Functional identification of the *prnABCD* operon and its regulation in *Serratia plymuthica*

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Abstract

The antibiotic pyrrolnitrin (PRN) is a tryptophan-derived secondary metabolite that plays an important role in the biocontrol of plant diseases due to its broad-spectrum of antimicrobial activities. The PRN biosynthetic gene cluster remains to be characterized in *Serratia plymuthica*, though it is highly conserved in PRN-producing bacteria. To better understand PRN biosynthesis and its regulation in *Serratia*, the *prnABCD* operon from *S. plymuthica* G3 was cloned, sequenced and expressed in *Escherichia coli* DH5α. Furthermore, an engineered strain *prn*ind which is a conditional mutant of G3 *prnABCD* under the control of the P*tac* promoter was constructed. This mutant was able to overproduce PRN with isopropylthiogalactoside (IPTG) induction by over-expressing *prnABCD*, whilst behaving as a conditional mutant of G3 *prnABCD* in the absence of IPTG. These results confirmed that *prnABCD* is responsible for PRN biosynthesis in strain G3. Further experiments involving

lux-/dsRed-based promoter fusions, combined with site-directed mutagenesis of the putative σ^S extended -10 region in the *prnA* promoter, and liquid chromatography-mass spectrometry (LC-MS) analysis extended our previous knowledge about G3, revealing that quorum sensing (QS) regulates PRN biosynthesis through cross talk with RpoS, which may directly activated *prnABCD* transcription. These findings suggest that PRN in *S. plymuthica* G3 is produced in a tightly controlled manner, and has diverse functions, such as modulation of cell motility, in addition to antimicrobial activities. Meanwhile, the construction of inducible mutants could be a powerful tool to improve PRN production, beyond its potential use for the investigation of the biological function of PRN.

Keywords: *Serratia plymuthica* G3, the *prnABCD* operon, a conditional mutant, over-expressing, pyrrolnitrin biosynthesis and regulation

Introduction

The antibiotic pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chloro-phenyl) pyrrole, PRN] is a secondary metabolite derived from tryptophan that was originally isolated from *Burkholderia pyrrocinia* (*Pseudomonas pyrrocinia*) (Arima et al., 1964). PRN displays a broad-spectrum of antimicrobial activities against a number of plant and human pathogenic bacteria and fungi (Chernin et al., 1996; Di Santo et al., 1998; Kirner et al., 1998), in addition to its nematicidal activity which was reported recently (Nandi et al., 2015). Additionally, PRN biosynthesis in rhizobacteria is known to play a key role in bacterial survival (Costa et al., 2009), and in biological control and induced systemic resistance in plants (Kim et al., 2014). Therefore, natural derivative products of PRN can be used as synthetic lead compounds for the production of novel agricultural fungicides, such as fludioxonil and fenpiclonil as promising alternatives to synthetic pesticides in sustainable agricultural practices (Keum et al., 2009).

The *prnABCD* gene cluster, first described in *Pseudomonas fluorescens* BL915, encodes four enzymes PrnA, PrnB, PrnC and PrnD, which are responsible for PRN biosynthesis (Hammer et al., 1997). Previous studies have established that the bacterial ability to produce PRN greatly affects their biocontrol activity against plant pathogens; thus, a number of studies have been carried out to elucidate PRN production and its regulatory mechanisms in bacteria (Costa et al., 2009; Mozes-Koch et al., 2012). For example, comparative analysis of DNA and protein sequences of the *prnABCD* gene cluster in PRN-producing bacteria revealed that it is highly conserved among strains of the genera *Pseudomonas*, *Burkholderia* and *Serratia* (Hammer et al., 1999; Costa et al., 2009).

Apart from nutritional clues which are known to affect PRN biosynthesis in *Burkhoderia* spp. O33 and *Pseudomonas chlororaphis* O6 (Keum et al., 2009; Park et al., 2011), PRN production in bacteria has been well documented to be modulated by several global regulators. For instance, quorum sensing (QS) is a cell-to-cell communication system that allows bacteria to coordinate gene expression and therefore the behavior, of the entire community in a population-dependent manner using small diffusible signal molecules, such as *N*-acyl homoserine lactones (AHLs) in gram-negative bacteria (Rutherford and Bassler, 2012). AHL signalling has been proven to control PRN biosynthesis in *S. plymuthica* (Liu et al., 2007) and

the *B. cepacia* complex (Schmidt et al., 2009). Several other global regulators, including the master regulator of the general stress response RpoS (σ^S) and the GacS/GacA two-component system (TCS) are also involved in controlling PRN biosynthesis in *S. plymuthica* IC1270 (Ovadis et al., 2004) and *Pseudomonas protegens* (*P. fluorescens*) Pf-5, although this strain is unable to produce AHLs (Sarniguet et al., 1995). However, whether this type of regulation applies generally, as well as the interactions among these regulators are still poorly understood. Furthermore, it is well known that the bacterial RpoD (σ^{70}) subclass of promoters contains two conserved sequence elements where the -10 TATACT box is absolutely essential to start transcription in prokaryotes, whilst the -35 TTGACA box affects the transcription rate. Although the σ^S and σ^{70} subunits recognize very similar promoter sequences, there is no clear -35 box for the σ^S-dependent promoters, which instead have an extended -10 consensus sequence **KCT**ATAC**T** (K=G/T, conserved positions in bold) where a T/GC motif at the -14/-13 positions is very important (Becker and Hengge-Aronis 2001; Weber et al., 2005; Umarov and Solovyev, 2017). This raises the question of whether RpoS directly controls the promoter activity of *prnABCD*, as well as interactions with QS. This remains to be investigated to better understand the mechanisms behind the integrated networks modulating PRN biosynthesis.

S. plymuthica has been used as a biocontrol agent (BCA) against several phytopathogenic fungi due to its ability to produce multiple antifungal factors, including the antibiotic PRN (de Vleeschauwer and Höfte, 2007). We isolated the strain *S. plymuthica* G3 from wheat stem (Liu et al., 2010, and identified two LuxI homologues, SplI and SpsI, which are responsible for synthesis of various AHL signal molecules. Interestingly, both AHL signalling and stationary-phase RpoS are implicated in controlling biofilm formation and cell motility in G3 (Liu et al., 2011; Liu et al., 2016). Analysis of the 5'-flanking region of *prnABCD* using the CNNPromoter (Umarov and Solovyev, 2017) allowed us to identify a putative σ^{70} -10 box TAATCT overlapping with a potential σ^S extended -10 element **TCT**AACA**T** (conserved positions in bold), similar to that found in *E. coli* (Weber et al., 2005), located at a position approximately 30 nucleotides upstream from the *prnA* start codon ATG. This finding implies that *prnABCD* might have an RpoS-dependent promoter, but this remains to be verified. In this

study, the *prnABCD* operon from *S. plymuthica* was characterized for the first time, and the results show that it is responsible for PRN biosynthesis in strain G3. Assay of *lux*-based promoter fusions, combined with LC-MS suggested that PRN biosynthesis is tightly controlled through interactions between AHL signalling and RpoS. Further site-directed mutagenesis of the putative extended -10 region in the *prnA* promoter revealed that RpoS may directly activate *prnABCD* transcription. In addition, a conditional mutant *prnind* of G3 *prnABCD* was constructed using a gene replacement strategy under the control of the inducible P*tac* promoter to improve PRN yield along with exploring the role of PRN in bacterial biology.

Materials and methods

Bacterial strains, plasmids, and growth conditions The bacterial strains, plasmids and primers used in this study are listed in Table 1. *S. plymuthica* G3 (CGMCC no. 4134) and its derivatives were grown at 30°C, and *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth (LB) or on LB agar plates. Antibiotics, when required, were added to the growth media at the following concentrations: ampicillin, 100 μg/ml; gentamicin, 20 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 25 μg/ml. The pathogenic fungus *Cryphonectria parasitica* (ATCC 38755), the causal agent of chestnut blight, was routinely grown on potato dextrose agar (PDA) at 25°C.

