart infusion (BHI) broth or on BHI agar containing, where appropriate, 5 μ g erythromycin (Em) ml⁻¹, 25 μ g lincomycin (Lm) ml⁻¹ and 10 μ g chloramphenicol (Cm) ml⁻¹.

E. coli was transformed by standard procedures (Sambrook & Russell, 2001) and *S. aureus* RN4220 by electroporation, as previously described (Grkovic *et al.*, 2003). Plasmids used to transform the *S. aureus* LH607 (protein A-deficient NCTC 8325 *S. aureus* strain) and SH1000 backgrounds were purified from RN4220 strains carrying the corresponding plasmids. Briefly, *S. aureus* RN4220 was first lysed with lysostaphin [0.2 mg ml⁻¹ (Sigma)] at 37 °C for 30 min, and then plasmid DNA was purified using the QIAprep Spin Miniprep kit (Qiagen). Purified DNA was then electroporated into the *S. aureus* LH607 and SH1000 backgrounds.

DNA isolation and manipulation. Plasmid DNA was isolated from *E. coli* using the QIAprep Spin Miniprep kit (Qiagen). Restriction enzymes, shrimp alkaline phosphatase (all New England Biolabs), T4 DNA ligase (Promega) and Pfx50 DNA polymerase for PCR amplification (Invitrogen) were each used according to the manufacturers' instructions. Oligonucleotides (Table 1) were purchased from Sigma, PCR products were purified with QIAquick PCR Purification columns (Qiagen), and DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction kit (Qiagen). Relevant sequences were verified by automated DNA sequencing, performed by the Australian Genome Research Facility (University of Queensland, Australia).

Construction of pLOW, pLOW-BgaB, pLOW-GFP-Control and pLOW-GFP. Construction of pLOW and pLOW-GFP is depicted in Supplementary Fig. S1. The *S. aureus* Em-resistance gene *ermC* was amplified from plasmid pNL9164 (Yao *et al.*, 2006) using primers *ermCf*-*Clal* and *ermCr*-*KpnI* (Table 1), producing a DNA fragment equivalent to basepairs 2067–3300 of the published sequence of

Table 1. Primer sequences

Underlined type, restriction endonuclease recognition sites; bold type, RBS sequences; italic type, stop codons changed to a tryptophan.

Primer	Sequence
<i>ermCf</i> - <i>Clal</i>	5'-TAAGATCGATGCTGTCTTGGTTCATTGATTGC-3'
<i>ermCr</i> - <i>KpnI</i>	5'-ATTAGGTACCGTTAAGGGATGCAGTTTATGC-3'
<i>bgaBf</i>	5'-GCTAGGGGGAATAATTATGAATGTG-3'
<i>bgaBr</i> - <i>EcoRI</i>	5'-GAACAGAATTCGACTTCTCTAAACCTTCC-3'
<i>gfpr</i> - <i>EcoRI</i>	5'-ATTACCCGGGATGAGTAAAGGAGAAG-3'
<i>gfpr</i> - <i>XmaI</i>	5'-TCTAGAATTCGTGGATCTGAAGTCTG-3'
<i>ftsZf</i> - <i>SalI</i>	5'-GATTGTCGACCAATAAACTAGGAGG-3'
<i>ftsZr</i> - <i>BamHI</i>	5'-TTAAGGATCCAACGTCCTTGTCTTCTTG-3'
<i>nusAf</i> - <i>SalI</i>	5'-CAGTGTTCGACTTAACGTGATGAGGAG-3'
<i>nusAr</i> - <i>BamHI</i>	5'-CCAAGGATCCATTCAGATTCTTTCTCTAC-3'
<i>GFP controlf</i> - <i>SalI</i>	5'-GATTGTCGACAACTAGGAGGAAATTTAAATGAGTAAAGGAGAAGAACT-3'
<i>GFP controlr</i> - <i>BamHI</i>	5'-AGAGGATCCATCTGAAGTCTGGACATTTA-3'

