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Development of shuttle vectors for evaluation of essential regulator regulated gene expression in *Staphylococcus aureus*

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Abstract

We describe the construction of a series of shuttle vectors for *Staphylococcus aureus*. In order to determine transcriptional regulation by essential regulators, we constructed promoterless *luxABCDE* reporter system using a TetR-regulated antisense RNA expression vector, pJYJ909, which is composed of *S. aureus* plasmid pE194, the Gram[−] plasmid pUC18, a TetR regulatory cassette, and *P_{xyl}/teto*-driven *yhcS* antisense expression construct. The reformed shuttle vector was utilized to construct an *opuCA* promoter-*luxABCDE* fusion and simultaneously examine transcriptional regulation by measuring bioluminescence intensity during down-regulating *yhcSR*. In addition, we utilized the same plasmid, pJYJ909, and constructed a *P_{spac}*-driven constant expression system, which allows us to determine the complementary effect of overexpression of *opuCA* operon modulated by *yhcSR*. These plasmids provide important tools for elucidating regulatory mechanisms for genes that are essential for bacterial growth in *S. aureus*.

1. INTRODUCTION

In *Staphylococcus aureus*, due to it being naturally nontransformable for foreign DNA, variant shuttle vectors that allow plasmid to replicate in both *E. coli* and *S. aureus* have been constructed and successfully utilized to knock out genes of interest by homogenous recombination (Arnaud et al, 2004), complement mutated gene, investigate regulatory mechanism using promoter-reporter fusions, and down-regulate endogenous gene expression by antisense RNA (Vagner et al, 1998; Zhang et al, 2000; Huang et al, 2004; Ji et al, 1999). Usually, these shuttle vectors are composed of partial *S. aureus* originated plasmid DNA and partial *E. coli* originated plasmid DNA. In *S. aureus*, pE194 is one of the small stable plasmids with multiple copies and erythromycin resistant selection marker, and has been used to construct different shuttle vectors (Ji et al, 1999; Zhang et al, 2000; Fan et al, 2001; Huang et al, 2004). In addition, pC194 based shuttle vectors have been successfully used to study complementation and construction of promoter-reporter fusions (Mahmood and Khan 1990; Palma et al, 2006).

Despite the fact that a variety of plasmids has been developed for gene cloning and expression in *S. aureus*, the plasmid used to investigate the functions of essential genes is lacking.

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Previously, we have identified many essential genes in *S. aureus* using the regulated expression of antisense RNA strategy and found that about 30% identified genes are of unknown function (Ji et al, 2001). In order to elucidate biological functions of these novel, essential, functionally unknown genes, it is necessary to create effective tools to manipulate these genes in *S. aureus*.

In this study, we created a shuttle vector that allows to downregulate an essential gene expression as well as simultaneously to determine its impact on other genes by measuring reporter gene expression using promoter-reporter fusions. LacZ and Gfp have been widely used for monitoring gene expression using promoter-*lacZ* and promoter-*gfp* fusion system in prokaryotic cells (Qazi et al, 2001a, 2001b; Cormack et al, 1996). It has been reported that a modified *luxABCDE* cassette can be used for transient expression in Gram⁺ bacteria, especially those with low G + C component in genomes (Qazi et al, 2001a). Considering the long half-time of degradation and high background produced by these two proteins, we chose luciferase (encoded by *lux*) as a reporter to monitor the activity of certain promoters in real time during bacterial growth in culture.

For proof of concept, we utilized the TetR-regulated *yhcS* antisense RNA expression plasmid, pJYJ909 (Sun et al, 2005), and constructed an *opuCA* promoter-*lux* fusion shuttle vector, because the *opuCA* is required for growth (Forsyth et al, 2001), and we found that that *opuCA* is modulated by *yhcSR* (unpublished data). In addition, we created a shuttle vector that allows us to determine the complementary effect of constitutive expression of OpuCA on growth inhibition during down-regulating *yhcS* expression.