DNA preparation and manipulations Standard methods were used for plasmid and genomic DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (Ausubel et al., 1994), or the manufacturer's instructions were followed.

Cloning and heterologous expression of the G3 *prnABCD* operon *S. plymuthica* G3 is able to produce the antibiotic PRN (Liu et al., 2010). Searches within the G3 genome (Liu and Chan, unpublished data) identified a *prnABCD* operon. To clone the *prnABCD* gene cluster, PCR was performed using the primer pair *prnA*-F and *prnD*-R (Table 2), the G3 genomic DNA as template and LA-Taq (TaKaRa, Dalian, China) for amplification of the entire *prnABCD*

operon with its promoter region including 574 nucleotides upstream of the start codon. The following program was used for thermal cycling: 94°C for 6 min; followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and extension at 72°C for 5 min; and a final extension at 72°C for 10 min. After purification, the resulting *ca.*6-kb PCR product was cloned into the vector pMD19-T (TaKaRa) to generate the plasmid pPRN, and transformed into *E. coli* DH5α for heterologous expression of the G3 *prnABCD* operon. Positive clones carrying the recombinant plasmid pPRN were selected by PCR and sequencing (Sangon Co. Ltd., Shanghai, China). The GenBank accession no. for the G3 *prnABCD* operon is JF274257.

Further phylogenetic analyses of 20 complete *prnABCD* sequences from *Pseudomonas*, *Serratia* and *Burkholderia* spp. currently available in the GenBank database, along with G3 *prnABCD* sequenced in this study, were carried out with *prnBCDA* from *Myxococcus fulvus* as the outgroup. A nucleic acid-based neighbour-joining tree was constructed, and a bootstrap test (1000 repetitions) was performed as previously described (Liu et al., 2011).

Construction of a conditional mutant *prnind* of G3 *prnABCD* To verify the biological role of the *prnABCD* gene loci, and obtain an engineered strain of *S. plymuthica* G3 with the ability to over-produce PRN, a G3 *prnind* conditional mutant was first constructed by replacing the native promoter of the *prnABCD* operon in the G3 chromosome with the *lacI*^{Q} repressor gene, along with the P*tac* inducible promoter as described by Rampioni et al., with minor modifications (Rampioni et al, 2010). The primers used are listed in Table 2. Briefly, the construction of the conditional mutant was performed as follows (Fig. 1-a): (a) a 662 bp fragment *prn*-LA, using as the left homologous arm from the upstream region of *prnABCD* was amplified by PCR using the primer pair *prnind*1-F/R, and included the *Sac*I/*Bam*HI sites for double digestion, (b) the 1264 bp fragment *prn*-RA, using as the right homologous arm carrying part of the *prn*A ORF was amplified by PCR using the primer pair *prnind*2-F/R and included the *Eco*RI site to allow cloning into pMD19-T for digestion with *Eco*RI, (c) the 853 bp Gm cassette was excised from plasmid p34S-Gm with *Bam*HI (Dennis and Zylstra, 1998) and inserted into the *Bam*HI site between the two fragments *prn*-LA and *prn*-RA, and (d) the 1.5 kb *lacI*^{Q} P*tac* inducible promoter fragment was excised from pME6032 with *Bam*HI/ *Eco*RI and,

then inserted into the same sites of pBluescript II SK(-) between the Gm cassette and the right arm *prn*-RA. These fragments mentioned above were assembled into pBluescript II SK(-) sequentially to generate pSK-YX. After being digested pSK-YX with *Sac*I/*Eco*RI, followed by Klenow blunting, the *ca*. 4300 bp fragment was ligated into the *Sma*I site of pDM4 to generate the suicide plasmid pDM4-YX, which was then conjugated into the parent strain G3. The inducible mutant *prnind* was obtained through crossover of the insert carried by this recombinant vector pDM4-YX into the G3 chromosome, followed by a second crossover triggered upon 5-15% sucrose selection and the isolation of Gm^R clones. The correct chromosomal insertion was verified by PCR and sequencing. Growth rates of the wild-type G3 and its inducible mutant, *prnind*, in the absence or presence of 0.5 mmol IPTG were monitored every three hours at OD_{600} nm.

PRN identification PRN was extracted from five-day-old bacterial cultures growing on PDA +1% glycerol and incubated at 30°C, followed by thin-layer chromatography (TLC) detection as previously described (Zhou et al., 2012) or LC-MS2 analysis as follows: an Agilent Technologies 1200 Series LC system (Waldbronn, Germany) was used for high-performance liquid chromatography (HPLC) analysis. The column oven was maintained at 50°C. The HPLC column used was a Phenomenex Gemini C18 column (3.0 µm, 100 x 3.0 mm) with an appropriate guard column. Mobile phase A was water, and mobile phase B was acetonitrile. The flow rate throughout the chromatographic separation was 450 µl/min. The binary gradient began initially at 10% B and increased linearly to 99% B in 4.5 min. This composition was maintained for a further 1.5 min, rapidly decreased to 10% B over 0.1 min, and stayed at this composition for 2.9 min. The total run time per sample was 10 min. In total, 10 µl of samples were injected per analysis. The MS system used was a Bruker HCT Plus ion trap mass spectrometer equipped with an electrospray ionisation (ESI) interface. The electrospray settings were as follows: nebuliser gas, 50 psi; drying gas, 9 L/min; and, drying temperature, 365°C. MS analysis was conducted under negative electrospray conditions (-ES) with the MS in MRM (multiple reaction monitoring) mode to screen the LC eluent specifically for PRN by comparing LC retention time of detected peaks with the available synthetic standard. The

precursor-product ion mass transition used for MRM detection was m/z 254.8-189.7.

Antifungal activity and cell motility Bioassays of the antifungal activity were performed in dual cultures on plates by measuring the diameter of the inhibition zone of bacterial strains against the chestnut blight pathogen *C*. *parasitica* as previously described (Liu et al., 2010). The bacterial swimming motility was assayed as described previously (Liu et al., 2016).

Construction and assay of *lux*-based promoter fusions To determine whether RpoS or the QS system also regulates G3 *prnABCD* expression, a *prnA*::*lux* transcriptional fusion was constructed and assayed as described previously (Zhou et al., 2012). Briefly, a *ca.* 550 bp PCR fragment including the promoter region of the *prnABCD* operon was first cloned into pMD19-T, digested with *Xba*I/*Sal*I, and ligated into the *Sma*I site of pBluelux (Atkinson et al., 2008) after Klenow blunting. Positive clones were selected according to bioluminescence and sequencing. Next, the plasmid pBluelux/*prnA::lux* was digested with *Pst*I and ligated into the same site of the broad-host-range vector pUCP26 to generate pP*prnA*::*lux*, which was used to transform *E.coli* /S17-1 to enable conjugation with the wild-type or the Δ*rpoS* mutant. A similar strategy was used to construct the *lux*-based promoter fusions pP*rpoS*::*lux* (*ca.* 580 bp *Eco*RI fragment), pP*splI*::*lux* (*ca.* 520 bp *Xba*I/*Sal*I fragment) and pP*spsI*::*lux* (*ca.* 590 bp *Xba*I/*Sal*I fragment) using the pair of primers *rpoS*-*lux*F/R, *splI*-*lux*F/R and *spsI*-*lux*F/R, respectively (Table 2).