pNL9164. This fragment was cloned into the *Clal* and *KpnI* sites of pAPNC213 (Morimoto *et al.*, 2002), resulting in plasmid pAPNC213-erm (Table 2). Plasmid pAPNC213-erm was digested with *EcoRI* and *BglII*, and the fragment containing the promoter *Pspac*, the Em-resistance determinant *ermC* and the gene encoding the repressor *LacI* was cloned into the *EcoRI* and *BamHI* sites of the pSK41 minireplicon pSK7700 (Ni *et al.*, 2009), resulting in plasmid pLOW (Table 2). The gene *bgaB*, encoding a thermostable β -galactosidase, was introduced in order to evaluate the capability of *LacI* to repress the *Pspac* promoter. The gene was amplified from plasmid pMAD (Arnaud *et al.*, 2004) using primers *bgaBf* and *bgaBr-EcoRI*. The DNA fragment was cloned into the *Sall* and *EcoRI* sites of pLOW, resulting in plasmid pLOW-BgaB. Plasmid pLOW expressing untagged GFP (pLOW-GFP-Control) was constructed by introducing the *gfpmut-1* gene PCR-amplified from plasmid pSG1164 (Lewis & Marston, 1999) using the *GFP controlf-Sall/GFP controlr-BamHI* primer pair. The PCR product was then cloned into plasmid pLOW using the *Sall* and *BamHI* restriction sites.

Plasmid pLOW-GFP was constructed by inserting the *gfpmut-1* gene downstream of the *Pspac* promoter. The gene *gfpmut-1*, encoding GFP, was amplified from plasmid pSG1164 (Lewis & Marston, 1999) using the primers *gfp-EcoRI* and *gfp-XmaI*. The amplicon was cloned into the *EcoRI* and *XmaI* sites of plasmid pLOW, resulting in plasmid pLOW-GFP (Fig. 1). The copy number of pLOW in *S. aureus* SH1000 was determined as described previously (Grkovic *et al.*, 2003) and found to be maintained at approximately 10 copies per cell.

Construction of *ftsZ-gfp* and *nusA-gfp* fusions on plasmid pLOW. To construct the C-terminal FtsZ-GFP fusion, the *ftsZ* gene was amplified from *S. aureus* SH1000 genomic DNA using the primer pair *ftsZf-Sall/ftsZr-BamHI*. The forward primer was designed to incorporate the native *ftsZ* RBS (Table 2) in the final *ftsZ-gfp* construct. The stop codon of the *ftsZ* gene was modified to encode a tryptophan residue, to create a translational fusion of *ftsZ* to GFP. The plasmid containing the in-frame *ftsZ-gfp* fusion was called pLOW-FtsZ-GFP. GFP was separated from the genes of interest by a short linker peptide (Trp-Ile-Pro-Gly).

The C-terminal NusA-GFP was constructed in a similar way to the FtsZ-GFP fusion, except that the primer pair *nusAf-Sall/nusAr-BamHI* was used to PCR-amplify the *nusA* gene from *S. aureus* SH100 genomic DNA.

β -Galactosidase reporter assay. *S. aureus* cells were grown to OD₆₀₀ 0.5. The expression of β -galactosidase was induced by the

addition of IPTG at concentrations of 50, 100, 200, 400, 800 and 1000 μ M. Cultures were incubated in the presence of inducer for an additional 3 h at 37 °C. Samples of ~500 μ l were collected from each culture. The number of cells in each sample was equalized based on the OD₆₀₀ readings. A quantitative ONPG β -galactosidase assay was performed as described by Bhavsar *et al.* (2001). The A₄₂₀ of each sample was determined with a PowerWave HT microplate spectrophotometer (BioTek).

Quantitative immunoblotting for FtsZ-GFP expression. An overnight culture of *S. aureus* LH607 cells carrying plasmid pLOW-FtsZ-GFP was diluted to a starting OD₆₀₀ of 0.05, and grown to mid-exponential phase (OD₆₀₀ ~0.40) with different inducer concentrations (0, 5, 10, 20, 50, 100, 200, 400 and 1000 μ M). The OD₆₀₀ of the different cultures was normalized to ensure that similar amounts of proteins were present in each sample. About 5 ml of each culture was harvested by centrifugation at 4000 r.p.m. for 5 min at room temperature. After removal of the supernatant, the bacterial cell pellet was resuspended in 150 μ l WL buffer [0.3 mg lysostaphin ml⁻¹ (Sigma), 1 $\times$ protease inhibitor (Roche), 25 mM Tris, 0.3 mg PMSF ml⁻¹]. The resuspension was then incubated at 37 °C for 30 min. A 50 μ l volume of 4 $\times$ NuPAGE buffer (Invitrogen) was then added to the cell lysate and the samples were boiled at 95 °C for 5 min.