2. RESULTS AND DISCUSSION

2. 1. Construction of a promoter-*lux* reporter fusion system using a TetR-regulated antisense RNA expression vector

In order to examine transcriptional regulations by an essential regulator, a promoterless-*lux* fusion was constructed. The bacterial strains and plasmids used in this study are listed in Table 1. The *luxABCDE* genes were obtained by PCR with pSB2025 as a template (Qazi et al, 2001b). Multiple clone sites including the enzymes *EcoR* I, *Not* I, *Fse* I and *Sca* I and a RBS site (GGAGG) were integrated into the pCY406/Luxfor1 (5' TAGAATTTCGCGCCGCGGCCGCGCCAGTACTAGGTAGGTAATAGGAGGACTCTCTATGAAATTTGGAACTT 3') and the pCY406/LuxRev (5' TAGAATTCTCAACTATCAAACGCTTCGGTTAAGCT 3') primers, and introduced into the recombinant plasmid DNA for further convenient cloning of promoter regions. The 6kb PCR fragment of *luxABCDE* was purified, digested with *EcoR* I, and ligated into the *EcoR* I site of pJYJ909 vector, which is a TetR-regulated *yhcS* antisense expression system (Sun et al., 2005). The recombinant plasmid DNA was electroporated into *E. coli* DH10B competent cells and selected with erythromycin (300 µg/ml). The reformed plasmid DNA was designated pCY606 (Fig. 1A).

To determine whether the plasmid pCY606 is suitable for monitoring gene transcription, we chose and constructed *opuCA* (SA2237) promoter-*lux* fusion since we found that the *opuCA* was regulated by *yhcSR* (unpublished data). The *opuCA* promoter region was amplified by PCR using the SA2237profor (5'AAGCGGCCGCGGACAAGCTAAACGCAGTGAA 3') and SA2237prore (5'ACAGTACTTCCAATAAATGCGATAAATTCC 3') primers, with the chromosome DNA of *S. aureus* WCUH29 as template. The bold nucleotide sequences of the primers represent *Not* I and *Sca* I recognizing site respectively. The PCR product was purified and digested with *Not* I and *Sca* I, and ligated into the corresponding sites of pCY606, resulting in plasmid pYJ2237 (Fig. 1A). The reformed plasmid pYJ2237 was electroporated into *S. aureus* RN4220, then into WCUH29 generating YJ2237 strain. The *opuCA* promoter driven-

lux expression was monitored by measuring bioluminescence intensity during different time of growth with a Chiron luminometer. Consistent with our previous finding (Sun et al., 2005), the down-regulation of *yhcS* expression with induced *yhcS* antisense RNA inhibited growth (Fig. 1B). More importantly, our results showed that the bioluminescence signal is detectable in the *S. aureus* YJ2237, indicating that the *opuCA* -driven *lux* expression is functional (Fig. 1C). Compared to the control (without ATc induction), the *opuCA* expression dramatically decreased by downregulating *yhcS* expression at early- and middle-log phases of growth (Fig. 1B, 1C). This is consistent with our unpublished data using microarray and quantitative RT-PCR assays.

2.2. Construction of a constitutive *Pspac*-driven expression system using the TetR-regulated antisense RNA expression vector