Site-direct mutagenesis To unravel whether RpoS may directly control the transcription of *prnABCD* in strain G3, site-directed mutagenesis of four nucleotide positions conserved in the extended -10 region in the *prnABCD* promoter was conducted. All primers used below are listed in Table 2. Briefly, PCR amplification of the shorter *prnA* promoter P*prnS* (61 bp upstream from ATG) from G3 genomic DNA, and the fluorescent reporter DsRed from plasmid pDsRed2 (Clontech) was performed with the primer pairs Ps-F/R and dsRed-F/R, followed by digested with *Eco*RI/*Bam*HI and *Bam*HI/*Hind*III, respectively. For site-directed mutagenesis, the primer pair Pm-F/R exchanging **TCT**AACA**T** into **CGA**AACA**A** within the extended -10

region (Fig. 1-b) was synthesized, followed by annealing 10 µM Pm-F and Pm-R at a temperature gradient from 95°C to 20°C gradually using a Biometra Thermocycler to obtain dsDNA with 5'-overhanging *Eco*RI and 3'- overhanging *Bam*HI. After validation by PCR and sequencing, both reporter fusions P*prnS*::*dsRed* and P*prnM*::*dsRed* were generated through triple ligation into pUCP26 which was first digested with *Eco*RI/*Hind*III, and then transformed into *E.coli* S17-1. Next, both plasmids pUCP26/*prnS::dsRed* and pUCP26/*prnM*::*dsRed* were separately conjugated into the wild-type G3 and Δ*rpoS* mutant. The promoter activities were monitored as red fluorescence signals at 535 nm excitation/ 595 nm emission, and the cell density OD_{600} was measured using micro-plate reader.

Statistical analysis All data were subjected to one-way-ANOVA analysis using SPSS Statistics 21 to compare treatment mean values. Each treatment was tested in at least triplicate. Experiments were performed at least two times independently.

Results

Cloning and phylogenetic analysis of the *prnABCD* operon from *S. plymuthica* G3 The complete *prnABCD* operon with its promoter region was PCR amplification from G3 chromosome DNA and cloned into pMD19-T as described in the Material and Methods. The resulting recombinant plasmid pPRN was sequenced, which carries *ca*. 6130 bp insert including a 475 bp upstream sequence from the *prnA* start codon (the GenBank accession no. JF274257).The BLAST program was used to identify homologues of *prnABCD* in the GenBank databases.

Phylogenetic analysis of the whole *prnABCD* sequence from *S. plymuthica* G3 and 20 representatives of PRN-producing strains retrieved from GenBank was performed. The results (Figure S1) showed that the *prnABCD* operons from strain G3 and other *Serratia* strains are closely related to *Pseudomonas* spp., clustering into one clade belonging to *Gammaproteobacteria*, but not with *Burkholderia* which is a member of *Betaproteobacteria*. This finding suggests that the clustering pattern of *prnABCD* among *Serratia*, *Pseudomonas*

and *Burkholderia* species reflects their taxonomic relationships.

Expression of G3 *prnABCD* operon and PRN identification To determine whether the G3 *prnABCD* operon is responsible for PRN production, two strategies were used: (a) heterologous expression of G3 *prnABCD* operon in *E. coli* DH5α, and (b) construction of a G3 conditional mutant of *prnABCD* under the control of the inducible P*tac* promoter. For heterologous expression, the plasmid pPRN harbouring G3 *prnABCD* with its native promoter was introduced into *E. coli* DH5α competent cells by heat shock. TLC analysis showed that positive clones of the recombinant *E.coli* DH5α (Fig. 2, Lane 2) expressed *prnABCD* successfully and synthesized the end product, PRN, as indicated by the presence of a purple spot after staining with 2% Ehrlich's reagent. Synthetic PRN was used as a positive control (Fig. 2, Lane S). As expected, no PRN production was observed for the negative control *E. coli* DH5α/pMD19-T with the empty plasmid (data not shown). Furthermore, to genetically manipulate the PRN production levels in strain G3, an IPTG-inducible *prnABCD* mutant, *prnind* was constructed and selected, where the native promoter of the G3 *prnABCD* was replaced with the *lacI*^{Q} P*tac* inducible promoter through gene replacement and homologous recombination, which was validated by PCR and sequencing (data not shown). In the absence of the inducer IPTG, the mutant *prnind* should function as a *prnABCD* mutant. Conversely, IPTG induction should over-express *prnABCD* to produce higher levels of PRN than the wild-type. As expected, the TLC assay verified the above scenario by showing that the mutant *prnind* was defective in PRN production without IPTG induction (Fig. 2, Lane 3), but accumulated PRN when induced with 0.5 mmol IPTG (Fig. 2, Lane 4).The wild-type G3 was used as a positive control (Fig. 2, Lane 2).

PRN is required for antifungal activity Dual culture bioassays of antifungal activity with *C. parasitica* on PDA plates showed a positive correlation between the PRN production levels and their antifungal activity among the different strains (Fig. 3). The recombinant *E. coli* DH5α/pPRN expressed *prnABCD* from G3, and secreted high levels of the end product PRN into PDA media generating an obvious inhibition zone compared with the *E. coli*

DH5α/pMD19-T as a negative control (Fig. 3-a). Meanwhile, the *prnind* mutant in the presence of 0.5 mmol IPTG (Fig. 3-b) exhibited higher antifungal activity due to PRN overproduction than the treatment in the absence of IPTG as a control (Fig. 3-c), where the expression of *prnABCD* was inhibited, no inhibition zone was observed. These findings suggest that PRN plays a central role in the suppression of the pathogenic fungus *C. parasitica* by strain G3.

PRN modulated swimming motility In addition to antimicrobial activity, PRN was found to modulate cell motility in strain G3. As shown in Fig. 4, in the conditional mutant *prnind*, 0.5 mmol IPTG induction greatly stimulated swimming ability, but the absence of IPTG decreased the swimming zone compared to the wild-type G3. Synthetic PRN at final concentration of 200 ng/ml was used as a positive control also stimulated the swimming motility of both wild-type G3 and the inducible mutant *prnind*, but 0.5 mmol IPTG had no effect on the swimming motility of the wild-type.Furthermore, no obvious difference in bacterial growth was observed between the wild type and the *prnind* mutant in the presence or absence of IPTG (Fig. S2), indicating that the impacts on cell motility by PRN are not due to differences in growth rate. These findings indicate that PRN, apart from being a secondary metabolite (antibiotic) with broad-spectrum antimicrobial activity, could also function as a signal molecule regulating bacterial behaviours.

Interplay between QS and RpoS regulates G3 *prnABCD* expression The global regulators QS and RpoS have been reported to affect PRN production in strains of *S. plymuthica* (Ovadis et al., 2004; Liu et al., 2007) or *P*. *protegens* (Sarniguet et al., 1995). To determine if this regulation generally applies, TLC or LC-MS assay was carried out to compare PRN production between the wild-type G3 and a quorum quenching strain expressing the lactonase AiiA or a Δ*rpoS* mutant. The results suggested that quorum quenching significantly reduced PRN production in strain G3 (Figure S3), which agrees with previous observations on the rhizospheric *S. plymuthica* HRO-C48 (Liu et al., 2007). Similarly, LC-MS2 analysis (Fig. 5) showed that the mutation in RpoS (peak area: 9.58 $\times 10^4$) also greatly decreased the PRN yield compared to the wild-type G3 (peak area: 2.28 $\times 10^6$) as observed in *P*. *protegens* Pf-5.

This shows that both QS and RpoS positively regulated PRN biosynthesis in *S. plymuthica* G3. The PRN MS2 spectra are shown in Figure S4. To better understand the mechanisms behind RpoS and AHL-mediated control of PRN biosynthesis, the expression of a *prnA*::*lux* promoter fusion in strain G3 supplemented with its major types of AHL signal molecules, 3-oxo-*N*-hexanoyl-homoserine lactone (3OC6-HSL, OHHL), the unsubstituted *N*-butyryl-homoserine lactone (C4-HSL, BHL) and *N*-hexanoyl-homoserine lactone (C6-HSL, HHL) (Liu et al., 2010) at a final concentration of 0.5 μm was performed (5 mM AHLs dissolved in methanol as stock solutions).1μl methanol added to 10 ml LB was used as a control, no bioluminescence difference was observed when compared with LB alone (data not shown). The results showed that AHL signalling positively modulated *prnABCD* transcription, and the maximum activity was induced by a mixture of three types of AHLs (1: 1: 1). 3OC6-HSL was the optimum single AHL signal for *prnABCD* transcriptional activity (Fig. 6-a). Similarly, the transcription of *rpoS*::*lux* promoter fusion was also stimulated by AHL signals, and the mixture of AHLs induced the highest level of activity. In contrast to the *prnA::lux* fusion, C4-HSL, not 3OC6-HSL, was the optimum single signal molecule for the transcription of *rpoS* (Fig. 6-a). Reciprocally, RpoS also has an impact on the transcription of the *luxI* homologous gene *spsI*, but not *spsI* (Fig. 6-b), which is known to encode the AHL synthase SpsI to produce the unsubstituted AHLs only (Liu et al., 2010).