Using the appropriate dilution of the sample, proteins were then separated on a 4–12% NuPAGE Novex Tris-Acetate precast gel (Invitrogen) under reducing conditions and blotted to nitrocellulose membranes using the iBlot Blotting System (Invitrogen). Blocking and incubation with primary (1:10 000) rabbit polyclonal anti-FtsZ antibodies and secondary (1:2500) horseradish peroxidase-conjugated anti-rabbit IgG (Promega) were done in 5% milk in PBS containing 0.05% Tween 20 for 2 h and 1 h, respectively, at room temperature. Protein visualization was performed with the ECL kit (GE Healthcare) as per the manufacturer's instructions, and densitometric analysis of the bands was performed with the Kodak 1D Molecular Imaging Software, version 4.5 (Kodak).

Establishing the linear range of detection for quantitative immunoblotting. To establish the linear range of detection, i.e. the range of band intensities over which a change in protein concentration generates a proportional change in intensity, a series of dilutions were prepared for one of the cell lysates (grown with 1000 μ M IPTG) and probed with anti-FtsZ antibodies (as described

Table 2. Plasmids

Plasmid	Relevant characteristics	Reference or source
pAPNC213	Ap ^R , Sp ^R , <i>B. subtilis</i> integrative vector, source of <i>Pspac</i> promoter and <i>lacI</i>	Morimoto <i>et al.</i> (2002)
pSK7700	Ap ^R , Tc ^R , pSK41 replication region and <i>tetA</i> (K) from pSK5413 cloned into pSK7698	Ni <i>et al.</i> (2009)
pSG1164	Ap ^R , Cm ^R , <i>B. subtilis</i> vector, source of <i>gfpmut-1</i> gene	Lewis & Marston (1999)
pNL9164	Ap ^R , Em ^R , source of Em ^R	Yao <i>et al.</i> (2006)
pGL485	Sp ^R , Cm ^R , <i>E. coli</i> - <i>S. aureus</i> RC shuttle vector, pE194 replication origin, constitutive expression of the repressor <i>LacI</i>	Cooper <i>et al.</i> (2009)
pMAD	Ap ^R , Em ^R , source of <i>bgaB</i> encoding a thermostable β -galactosidase enzyme	Arnaud <i>et al.</i> (2004)
pAPNC213-erm	<i>ermC</i> cloned into <i>Clal</i> and <i>KpnI</i> sites of pAPNC213	This study
pLOW	pAPNC213-erm fragment encoding the <i>Pspac</i> promoter, <i>ermC</i> , and <i>lacI</i> cloned into <i>EcoRI</i> and <i>BamHI</i> sites of pSK7700	This study
pLOW-BgaB	<i>bgaB</i> cloned into <i>Sall</i> and <i>EcoRI</i> sites of pLOW	This study
pLOW-GFP	<i>gfpmut-1</i> cloned into <i>XmaI</i> and <i>EcoRI</i> sites of pLOW	This study
pLOW-FtsZ-GFP	<i>ftsZ</i> cloned into <i>Sall</i> and <i>BamHI</i> sites of pLOW-GFP	This study
pLOW-NusA-GFP	<i>nusA</i> cloned into <i>Sall</i> and <i>BamHI</i> sites of pLOW-GFP	This study
pLOW-GFP-Control	Plasmid pLOW expressing untagged GFP	This study

Fig. 1. (a) Plasmid pLOW, designed for regulated expression of *S. aureus* proteins. The MCS is shown in full, with the reading frame indicated by the triplet arrangement of the nucleotide sequence. Only unique restriction sites are shown. Genes: ApR, *E. coli blaM* gene for β -lactamase; ColEI-HC, high-copy-number *E. coli* plasmid origin of replication; EmR, *S. aureus* gene originating from plasmid pE194 and encoding Em-resistance gene *ermC*; pSK41 ori, low-copy-number *S. aureus* origin of replication; LacI, *lac* repressor protein constitutively expressed from the penicillinase promoter *Ppcn*. (b) Plasmid pLOW-GFP, designed for producing protein-GFP fusions in *S. aureus*. GFP, *gfpmut-1* gene encoding GFP.

Growth rate analysis of *S. aureus*. A 50 ml volume of BHI medium containing the appropriate antibiotics was inoculated with a stationary phase culture of *S. aureus* to a final OD₆₀₀ of 0.005. Subsequently, cell growth was monitored by OD₆₀₀ measurements every 30 min. Generation times were calculated, and data were analysed for statistical significance using a Student's paired *t* test with a two-tailed distribution.