In order to evaluate complementary effect of expression of gene regulated by an essential regulator, we created a constitutive *Pspac*-driven expression system using the TetR-regulated *yhcS* antisense expression vector, pJYJ909. The *Pspac* promoter region was obtained by PCR using Pspacfor (5' GCTTGAATTCATTTCAGAACGCTCGGTTGCC 3') and Pspacrev (5' GCTTGAATTCCTCGGGCGGCCGCGGCCGCGGCC AATTGTTATCCGCTCACAATTC 3') primers with pFF71 as the template (Fan et al, 2001). The bold nucleotide sequences recognized by *EcoR* I and *Not* I, *Sac* II, and *Fse* I, respectively were introduced for subcloning purpose. The PCR product was digested with *EcoR* I and ligated into the *EcoR* I site of pCY606. The resulting plasmid was designed pMY107 (Fig. 2A). Due to the lack of *lacI* gene, any gene cloned downstream of *Pspac* promoter region would be constitutively expressed. To evaluate complementary effect, we first validated whether inserted *Pspac* promoter mediates expression of target gene in *S. aureus* using Gfp as a reporter. The *gfp* gene was amplified from pSB2019 (Qazi et al, 2001b) using gfp-NotFor (5' TAGTGCGGCCGCTTAACAAGGAGGAATAAAAAATGAGTAAAGGCGAAGAAGT TTTCACTGGAG 3') and gfp-NotRev (5' TAGTGCGG CCGCACAT TTATTG TATAGTTC 3') primers. The bold nucleotide sequence represents the *Not* I recognizing site, and the underlined nucleotide sequence represents a RBS site). The PCR product was purified, digested with *Not* I, and cloned into *Not* I site of pMY107, generating pMY507 (Fig. 2B). The reformed plasmid pMY507 was electroporated into RN4220, and then into WCUH29, resulting in strain YJ507. YJ107 (which is WCUH29 carrying the parental plasmid pMY107) was used as negative control.

The *gfp* expression in *S. aureus* was monitored under the fluorescence microscope. As shown in Fig. 2B, no apparent fluorescence signal was detected for the control strain (YJ107). In contrast, all the bacteria cells (YJ507) displayed with green color, indicating the 352bp *Pspac* promoter region is sufficient to initiate *gfp* expression in *S. aureus* (Fig. 2C). This is consistent with previous findings the Gfp signal is detectable under fluorescence microscope in *S. aureus* carrying the plasmid pSB2019 (Qazi et al 2001a;Liang and Ji, 2006).

To elucidate the complementary effect of an essential gene, we selected *opuCA* operon since it is essential for *S. aureus* (Forsyth et al, 2002) and modulated by the *yhcSR* system (unpublished data). The 3.8kb *opuCA* operon was obtained by PCR using the primers, SA2237RBSNotFsefor (5' TAGTGCGGCCGCGGCCGCGGCCAGGAGGGGAAGGAGAAATGATTATGTTAAGTA T 3') and SA2234-37ORFNotrev (5'TAGTGCGGCCGCAAACGCATACAAACACAACC 3'). The PCR product was purified and digested with *Not* I, and then cloned into the downstream of *Pspac* promoter region of pMY107 (Fig. 2A). The reformed plasmid was designated as pMY207. The plasmid pMY207 was electroporated into RN4220, and then into WCUH29. The WCUH29 carrying pMY207 was denoted YJ207. The complementary effect of SA2237

operon on growth by down-regulating *yhcSR* was examined by monitoring the growth of YJ207. Consistent with our previous finding (Sun et al 2005), the down-regulation of *yhcS* expression with induced *yhcS* antisense RNA inhibited the growth of *S. aureus* YJ107 in a dose-dependent manner (Fig. 3A). In contrast, during down-regulation of *yhcS* expression the growth was dramatically improved for *S. aureus* YJ207 carrying constitutive expressed *opuCA* operon (Fig. 3B), indicating that the *Pspac*-driven expression of *opuCA* operon partially complements the growth defect during down-regulation of the essential *yhcSR* regulator.

To assure the over-expression of SA2237 operon in the complementary construct pMY207, we performed quantitative RT-PCR. The results showed that the transcriptional level of *opuCA* in the culture of YJ207 was 4-fold higher than that in the culture of control YJ107 (data not shown). Taken together, the above data suggest that the *Pspac* mediated constitutive expression system is useful for complementation analysis of genes of interest, especially essential genes in *S. aureus*. We cannot exclude that the overexpression of certain genes may be toxic to bacterial growth. Therefore, an alternative approach, such as integrating a regulated promoter-driven gene construct into the chromosome of *S. aureus* may be employed.