Expression of the *prnABCD* operon is RpoS-dependent The expression of the *prnA*::*lux* promoter fusion (~500 bp *prnA* upstream sequence) in the wild-type or the Δ*rpoS* mutant background was also measured. As shown in Fig. 7-a, the relative bioluminescence unit (RLU) of the *prnA*::*lux* reporter fusion significantly decreased in the Δ*rpoS* mutant compared to the wild-type G3, suggesting that *prnABCD* transcription might be RpoS-dependent. To validate this hypothesis, the fluorescent reporter *dsRed* was fused, in combination with site-directed mutagenesis of the conserved nucleotides in the extended -10 region (TCT….T replaced with CGA….A) to the shorter (~60 bp) *prnA* upstream sequence. Assays of the *dsRed*-based reporter fusions demonstrated the *ca*. 60 bp *prnA* upstream sequence (*prnS*::*dsRed*) does have promoter activity, but its activity decreased in the Δ*rpoS* mutant compared to the

wild-type (Fig.7-b), the same trend as the *prnA*::*lux* promoter fusion (Fig. 7-a). Meanwhile, the relative fluorescent unit (RFU) of *prnM*::*dsRed* promoter fusion in both wild-type and the Δ*rpoS* mutant was also greatly reduced due to mutation in the conserved nucleotide positions compared to the native *prnS*::*dsRed* (Fig. 7-b). The results indicated the *prnABCD* upstream sequence includes the σ^S-selective promoter, revealing that RpoS could directly control the transcription of *prnABCD*, in addition to affecting PRN yield. Taken together, these findings suggest that RpoS may play a key role in fine tuning both transcription and translation of the *prnABCD* loci through cross talk with QS, which could also have impacts on the antifungal activity and cell motility of *S. plymuthica* G3 (Fig. 8).

Discussion

PRN-producing strains have been described mainly in a narrow range of gram-negative bacteria of the genera *Burkholderia*, *Pseudomonas*, and *Serratia*, and are key players in the biocontrol of plant diseases (Hill et al., 1994; Kalbe et al., 1996; Hammer et al., 1999; Ligon et al., 2000; Costa et al., 2009). For example, PRN production from *S. plymuthica* was required for the suppression of a broad range of fungal pathogens, such as soil-borne *Rhizoctonia solani* and *Pythium aphanidermatum* in vegetables (Ovadis et al., 2004), airborne *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Kamensky et al., 2003), and post-harvest diseases caused by *Penicillium* spp. in peaches and apples (de Vleeschauwer and Höfte, 2007). To improve its biocontrol efficacy, we sought to gain a better insight into PRN biosynthesis and regulation in *S. plymuthica* by cloning the *prnABCD* loci from *S. plymuthica* G3 into *E. coli*. TLC detection confirmed that the four genes *prnA*, *prnB*, *prnC* and *prnD* are sufficient for the production of the antibiotic PRN without the need for the surrounding genes, similar to *P. fluorescens* BL915 (Hammer et al., 1997). This finding was further verified by constructing a G3 conditional mutant *prnind* under the control of the inducible P*tac* promoter, which behaved like a *prnABCD* mutant in the absence of IPTG by barely producing PRN (Fig. 2 Lane 3). More importantly, the *prnind* mutant induced with 0.05 mmol IPTG also behaved like an over-expressing strain of the *prnABCD* operon, which has potential for PRN overproduction

(Fig. 2, Lane 4). Correspondingly, PRN contents secreted by the wild-type G3 and its derivatives are positively correlated with their antifungal activity against *C. parasitica* (Fig. 3), consistent with previous studies in various biocontrol strains of *Pseudomonas* (Hill et al., 1994; Sarniguet et al., 1995), *Burkhoderia* (Hwang et al., 2002) and *Serratia* (Ovadis et al., 2004). Recently, the entire *prnABCD* operon from *P. protegens* Pf-5 was expressed successfully in tomato plants through the plant universal vector IL-60, resulting in the appearance of a unique plant phenotype with resistance to damping-off disease caused by *R. solani* (Mozes-Koch et al., 2012).These findings indicate that the role played by PRN in biological control of plant diseases is important and conserved in bacteria.

Apart from their antimicrobial activities, the antibiotics phenazine (Dietrich et al., 2006; Pierson et al., 2010) and 2,4-diacetylphloroglucinol (DAPG) (Maurhofer et al., 2004; Combes-Meynet et al., 2011) both serve as signal molecules to induce cross-talk among rhizoplane bacterial communities and have impacts on bacterial behaviours. Similarly, our investigation of swimming motility of the conditional mutant *prnind* with or without IPTG induction showed that PRN also affected cell motility which was confirmed by using a synthetic PRN standard as a positive control (Fig. 4). Thus, PRN may also function as a signal molecule, beyond its role as an antibiotic to suppress fungal pathogens. However, the non-IPTG-induced *prnind* could still swim; one possible explanation may be due to PRN-mediated alteration of gene expression such as motility-related genes encoding chemotaxis, flagellar etc., and/or their regulatory genes, but still to be investigated. For instance, RNA-seq based transcriptomic studies, together with qRT-PCR and phenotypic analyses could shed light on the molecular basis for PRN-driven regulation of gene expression, and unravel the reasons why PRN can affect cell motility on a whole genome scale. Altogether, the construction of the inducible mutant could be a powerful tool that can be used not only for improving the PRN yield, but also for exploring the role of PRN in bacterial physiology and behaviour. It is worth noting that the target gene or operon suitable for the construction of the P*tac* inducible mutant must be a single copy with no polar effects. Fortunately, sequencing the whole genome of G3 (Liu and Chan unpublished data) allowed us to discover only one copy of the *prnABCD* loci in strain G3, further analysis of its genomic context by Virtual Footprint 3.0 (Münch et al., 2005) predicted a 171 bp ORF

encoding hypothetical protein flanked the *prnABCD* operon, but with its own promoter approximately 50 bp upstream from the start codon ATG, and within *prnD* ORF. Therefore, we do not expect any polar effect on surrounding genes.

Increasing our knowledge of regulatory mechanisms governing the biosynthesis of antibiotics may help in the construction of strains with enhanced biocontrol activity (Chet and Chernin, 2002). The manipulation of bacterial regulatory systems, such as GacS/GacA TCS or alternative sigma factors in *P. fluorescens* has resulted in a significant improvement of the biocontrol potential of this organism (Schnider et al., 1995; Haas et al., 2000). QS or RpoS positively regulate PRN biosynthesis in the rhizospheric *S. plymuthica* HRO-C48 (Liu et al., 2007), the *Burkholderia cepacia* complex (Schmidt et al., 2009), and *P*. *protegens* Pf-5 (Sarniguet et al., 1995). We have provided evidence that both AHL signalling and the sigma factor RpoS can stimulate *prnABCD* transcription and the secretion of the end product PRN in *S. plymuthica* G3. Bioluminescence assay of the *lux*-based promoter fusions suggest that the transcription of both *prnABCD* and *rpoS* from G3 is significantly induced by the mixture of AHL signals in a cell density-dependent manner under the control of QS systems (Fig. 6-a). Interestingly, mutation in RpoS also had a considerable impact on the transcription of the *luxI* homologue *spsI*, but not *splI* (Fig. 6-b), which is responsible for the synthesis of unsubstituted AHLs, especially C4-HSL in strain G3 (Liu et al., 2011). These findings indicate that RpoS selectively controls the expression of SpsI/SplI QS regulators, besides the cross-link between the QS systems and RpoS, which is similar to previous reports in several *Pseudomonas* spp. For instance, there are two AHL-mediated QS systems, LasIR/RhlIR, in *P. aeruginosa*, and RpoS negatively regulates QS through repression of the transcription of *rhlI*, but not *lasI*; on the other hand, QS activates *rpoS* expression through RhlIR (Venturi 2006). Further sequence comparison revealed a potential σ^S extended -10 region approximately 30 bp upstream of the *prnA* start codon which was verified through site-directed mutagenesis together with *dsRed*-based promoter fusion assays (Fig. 7-b). To the best of our knowledge, this is the first experimental evidence showing that *prnABCD* is an RpoS-dependent operon; therefore, QS may at least indirectly affect PRN biosynthesis via RpoS-driven direct control of the transcription of *prnABCD* since no *lux* box-like element was found in the upstream sequence of

prnA (data not shown), which is the binding site for the AHL/LuxR complex to activate/repress the transcription of target genes (Rutherford and Bassler, 2012).

