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.

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

†These authors contributed equally to this work.

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* (Zhang *et al.*, 1997; Muchova 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 DH5α (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 μg 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 μg erythromycin (Em) ml⁻¹, 25 μg lincomycin (Lm) ml⁻¹ and 10 μg chloramphenicol (Cm) ml⁻¹.

*E. coli* was transformed by standard procedures (Sambrook & Russell, 2001) and *S. aureus* RN4220 by electroporation, as previously described (Gkovic *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 PstI 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* strain LH607 and SH1000 backgrounds.

### Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>ermCt-Clal</td>
<td>5'-TAAGATGATGATGCTGTTCCTGTGTCATTTGATTGC-3'</td>
</tr>
<tr>
<td>ermCt-Kpnl</td>
<td>5'-ATTTAGTACGCTTAAGGATGACTTATATGC-3'</td>
</tr>
<tr>
<td>bgaBf</td>
<td>5'-GGCTAGGGAATTATTTATGATGTTG-3'</td>
</tr>
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<td>bgaBr-EcoRI</td>
<td>5'-GAAACAGATTCCACTTCTCTACAACCTCC-3'</td>
</tr>
<tr>
<td>gpr- EcoRI</td>
<td>5'-ATTACCCGGGATGATGAAAGGAGAAG-3'</td>
</tr>
<tr>
<td>gpr- Xmal</td>
<td>5'-TCTAGAATTCTGGATCTGATGATG-3'</td>
</tr>
<tr>
<td>ftsZ-Ac-SalI</td>
<td>5'-GATGGTCCGCAATTAAACATAGGAGG-3'</td>
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<tr>
<td>ftsZr- BamHI</td>
<td>5'-TTAGGGATCAGCTTCTTCTTCTTG-3'</td>
</tr>
<tr>
<td>nusA-SalI</td>
<td>5'-AGCTTGCGATCTGCTGAGATGAGGAG-3'</td>
</tr>
<tr>
<td>nusAr-BamHI</td>
<td>5'-GATGGTCCGCAAATGAGGAGAAG-3'</td>
</tr>
<tr>
<td>GFP controlf-SalI</td>
<td>5'-AGAGGATCCACATTGCTGAGATGAGGAG-3'</td>
</tr>
<tr>
<td>GFP control-BamHI</td>
<td>5'-AGAGGATCCACATTGCTGAGATGAGGAG-3'</td>
</tr>
</tbody>
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The addition of IPTG at concentrations of 50, 100, 200, 400, 800 and 1000 μM. Cultures were incubated in the presence of inducer for an additional 3 h at 37 °C. Samples of ~500 μl were collected from each culture. The number of cells in each sample was equalized based on the OD_{600} readings. A quantitative ONPG β-galactosidase assay was performed as described by Bhavsar et al. (2001). The A_{420} 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 OD_{600} of 0.05, and grown to mid-exponential phase (OD_{600} =0.40) with different inducer concentrations (0, 5, 10, 20, 50, 100, 200, 400 and 1000 μM). The OD_{600} 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 μl WL buffer (0.3 mg lysostaphin ml⁻¹ (Sigma), 1 x 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 μl volume of 4 x 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 μM IPTG) and probed with anti-FtsZ antibodies (as described

### Table 2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>pSK7700</td>
<td>Ap^R^, Tc^R^, pSK41 replication region and tet(AK) from pSK541 cloned into pSK7698</td>
<td>Ni et al. (2009)</td>
</tr>
<tr>
<td>pAPNC213-erm</td>
<td>ermC cloned into <em>Clah</em> and <em>Kpnl</em> sites of pAPNC213</td>
<td>This study</td>
</tr>
<tr>
<td>pLOW</td>
<td>pAPNC213-erm fragment encoding the Psac promoter, ermC, and lacI cloned into <em>EcoRI</em> and <em>BamHI</em> sites of pSK7700</td>
<td>This study</td>
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<tr>
<td>pLOW-BgaB</td>
<td>bgaB cloned into <em>SalI</em> and <em>EcoRI</em> sites of pLOW</td>
<td>This study</td>
</tr>
<tr>
<td>pLOW-GFP</td>
<td>gfpmut-1 cloned into <em>Xmal</em> and <em>EcoRI</em> sites of pLOW</td>
<td>This study</td>
</tr>
<tr>
<td>pLOW-FtsZ-GFP</td>
<td>ftsZ cloned into <em>SalI</em> and <em>BamHI</em> sites of pLOW-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>pLOW-NusA-GFP</td>
<td>nusA cloned into <em>SalI</em> and <em>BamHI</em> sites of pLOW-GFP</td>
<td>This study</td>
</tr>
<tr>
<td>pLOW-GFP-Control</td>
<td>Plasmid pLOW expressing untagged GFP</td>
<td>This study</td>
</tr>
</tbody>
</table>
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.

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 ml LB broth. A 3 ml 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 ml Gene Frame (Integrated Sciences). Where necessary, S. aureus cells were stained with 1 mg 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 ml 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× 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,
i.e., a high-copy-number origin of replication, ColE1HC, 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 DH5α 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 μM 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 μM). 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.

<table>
<thead>
<tr>
<th>IPTG concentration (μM)</th>
<th>β-Galactosidase activity (Miller units)</th>
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<tr>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>50</td>
<td>0.1</td>
</tr>
<tr>
<td>100</td>
<td>0.3</td>
</tr>
<tr>
<td>200</td>
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<td>2.0</td>
</tr>
<tr>
<td>1000</td>
<td>2.5</td>
</tr>
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</table>

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 μM. The repressor protein LacI was constitutively expressed either from plasmid pLOW-BgaB alone (○) or from pLOW-BgaB and pGL485 (●). 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 gpmut-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 μM), 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 μM). This protein likely corresponds to a proteolytically cleaved FtsZ–GFP (Cristea et al., 2005), and was no more than 11 ± 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 μM 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 μM IPTG. At 1000 μM IPTG, a maximal sixfold overproduction was 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 using anti-FtsZ antibodies. Lanes contained the following IPTG concentrations: 1, 0 μM; 2, 5 μM; 3, 10 μM; 4, 20 μM; 5, 50 μM; 6, 100 μM; 7, 200 μM; 8, 400 μM; 9, 1000 μM. 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 μM; 2, 5 μM; 3, 10 μM; 4, 20 μM; 5, 50 μM IPTG. The contrast of the image was enhanced to visualize faint FtsZ–GFP bands in lanes 2–4.
100 µM (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 µM) 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

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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.
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...
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 μM), 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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