Fluorescence microscopy. Live-cell microscopy was performed as described previously for *B. subtilis* (Peters *et al.*, 2007). Exponentially growing *S. aureus* cells from 1 ml culture were collected by centrifugation and resuspended in 20 μ l LB broth. A 3 μ l volume of the cell suspension was transferred onto a 2% (w/v) agarose pad [prepared by dissolving type 1 agarose (low electroendosmosis, Sigma) in LB broth] within a 65 μ l Gene Frame (Integrated Sciences). Where necessary, *S. aureus* cells were stained with 1 μ g ml⁻¹ 4',6-diamidino-2-phenylindole (DAPI) to visualize nucleoids.

Immunofluorescence microscopy (IFM) was performed as described previously (Peters *et al.*, 2007; Pinho & Errington, 2003), with modifications. Briefly, 0.5 ml of mid-exponential culture was first harvested and fixed with ice-cold methanol for 1 h at -20°C . After washing with PBS, cells were resuspended in 90 μ l GTE buffer (50 mM glucose, 20 mM Tris/HCl, pH 7.5, 10 mM EDTA). A gentle lysis was performed using lysostaphin (Sigma) at a final concentration of 30 ng ml⁻¹ for 1 min on a polylysine-treated slide. Cells were then treated with 2% BSA/PBS Tween 20 for 15 min and washed with PBS. Primary anti-FtsZ antibodies (raised against *B. subtilis* FtsZ in rabbits) were then added and incubated overnight at 4°C . Following this, the cells were washed 10 times with PBS and incubated with 5% goat serum in PBS for 30 min. Secondary antibody (donkey anti-rabbit FITC-conjugated antibody) was then added at a dilution of 1:100 and incubated for 1 h in the dark at room temperature. Finally, cells were washed again with PBS and mounted in 50% (v/v) glycerol.

Phase contrast and fluorescence microscopy were performed using a Zeiss Axioplan 2 fluorescence microscope equipped with a 100 $\times$ phase contrast objective and an AxioCam MRm cooled charge-coupled device (CCD) camera controlled through AxioVision software (version 4.5, Carl Zeiss). GFP and DAPI fluorescence was visualized with filter sets 488009 and 488002 (Carl Zeiss), respectively. Image analysis, processing and preparation for publication were performed using AxioVision version 4.5. Data from the cell-size measurements were analysed for statistical significance using a Student's paired *t* test with a two-tailed distribution.

RESULTS AND DISCUSSION

Features of plasmid pLOW

As depicted in Fig. 1, plasmid pLOW features pUC19 components that allow replication and selection in *E. coli*,

above). The observed band intensities, measured by densitometry, were plotted against the lysate concentration to determine the linear range of detection for FtsZ-GFP and native FtsZ, which was subsequently used for quantification of FtsZ-GFP and native FtsZ from immunoblots.

i.e., a high-copy-number origin of replication, ColEI^{HC}, and an Ap-resistance determinant (Ni *et al.*, 2009; Yanisch-Perron *et al.*, 1985). Replication of pLOW in *S. aureus* occurs by the theta mode of replication from sequences derived from the low-copy-number multiresistance plasmid pSK41 (Firth *et al.*, 2000). Furthermore, the plasmid contains a number of restriction sites located downstream from the *Pspac* promoter to facilitate the introduction of the gene under investigation (Fig. 1a). The gene encoding LacI, constitutively expressed from the penicillinase promoter *Ppcn*, was introduced into pLOW to limit the detrimental effects of low-level expression of *S. aureus* genes in the cloning host *E. coli* DH5 α as well as to effectively repress the expression of genes placed under the control of *Pspac* in *S. aureus* in the absence of IPTG. A selectable resistance marker for use in *S. aureus* is provided by the *ermC* gene. This marker was chosen to allow for the use of the pLOW vector together with the tetracycline-selectable *S. aureus* integrative vectors, which can be used to ectopically introduce other gene constructs onto the *S. aureus* chromosome (Lee *et al.*, 1991).