In summary, PRN biosynthesis in *S. plymuthica* could be controlled at multiple levels through an integrated hierarchical network of several global regulators. For example, the interplay between QS and RpoS was involved in controlling the transcription of *prnABCD* and PRN production in strain G3. Similarly, in strain IC1270 where the GrrS/GrrA TCS is required for AHL accumulation, besides PRN production (Ovadis et al. 2004), showing the link between QS and GacS/GacA, which is known to regulate the transcription of small RNAs of RsmB family in bacteria. On the other hand, the RNA chaperon Hfq of *S. plymuthica* is a post-transcriptional regulator to positively modulate PRN production, in addition to stimulating RpoS translation (Zhou et al., 2012). All these findings in *S. plymuthica* implicate that RpoS as a central player in the control of PRN biosynthesis at both transcriptional and translational levels through integrated multiple regulators into a complex network, whilst the tightly regulated PRN may play a more important role in bacterial physiology and adaption to the environment, which is far beyond our current knowledge.

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Compliance with ethical standards

Conflict of Interest: All authors declare that he/she has no conflict of interest.

Ethical Statement: This article does not contain any studies with human participants or animals performed by any of the authors

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Tables and Figures' Legends

Table 1 Bacterial strains and plasmids used in this study

Table 2 Primers used in this study

Fig. 1 Schematic representation of the *prnABCD* loci in S. *plymuthica* G3 wild-type and its IPTG-inducible mutant *prnind* (A); and the *prnA* upstream DNA sequence (B)

Panel a: The gentamycin (Gm) cassette is excised with *Bam*HI from plasmid p34S-Gm, and the *lacI*^{Q} repressor with the P*tac* promoter is derived from plasmid pME6032. The organization of the *prnABCD* operon is shown, which is followed by a 171 bp hypothetical protein (hp) with its own promoter P_{hp} located in the *prnD* ORF. Panel b: The putative σ^{70} -10 box is indicated by a black bar, and the putative σ^{S} extended -10 region in grey box, and the conserved nucleotides in bold are replaced (short vertical lines) through site-directed mutagenesis, the primer pairs Ps-F/R are underlined. The proposed ribosome binding site (RBS) and the ATG start codon are in grey box.

Fig. 2 TLC detection of PRN production

Lane S：synthetic PRN standard (4 μg from Sigma); Lane 1: wild-type G3; Lane 2: *E. coli* DH5α/pPRN carrying pMD19-T-*prnABCD*; Lane 3: non-IPTG induced mutant *prnind* ; Lane 4: *prnind* + 0.5 mmol IPTG

Fig. 3 Effects of the PRN production on antifungal activity in dual cultures with the pathogenic *C. parasitica.*

Panel a: the recombinant *E. coli* DH5α/pPRN carrying the G3 *prnABCD* operon (A); *E.coli* DH5α/pMD19-T with the empty plasmid as a negative control (B); and the wild-type G3 as a positive control. Panel b: IPTG induced mutant *prnind*. Panel C: non-IPTG induced mutant *prnind*.

Fig. 4 Effects of the PRN production on swimming motility

0.5 mmol IPTG was used for induction of the P*tac* promoter expression ; 2 μl of overnight bacterial suspension was inoculated on swimming plates containing 0.2 μg/ml concentration of synthetic PRN and incubated at 30 °C for 16 h. 2 μl methanol (MeOH) as the solvent control.

Fig. 5 LC-MS2 identification of PRN production

Panel a: HPLC profile of 10 μm synthetic PRN as a positive control. Panel b: HPLC profiles of PRN extracts from the wild-type G3 in black and the mutant Δ*rpoS* in grey, respectively.

Fig.6 Assay of *lux*-based promoter fusions

Panel a: Impacts of 0.5 μM AHLs on the transcription of *prnABCD* and *rpoS* in strain G3. Panel b: Impacts of RpoS on the transcription of the *luxI* homologues *splI* and *spsI* in strain G3. 5 mM AHLs dissolved in MeOH were used as stock solutions, 1μl MeOH diluted with 10 ml LB as a control. BHL: *N*-butyryl-homoserine lactone (C4-HSL); HHL: *N*-hexanoyl-homoserine lactone (C6-HSL); OHHL: 3-oxo-*N*-hexanoyl-homoserine lactone (3OC6-HSL); Mix: the mixture of BHL, HHL and OHHL (1:1:1).

Fig. 7 RpoS-dependent transcription of the *prnABCD* operon

Panel a: Assay of *lux*-based promoter fusion to *prnA*. Panel b: RpoS directly activates the *prnA*

transcription determined by site-directed mutagenesis.

Fig. 8 Schematic model describing the interplay between AHL signaling and RpoS involved in regulation of PRN biosynthesis in *S. plymuthica* G3

Supplementary materials (Figure S1-S4)

Table 1

Table 1 Bacterial strains, plasmids and primers used in this study

Strain or plasmid	Description	Reference
S. plymuthica G3	Wild type, Rif ^{R}	Liu et al. 2011
*prn*ind	G3 derivative in which *prnABCD* expression is under the control of *lacI*^{q} P*tac* inducible promoter, Gm^{R}	This study
Δ*rpoS*	A *rpoS*::Km mutant of G3, Km^{R}	Liu et al., 2016
E. coil DH5α	A host strain for cloning	Ausubel et al., 1994
E. coil S17-1	*Pro thi hsdR recA*$^{-}$Tp^{r} Sm^{r}; chromosome::RP4-2 Tc::Mu-Km::Tn7	Ausubel et al., 1994
pMD19-T	Cloning vector, Amp^{R}	Takara
pPRN	Plasmid pMD19-T harboring G3 *prnABCD* operon	This study
pME6032	Shuttle vector with *lacI*^{Q} P*tac* inducible promoter; Tc^{R}	Heeb et al., 2002
p34S-Gm	Source of Gm^{R} cassette	Dennis et al., 1998
pBluescript II SK(-)	Cloning vector, Amp^{R}	Stratagene
pDM4	Suicide vector; sacBR, oriR6K; Cm^{R}	Milton et al. 1996
pDM4-YX	pDM4 carrying *lac*I^{Q} P*tac-prnA,* Gm^{R}, Cm^{R}	This study
pUCP26	Broad-host-range cloning vector; Tc ^{R}	West et al., 1994
pBluelux	a promoter-less *luxCDABE* cassette in pBluescript II, Amp^{R}	Atkinson et al., 2008
pP*prnA*::*lux*	pUCP26 with a *prnABCD* promoter fusion to *luxCDABE*, Tc^{R}	Zhou et al., 2012
pP*rpoS*::*lux*	pUCP26 with a *rpoS* promoter fusion to *luxCDABE*, Tc^{R}	This study
pP*spII*::*lux*	pUCP26 with a *spII* promoter fusion to *luxCDABE*, Tc^{R}	This study
pP*spsI*::*lux*	pUCP26 with a *spsI* promoter fusion to *luxCDABE*, Tc^{R}	This study
pDsRed2	pDsRed-express2 vector, Amp^{R}	Clontech
pP*prnS*::dsRed	pUCP26 carrying a shorter *prnA* promoter with a – 10 box, Tc^{R}	This study
pP*prnM*::dsRed	pUCP26 carrying mutation in -10 box of the *prnA* promoter, Tc^{R}	This study