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Figure 1.

Construction of promoter-*lux* reporter fusion plasmid (A) and examination of the *opuCA* driven-*luxABCDE* expression in *S. aureus* (B, C). **A.** The 6kb fragment of *luxABCDE* with a multiple clone sites (MCS) including *EcoR* I, *Not* I, *Fse* I, and *Sca* I was digested with *EcoR* I and cloned into the *EcoR* I site of pJYJ909 vector, resulting in plasmid pCY606; The promoter region of *opuCA* was amplified by PCR followed by digestion with *Not* I and *Sca* I, and cloned into pCY606 resulting in plasmid pYJ2237. The *S. aureus* strain YJ2237 was inoculated into fresh TSB medium with appropriate antibiotics. **B.** The growth of YJ2237 was determined by measuring optical density of culture with or without inducer ATc (anhydrotetracycline, 250 ng/ml). **C.** The *lux* expression level of YJ2237 was monitored by measuring the bioluminescence intensity during different time of growth. To eliminate the impact of bacterial density, the bioluminescence intensity was divided by optical density at 600nm. RLU: relative light unit.

Figure 2.

Construction and validation of a constitutive *Pspac*-driven expression system using the TetR-regulated *yhcS* antisense expression vector. **A.** The *Pspac* promoter region with a multiple clone sites (MCS) including *EcoR* I, *Not* I, *Sac* II, and *Fse* I was digested with *EcoR* I and cloned into the *EcoR* I site of pCY606, generating plasmid pMY107; The *gfp* gene was obtained by PCR, digested with *Not* I, and cloned into the *Not* I site of pMY107 generating pMY507; The 3.8k b *opuCA* operon was amplified by PCR, digested with *Not* I, and cloned into the downstream of *Pspac* promoter region of pMY107 resulting pMY207. **B & C.** Examination of *gfp* expression in *S. aureus* using a fluorescence microscopy at 100 ×magnitude. **B.** YJ107

(*S. aureus* control strain carrying parental plasmid pMY107); C. YJ507 (*S. aureus* strain carrying pYM507).

Figure 3.

Examination of complementary effects of SA2237 operon on inhibitory growth during the down-regulation of *yhcSR* expression in *S. aureus*. Growth curves of control strain YJ107(A) and *opuCA* complemented strain YJ207 (B) were determined in TSB containing 5 μ g/ml erythromycin and various concentration of ATc (0, 100, 250, 500, 1000 ng/ml) using a 96 wells plate. Cell growth was monitored at 37°C by measuring the optical density at 600nm every 15 min using a SpectraMax plus spectrophotometer. The growth curves represent one of three repeated experiments.

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>S. aureus</i> strains		
RN4220	Laboratory strain: <i>rsbU</i> ⁻	Kreiswirth et al, 1983
WCUH29	Clinical human <i>S. aureus</i> isolate: <i>rsbU</i> ⁺	
YJ606	WCUH29 containing plasmid pCY606; Erm ^r	This study
YJ2237	WCUH29 containing plasmid pYJ2237; Erm ^r	This study
YJ107	WCUH29 containing plasmid pMY107; Erm ^r	This study
YJ207	WCUH29 containing plasmid pMY207; Erm ^r	This study
YJ507	WCUH29 containing plasmid pMY507; Erm ^r	This study
Plasmids		
pCY606	Shuttle vector, derived from pJYJ909, containing <i>luxABCDE</i> and <i>yhcS</i> antisense; Erm ^r	This study
pYJ2237	Derived from pCY606, with SA2237 promoter - <i>luxABCDE</i> reporter fusion; Erm ^r	This study
pMY107	Derived from pCY606, with <i>luxABCDE</i> replaced by Pspac promoter region; Erm ^r	This study
pMY207	Derived from pMY107, with SA2237 operon under the Pspac; Erm ^r	This study
pMY507	Derived from pMY107, with <i>gfp</i> gene under the Pspac; Erm ^r	This study