Construction of plasmid pLOW

Plasmid pLOW was constructed using two different plasmids and is depicted in Supplementary Fig. S1. The first plasmid, pAPNC213, is a *B. subtilis* integrative plasmid containing the *Pspac* promoter, a multiple cloning site (MCS) and the gene encoding the *lacI* repressor. The second plasmid (pSK7700) contains the staphylococcal pSK41 minireplicon. Construction of plasmid pLOW first involved the introduction of the resistance determinant *ermC* for selection of the plasmid in *S. aureus* into plasmid pAPNC213. The *Pspac-ermC-lacI* cassette was then excised from plasmid pAPNC213 and cloned into plasmid pSK7700 containing the staphylococcal pSK41 minireplicon, creating plasmid pLOW. Plasmid pLOW-GFP was constructed by inserting the PCR-amplified *gfpmut-1* gene downstream of the *Pspac* promoter. For complete details of pLOW plasmid construction see Methods and Supplementary Fig. S1.

Regulated and titratable induction of protein expression with plasmid pLOW

It was important to ensure that genes placed under the control of the IPTG-regulated promoter *Pspac* of pLOW showed titratable gene expression in response to the level of IPTG. To test this, we placed the *bgaB* gene, encoding the β -galactosidase enzyme from *Bacillus stearothermophilus*, under the control of the *Pspac* promoter of plasmid pLOW and assayed β -galactosidase activity using different inducer concentrations. This assay has been shown to be a reliable measure of promoter induction in *S. aureus* (Zhang *et al.*, 2000). However, no transcriptional control in response to different IPTG concentrations was detected even though *lacI* was expressed from the pLOW construct, suggesting that the repressor concentration was too low to effectively

repress *Pspac* (Fig. 2). This is not surprising as earlier studies have shown the need for a multi-copy plasmid containing constitutively expressed *lacI* to repress the *Pspac* promoter (Jana *et al.*, 2000). Therefore, a second copy of *lacI* was introduced on a co-resident rolling circle (RC) plasmid, pGL485 (Table 2). This plasmid has been shown to exist at a copy number of about 20 copies per cell in *S. aureus* (Weisblum *et al.*, 1979). The additional copy of *lacI* provided by pGL485 resulted in titratable and IPTG-dependent *bgaB* expression from *Pspac*, with a basal level of ~4% of the maximum β -galactosidase activity. Maximal enzyme activity was achieved between 200 and 400 μ M IPTG (Fig. 2), which resulted in a 20-fold increase in activity compared with the uninduced sample. Interestingly, maximal enzyme activity, in the presence of *lacI* overexpression (from plasmid pGL485), was slightly lower than the activity measured in the absence of plasmid pGL485. This is likely due to *lacI* expression from pGL485 preventing full release of LacI from the *Pspac* promoter in pLOW, even at high IPTG concentrations (400–1000 μ M). Nevertheless, this assay demonstrates the utility of this two-plasmid system for controlled high through to very low level expression of genes.

Quantification and phenotypic effects of protein overexpression: FtsZ

Having established that the *Pspac* system of plasmid pLOW/pGL485 was capable of titratable induction of gene expression utilizing the β -galactosidase assay, it was important to demonstrate the utility of the plasmid for the tightly controlled expression of natural *S. aureus* genes.

Fig. 2. Titratable induction of the *Pspac* promoter system in *S. aureus*, using the thermostable β -galactosidase *bgaB* from *B. stearothermophilus* as a reporter gene. IPTG concentrations tested were 0, 50, 100, 200, 400, 800 and 1000 μ M. The repressor protein LacI was constitutively expressed either from plasmid pLOW-BgaB alone ($\circ$) or from pLOW-BgaB and pGL485 ($\bullet$). Points and error bars represent the mean of three measurements and the SEM, respectively.

We chose the cell division protein FtsZ (Haydon *et al.*, 2008) as an example to demonstrate the applicability of our plasmid system for controlled gene expression. FtsZ is a highly conserved, tubulin-like protein which polymerizes into a ring called the Z ring at the midcell division site, and has been shown to be essential for cell viability in *S. aureus* (Pinho & Errington, 2003). We chose this protein for this study because FtsZ is currently a promising candidate for anti-staphylococcal therapy (Singh & Panda, 2010), and therefore a tool to evaluate the effects of regulated *ftsZ* expression in live cells in response to potential FtsZ inhibitors may be useful in drug-protein interaction studies. Secondly, the concentration of FtsZ has been shown to be important for proper cell division to occur in *E. coli* and *B. subtilis*. High-level overexpression (two- to fivefold overexpression) of *ftsZ* in these organisms results in a perturbation of cell growth (Ma *et al.*, 1996; Ward & Lutkenhaus, 1985; Weart & Levin, 2003), indicating that cellular levels of FtsZ have to be tightly regulated.