Table 2

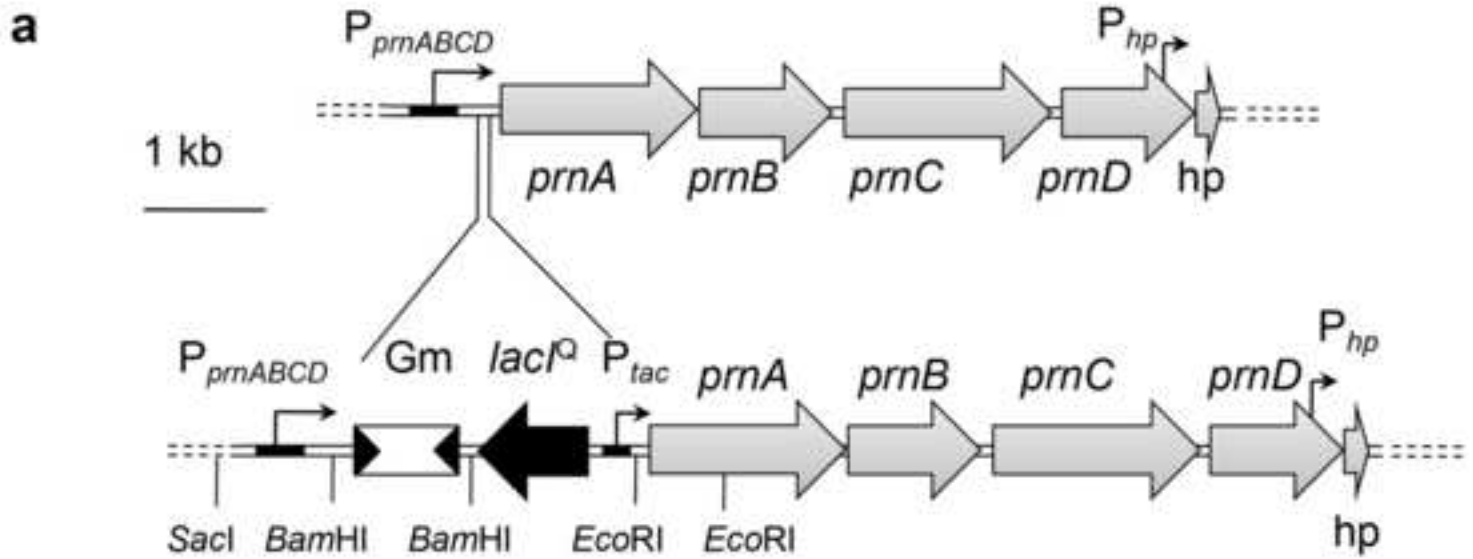
Table 2 Primers used in this study

Primer	5'-3' sequences
prnA-F	GGTGTTCGATTTATAGGGT
prnD-R	CGCCATGATGACAGTGA
*prn*ind$_1$-F	CGAGCTCTTTCCGAATGTTGTTGA (*Sac*I)
*prn*ind$_1$-R	CGGGATCCTTGCTCATGACACTCT (*Bam*HI)
*prn*ind$_2$-F	GGGAATTCATGAGCAAACCGATC (*Eco*RI)
*prn*ind$_2$-R	TTGATGGAGTCAGAGAG
*prn-lux*F	CGTAAGTAACGAATGAATC
*prn-lux*R	CAGGCTAGACTCTCGTCT
*rpoS-lux*F	GAATTCACTGCAACGGTTGATTCT (*Eco*RI)
*rpoS-lux*R	ATACCGCGAGCAGAATATC
spII-luxF	CAGCGACTTCGACAGCAT
spII-luxR	TTGGCGCAAATATATAGCG
spsI-luxF	TGTATTGGTCGGTGGTGA
spsI-luxR	GTCTTTCGGTATTGGTGAGT
Ps-F	GAATTCAGAGTCTAGCCTGATTAGAAC (*Eco*RI)
Ps-R	GGATCC ATAGGAGGATAGTGGAGATG (*Bam*HI)
Pm-F	AATTCAGAGTCTAGCCTGATTAGAACCTAACGAAACAACTCCACTATCCTCCTATG (*Eco*RI)
Pm-R	GATCCATAGGAGGATAGTGGAGTTGTTTCGTTAGGTTCTAATCAGGCTAGACTCTG (*Bam*HI)
dsRed-F	GGATCC**AGGAAACAG**ATGGATAGCACTGAGAACGT (*Bam*HI, **RBS**)
dsRed-R	AAGCTTCTACTGGAACAGGTGGTG(*Hind*III)

* Restriction sites are underlined, Ribosome binding site RBS in bold. Annealing the primer pair Pm-F/Pm-R was completed from 95 to 20°C gradually in Biometra gradient PCR apparatus to obtain dsDNA with 5'-*EcoR*I and 3-*Bam*HI cohesive end (in shade), respectively for triple ligation.

Figure 1

Click here to download Figure Fig 1.tif



b

GATATATCGCTGAGTTAATTCTAAAACGGAATGTGGTAATTTTCAGACGAGAGTCTAGCCTGATTAGAACC

σ^S extended -10 box

RBS

prnA start

Primer Ps-F

CGA A

TAATCTAACATCTCCACTATCCTCCTATGGGAGAGTGTCATGAGCAAA

M S K

σ^{70} -10 box

Primer Ps-R

Figure 2

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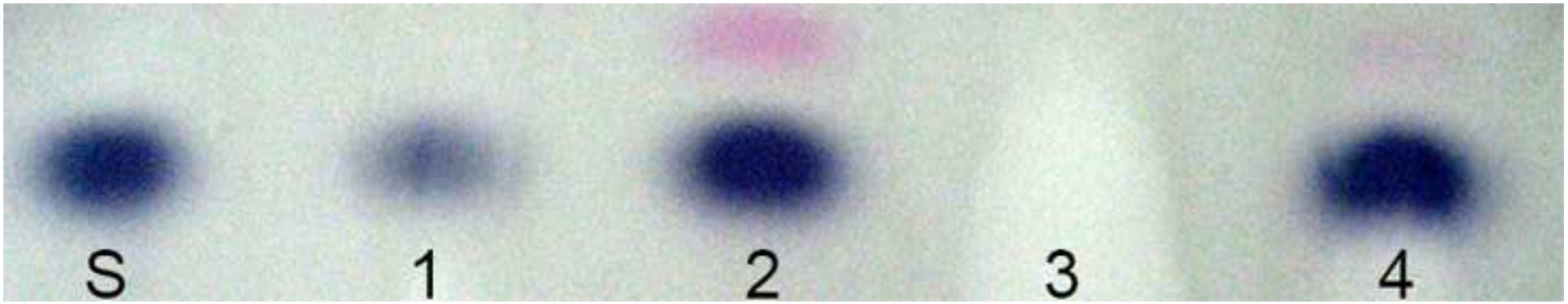

Figure 3

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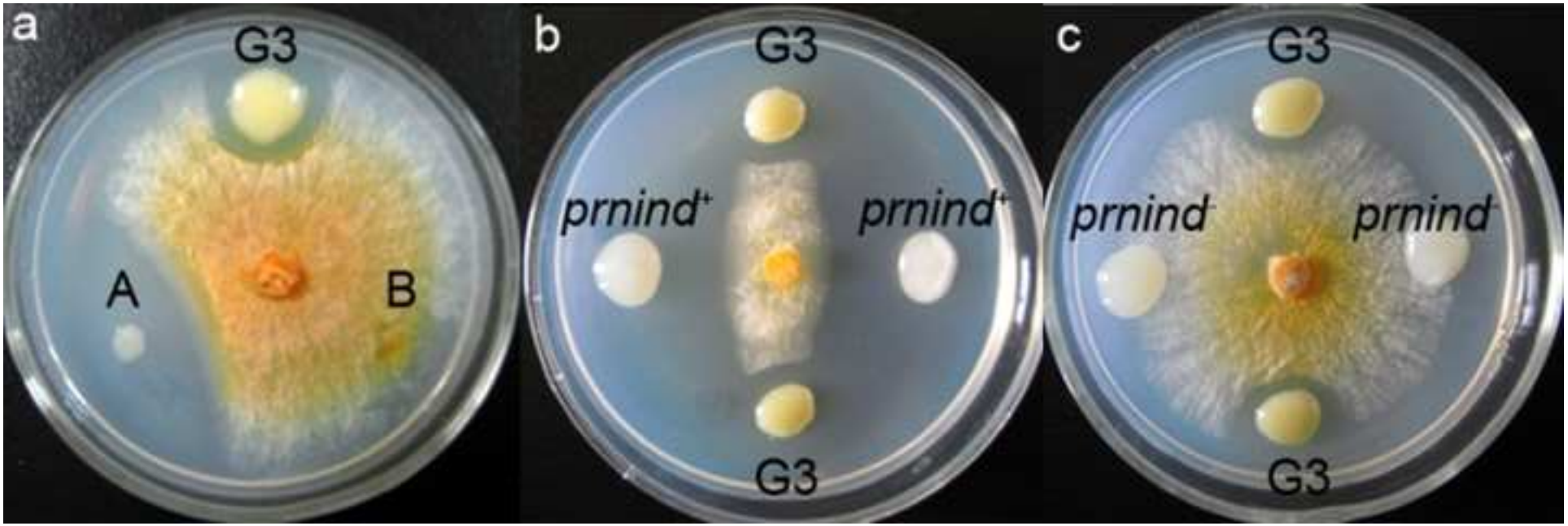

Figure 4

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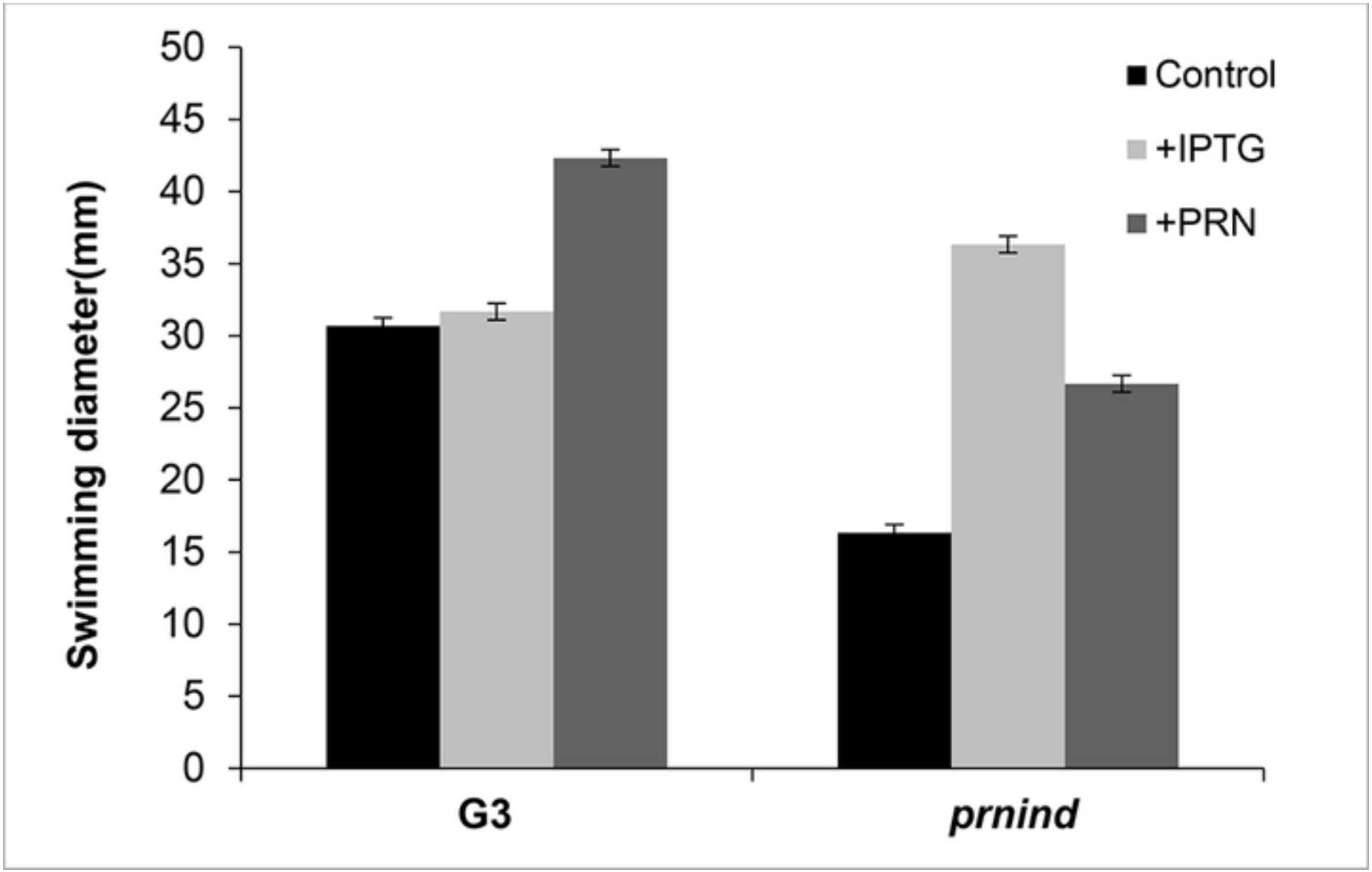

Figure 5

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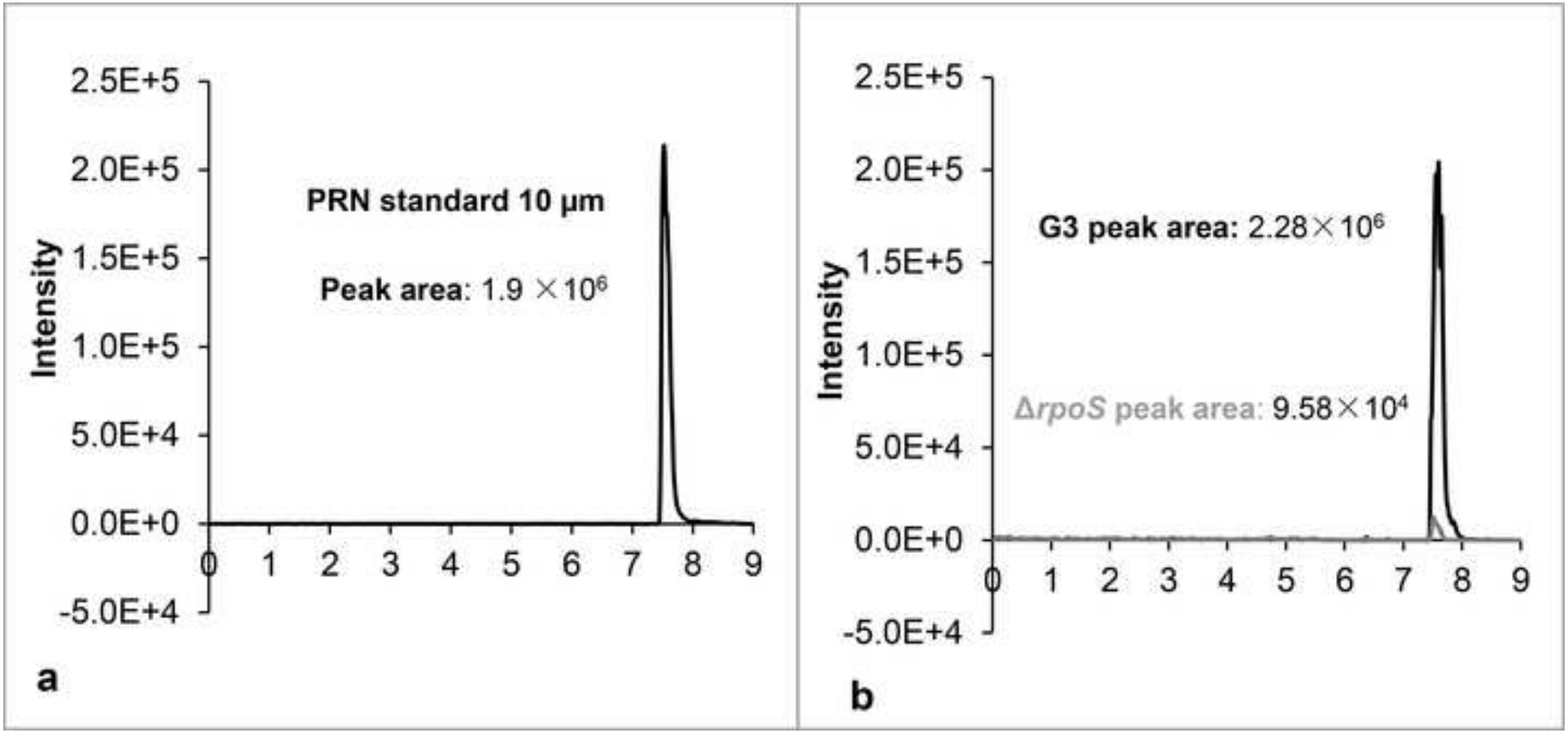