To demonstrate tightly controlled *ftsZ* expression using the pLOW *Pspac* system, we quantified the cellular FtsZ levels present when different inducer concentrations were used. We used an FtsZ–GFP fusion so that we could visualize FtsZ production *in vivo*. We introduced the *gfpmut-1* gene into plasmid pLOW downstream of the *Pspac* promoter, creating plasmid pLOW–GFP (Fig. 1b). The *S. aureus ftsZ* gene was then PCR-amplified and inserted upstream of the GFP tag, creating an *ftsZ–gfp* fusion in the plasmid.

To determine the level of FtsZ–GFP produced from plasmid pLOW, we used quantitative immunoblotting and anti-FtsZ antibodies. Cells were incubated with different IPTG concentrations (5, 10, 20, 50, 100, 200, 400 and 1000 μ M), harvested and probed with anti-FtsZ antibodies (Fig. 3a). Two bands corresponding to the molecular mass of FtsZ–GFP (~68 kDa) and native FtsZ (~41 kDa) were observed in the immunoblot. A third band with a higher molecular mass than that of native FtsZ (~49 kDa) was detectable in cells incubated with higher IPTG levels (100–1000 μ M). This protein likely corresponds to a proteolytically cleaved FtsZ–GFP (Cristea *et al.*, 2005), and was no more than $11 \pm 1\%$ of total FtsZ–GFP. Whilst the amount of cleaved FtsZ–GFP product was small, we combined the levels of cleaved FtsZ–GFP and FtsZ–GFP and utilized this value to calculate the ratio of FtsZ–GFP levels to those of native FtsZ to quantify the protein expression level from the *Pspac* promoter.

As depicted in Fig. 3(b, c), at concentrations between 0 and 20 μ M IPTG, the cellular level of FtsZ–GFP was only about 4 % of the level of native FtsZ (that is, the level of total FtsZ in the FtsZ–GFP-expressing strain was ~0.04-fold higher than wild-type FtsZ levels). In contrast, the level of FtsZ–GFP increased to 80 % of the native FtsZ level (that is the level of total FtsZ in the FtsZ–GFP-expressing strain was ~1.8-fold higher than wild-type FtsZ levels) at 50 μ M IPTG. At 1000 μ M IPTG, a maximal sixfold overproduction was

Fig. 3. (a) Quantitative immunoblot showing different levels of FtsZ–GFP overexpression in *S. aureus* using anti-FtsZ antibodies. Lanes contained the following IPTG concentrations: 1, 0 μ M; 2, 5 μ M; 3, 10 μ M; 4, 20 μ M; 5, 50 μ M; 6, 100 μ M; 7, 200 μ M; 8, 400 μ M; 9, 1000 μ M. WT, wild-type *S. aureus* LH607; FtsZ–GFP' refers to a possible proteolysed product of FtsZ–GFP. (b) Fold overexpression of FtsZ in *S. aureus* obtained by calculating the ratio of FtsZ levels (FtsZ–GFP + native FtsZ) in the FtsZ–GFP-expressing strain to native FtsZ levels of wild-type LH607. IPTG concentrations tested were as for (a). Points and error bars represent the mean value obtained from three separate experiments and the SEM, respectively. (c) Longer exposure of a partial immunoblot from (a) showing the low but detectable levels of FtsZ–GFP in lanes 2–4. Lanes: 1, 0 μ M; 2, 5 μ M; 3, 10 μ M; 4, 20 μ M; 5, 50 μ M IPTG. The contrast of the image was enhanced to visualize faint FtsZ–GFP bands in lanes 2–4.

achieved. These results demonstrate both the tight regulation of gene expression at low levels of IPTG and the substantial overexpression, in this case sixfold overproduction of FtsZ, at high levels of inducer.

We then determined the phenotypic effect of the various levels of FtsZ–GFP expression in *S. aureus* SH1000 cells by examining growth rate (Fig. 4a), cell size (Fig. 4b) and FtsZ–GFP localization (Fig. 5). Carriage of pLOW–GFP and pGL485 had no significant effect on the size of *S. aureus* cells ($P > 0.05$ compared with *S. aureus* SH1000). However, the cells grew slightly slower ($P < 0.05$ compared with *S. aureus* SH1000), probably due to plasmid replication and gene expression. In the absence of IPTG (Fig. 5a), FtsZ–GFP could not be visualized, which is consistent with the tight repression of gene expression of the *Pspac* promoter of plasmid pLOW in the presence of pGL485 in the absence of inducer. Expression of *ftsZ–gfp* at IPTG levels up to

Fig. 4. Generation times (a) and cell diameters (b) of *S. aureus* strains expressing different levels of FtsZ–GFP and NusA–GFP from the plasmids pLOW-FtsZ-GFP and pLOW-NusA-GFP, respectively. Except for SH1000, all *S. aureus* strains analysed for generation time and cell diameter carried the RC plasmid pGL485 from which an additional copy of *lacI* was constitutively expressed. (a) Bars represent the mean of three measurements; (b) bars represent the mean of at least 100 measurements. Error bars, SEM.

100 μ M (3.5-fold higher than cellular levels of FtsZ in wild-type cells) had no significant effect on either cell size or generation time ($P > 0.05$ compared with *S. aureus* carrying pLOW-GFP and pGL485), as shown in Fig. 4(a, b). Consistent with this, FtsZ–GFP could be seen localizing as a ring at the future division site (Fig. 5b–d). The localization pattern looked identical to that of *S. aureus* FtsZ observed with IFM (Fig. 5g) and previously published FtsZ immunolocalization data (Pinho & Errington, 2003).

Pspac derepression at higher IPTG concentrations ($>200 \mu$ M) resulted in severe cell division defects. We observed a 40 % increase in cell size (Fig. 4b) and generation times of up to 126 min compared with 35 min for *S. aureus*

strains carrying pLOW-GFP/pGL485 (Fig. 4a). In addition, cell lysis was frequently observed (yellow arrows in Fig. 5e, f). These observations are consistent with the cell division defects observed with high-level FtsZ overproduction in *B. subtilis* and *E. coli* (Ma *et al.*, 1996; Ward & Lutkenhaus, 1985; Weart & Levin, 2003). This increase in cell size has also been observed with FtsZ-depleted *S. aureus* cells (Pinho & Errington, 2003). FtsZ also appeared delocalized and formed non-ring-like structures (blue and red arrows in Fig. 5e, f). The large increase in cell size and delocalized FtsZ signal are likely to be associated with aberrant (non-septal) peptidoglycan synthesis caused by the delocalization of the peptidoglycan synthetic machinery when FtsZ is delocalized (Pinho & Errington, 2003). Our results suggest that this high

Fig. 5. (a–f) Live-cell microscopy of a strain expressing FtsZ–GFP grown with different IPTG concentrations (shown in μM). Yellow arrows indicate cell lysis, red arrows show non-ring-like structures and blue arrows show delocalized FtsZ–GFP. A phase/GFP fluorescence overlay is shown in the left-hand panel and a corresponding phase image in the right-hand panel. (g) IFM of *S. aureus* FtsZ. Bars, 1 μm .

level of FtsZ production results in a phenotype similar to FtsZ depletion in *S. aureus*.

Phenotypic effects of protein overexpression: NusA

We also examined the localization pattern of a transcription protein of *S. aureus*, NusA. NusA is an RNA polymerase (RNAP)-associated protein that is highly conserved across many bacterial species (Davies *et al.*, 2005). It has been shown to play a role in both transcription antitermination and pausing (Quan *et al.*, 2005). In *E. coli* and *B. subtilis*, NusA acts to stimulate intrinsic termination of RNA transcription (Yakhnin *et al.*, 2008), and has been shown to localize to the nucleoid due to its interaction with RNAP (Davies *et al.*, 2005; Watt *et al.*, 2007).

Interestingly, unlike FtsZ–GFP, maximal expression of NusA–GFP only resulted in a very mild phenotype. As depicted in Fig. 4(a, b), there was no significant change in the generation times ($P > 0.05$ compared with *S. aureus* maintaining pLOW–GFP and pGL485), and only a slight, but statistically significant, increase in cell size ($P < 0.05$ compared with *S. aureus* maintaining pLOW–GFP and pGL485).