Figure 6

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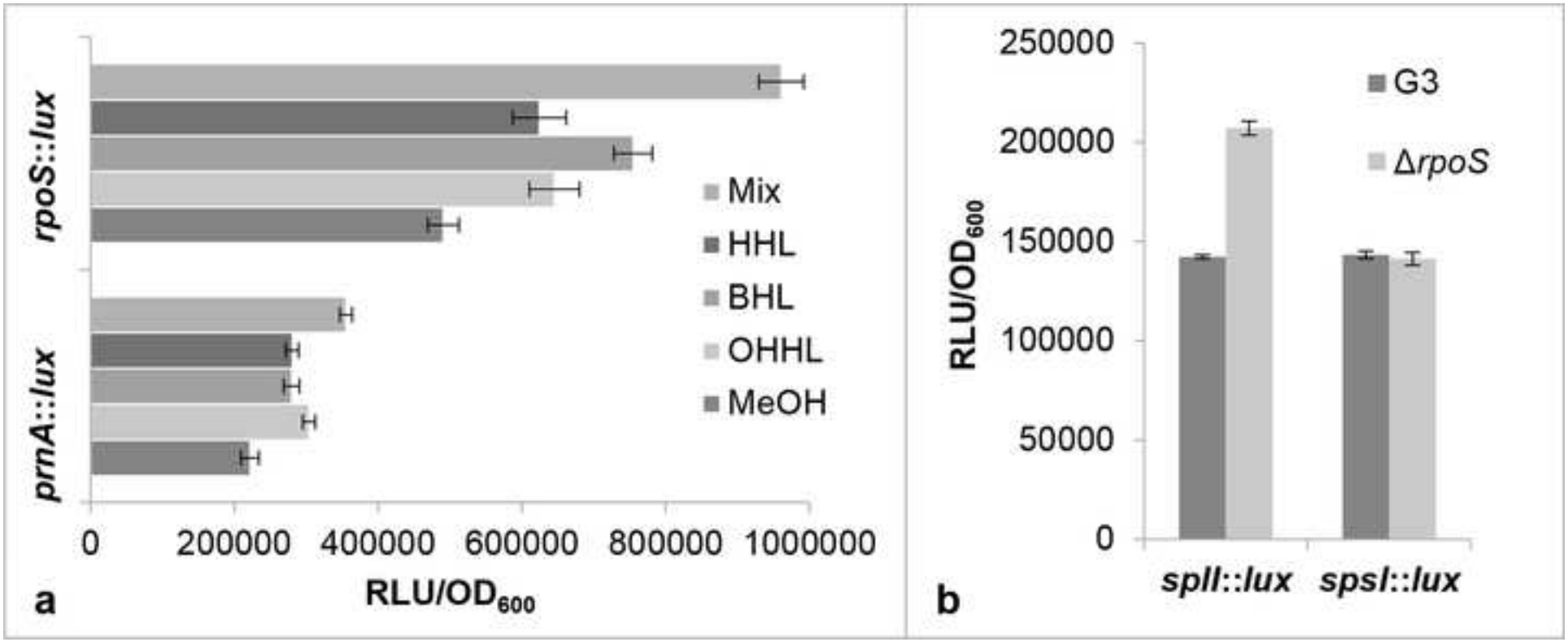

Figure 7

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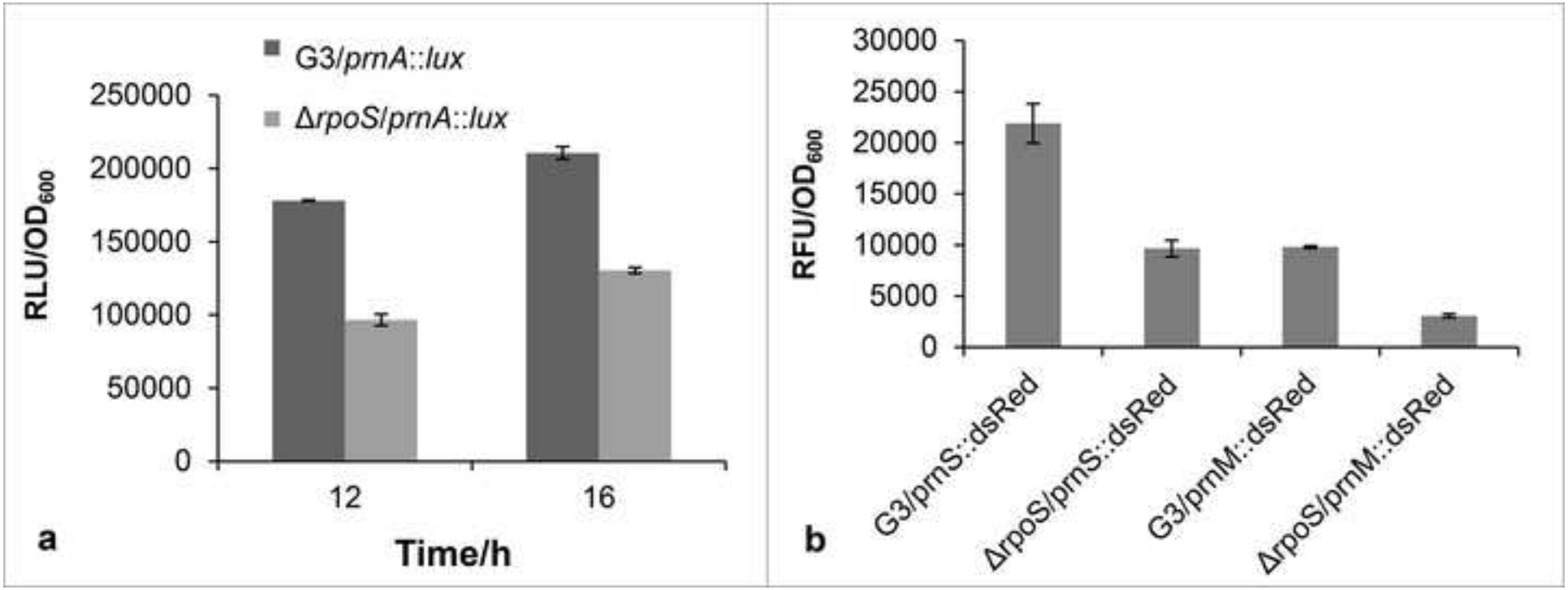

Figure 8

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