The localization pattern of NusA–GFP was consistent with the staining pattern observed with the DNA stain DAPI (Fig. 6a, b), suggesting that NusA–GFP in *S. aureus* also localizes to the nucleoid. To ensure that the NusA–GFP localization in *S. aureus* was specific for the nucleoid and not just localizing within the cytoplasm, we compared the localization pattern of NusA–GFP with that of untagged GFP expressed at the same inducer concentration (500 μM IPTG) from plasmid pLOW–GFP–Control (Fig. 6d, e). Although untagged GFP had a uniform staining pattern like that of NusA–GFP, it consistently occupied more of the cell and extended outside the nucleoid-staining region. Taken together, this shows that like *B. subtilis* NusA–GFP, *S. aureus* NusA–GFP expressed from plasmid pLOW localizes to the nucleoid.

Similarities in NusA localization between *B. subtilis* and *S. aureus* indicate that NusA was being correctly localized in *S. aureus*. However, in *B. subtilis*, NusA appears to concentrate into discrete foci, which represent sites of rRNA transcription (Davies *et al.*, 2005). Utilizing various inducer concentrations (5–500 μM), no such transcription foci were detected in *S. aureus* expressing NusA–GFP. We attribute the lack of transcriptional foci to differences in

Fig. 6. Localization of NusA (a–c) and a GFP control (d–f) in *S. aureus* grown in the presence of 50 μ M IPTG. Exponentially growing cells of *S. aureus* harbouring the plasmids pLOW-NusA-GFP (a–c) and pLOW-GFP (d–f) were examined by fluorescence microscopy. DNA staining using DAPI (a, d), GFP fluorescence (b, e) and an overlay of the two images (c, f) corresponding to the same cells are shown. Bars, 1 μ m. Arrows in (d) and (e) show that, unlike NusA-GFP, the untagged GFP shows no clear gap in GFP fluorescence when there is a clear separation of nucleoids. This indicates that NusA localizes specifically to the nucleoid in these cells.

rRNA operon number and distribution over the genome. *B. subtilis* has 10 rRNA operons, and seven of these are clustered near *oriC*, leading to the observed transcription foci in the presence of NusA-GFP. *S. aureus* has only five rRNA operons, located between approximately 60° and 270° on the 360° map of the chromosome. Fewer rRNA operons and the absence of rRNA operon clusters are most probably the reasons for no clearly defined NusA-GFP foci in *S. aureus*. We cannot rule out the possibility that the increase in cellular NusA, even at the lowest levels of IPTG (5 μ M), prevents the formation of these foci. Regardless of this, the presence of NusA over the entire nucleoid in *S. aureus* is consistent with its localization in other organisms.

Conclusion

We have shown that the plasmid pLOW system allows quick and easy titratable expression of genes in *S. aureus*, demonstrating the utility of the plasmid for high- and low-level gene expression. Although several overexpression systems are available for *S. aureus* (Lindsay, 2008; Zhang *et al.*, 2000), we have shown that our plasmid system is capable of tight repression of gene expression in the absence of inducer, through the use of a second high-copy-number plasmid constitutively expressing *lacI* (pGL485), while

allowing up to a sixfold increase in protein overproduction. We have also demonstrated the usefulness of the pLOW-GFP plasmid for protein localization in live *S. aureus* cells without perturbing cellular homeostasis when low inducer concentrations are used.

We envisage that this system would be useful for studying drug–protein interaction dynamics, namely to determine the cellular targets of inhibitory compounds either by determining the localization of the protein in the presence of the compound or by determining whether overexpression of the protein target confers resistance to the chemical (Haydon *et al.*, 2008; Stokes *et al.*, 2005). This system would be especially useful for low-level expression of GFP-fused proteins that may not be fully functional, but are used to ‘decorate’ the chromosomally encoded native protein and allow its accurate localization. Since expression of GFP on a plasmid in addition to the wild-type protein might result in artefacts in terms of protein localization, the localization should be confirmed with further experiments. In fact, this plasmid-based system would be an ideal first-pass approach for high-throughput, genome-wide analysis of protein localization and protein interaction networks in *S. aureus*, similar to the impressive study conducted in *Saccharomyces cerevisiae* (Huh *et al.*, 2003).

The localization patterns of candidate proteins identified in such a study should then be further evaluated/confirmed by chromosomally tagging these proteins with fluorescent tags (Pereira *et al.*, 2010). Overall, we present here a useful tool for studying the effects of protein overexpression as well as localizing proteins in *S. aureus*.

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