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PsrA is a novel regulator contributes to antibiotic synthesis, bacterial virulence, cell motility and extracellular polysaccharides production in *Serratia marcescens*

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ABSTRACT

Serratia marcescens **is a Gram-negative bacterium of the** ***Enterobacteriaceae*** **family that can produce numbers of biologically active secondary metabolites. However, our understanding of the regulatory mechanisms behind secondary metabolites biosynthesis in** ***S. marcescens*** **remains limited. In this study, we identified an uncharacterized LysR family transcriptional regulator, encoding gene** ***BVG90_12635*****, here we named** ***psrA*****, that positively controlled prodigiosin synthesis in** ***S. marcescens*****. This phenotype corresponded to PsrA positive control of transcriptional of the prodigiosin-associated** ***pig*** **operon by directly binding to a regulatory binding site (RBS) and an activating binding site (ABS) in the promoter region of the** ***pig*** **operon. We demonstrated that L-proline is an effector for the PsrA, which enhances the binding affinity of PsrA to its target promoters. Using transcriptomics and further experiments, we show that PsrA indirectly regulates pleiotropic phenotypes, including serrawettin W1 biosynthesis, extracellular polysaccharide production, biofilm formation, swarming motility and T6SS-mediated antibacterial activity in** ***S. marcescens*****. Collectively, this study proposes that PsrA is a novel regulator that contributes to antibiotic synthesis, bacterial virulence, cell motility and extracellular polysaccharides production in** ***S. marcescens*** **and provides important clues for future studies exploring the function of the PsrA and PsrA-like proteins which are widely present in many other bacteria.**

INTRODUCTION

Serratia marcescens, a Gram-negative rod-shaped bacterium of the *Enterobacteriaceae* family, is found in a wide range of ecological niches like soil, water, plants, insects, and foods. *S. marcescens* can produce many high-value secondary metabolites like prodigiosin (1), althiomycin (2), serratamolide (3), acetoin (4) and 2,3-butanediol (5). Prodigiosin (PG), a red linear tripyrrole pigment and the most prominent member of the prodiginine family, has received widespread attention for its reported antimalarial, antibacterial, antifungal, antiprotozoal and immunosuppressant activities (1). Previous studies on the metabolic regulation network of prodigiosin biosynthesis in *S. marcescens* have revealed that *pigABCDEFGHIJKLMN* genes were involved in the biosynthetic pathway of prodigiosin synthesis (Supplementary Figure S1) (1). Also, it is reported that prodigiosin biosynthesis in *S. marcescens* was widely regulated by a number of transcription factors including positive regulators EepR (6), PigP (7), GumB (8) and RbsR (9), and negative regulators MetR (10), SpnR (11), CopA (12), CRP (13), HexS (14), RssB (15), RcsB (16,17) and SmaR (18).

Unfortunately, although several regulator-encoding genes have been shown to be essential for prodigiosin synthesis in *S. marcescens*, our knowledge of the regulatory mechanisms behind prodigiosin production and the functions of these regulators in *S. marcescens* is still limited.

S. marcescens JNB5-1 is a prodigiosin production strain isolated from soil samples (10). In this study, a Tn5G transposon library of strain JNB5-1 was constructed to identify the genes required for prodigiosin synthesis, and the LysR type regulator PsrA encoding gene *psrA* (*BVG90_12635*) was confirmed to positively regulate prodigiosin production in strain JNB5-1. The mechanism for positive regulation

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of prodigiosin production by PsrA protein was explored. Moreover, our study investigated the regulatory role of the PsrA protein in cell motility, biofilm formation, extracellular polysaccharide production, serrawettin W1 biosynthesis, and type VI secretion system (T6SS) meditated antibacterial activity in strain JNB5-1. Our data showed a novel regulator PsrA, which is important for various cellular processes in *S. marcescens*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Serratia marcescens JNB5-1 is a prodigiosin producing strain isolated from soil sample (10). Mutant SK8-37 is a prodigiosin production mutant isolated from a Tn5G transposon insertion mutant library of strain JNB5-1. *Escherichia coli* DH5α and S17-1 were used for plasmid construction and *E. coli* BL21 (DE3) was used for protein expression. For *E. coli*, bacterial cultures were incubated in LB medium at 37°C and for *S. marcescens* and *Enterobacter cloacae*, strains were incubated in LB medium at 30°C. Whenever necessary, appropriate antibiotics were added at defined concentrations for strains cultivation. For the cultivation of *E. coli* strains, spectinomycin at 25 μg/ml, streptomycin at 25 μg/ml, kanamycin at 50 μg/ml, ampicillin at 50 μg/ml, or gentamicin at 10 μg/ml were used. For the cultivation of *S. marcescens* strains, spectinomycin at 50 μg/ml, streptomycin at 50 μg/ml, ampicillin at 150 μg/ml, apramycin at 50 μg/ml, or gentamicin at 50 μg/ml were used. Bacterial strains and plasmids used in this study are listed in Table 1.

Identification of the Tn5G inserted gene in mutant SK8-37

A mutant strain SK8-37, with significantly decreased prodigiosin production, was isolated from a transposon Tn5G insertion mutant library of strain JNB5-1 conducted by the method described previously (10). Inverse PCR was performed to identify Tn5G insertion site in mutant SK8-37 as described previously (19). In brief, genomic DNA of mutant SK8-37 was digested completely by the restriction nuclease *Taq*I, and subjected to self-ligation. The ligated molecules were then amplified using the primers OTn1 and OTn2 (Supplementary Table S1), and amplified PCR product was cloned into pMD18T vector for sequencing. The obtained sequences were analyzed by searching the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/) to identify the insertion site. The domains of the regulatory protein PsrA (BVG90_12635) was identified using the online software CD-search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). For complementation experiment, the intact *psrA* gene was amplified using the primer pairs PsrA-F1 and PsrA-R1 listed in Supplementary Table S1 to construct the recombinant plasmid pXW1903 and transformed into the mutant SK8-37 by electroporation.

Construction of *psrA* and *tssE* deletion mutants

The *psrA* and *tssE* genes of strain JNB5-1 were deleted by the method described previously (10). Briefly, the upstream and downstream DNA fragments of *psrA* gene or *tssE* gene and DNA fragments of *aacC3* resistance gene were amplified by PCR method. The *aacC3* gene was then integrated into the middle of the upstream and downstream fragments of the *psrA* gene or the *tssE* gene by overlap extension PCR. The obtained PCR products were cloned into the pUTKm vector. The resulted plasmids were then transformed into *E. coli* S17-1, and introduced into the strain JNB5-1 by conjugation to knock out the *psrA* gene and *tssE* gene, respectively. These deletions removed M1 to Q295 of the total 299 amino acids in PsrA, and P6 to K188 of the total 191 amino acids in TssE. The *psrA* gene deleted mutant ΔPsrA and *tssE* gene deleted mutant ΔTssE were confirmed by PCR and DNA sequencing analysis. Primers used for gene deletion are listed in Supplementary Table S1 in the supplemental material.

Prodigiosin production assays

The method for the determination of prodigiosin yield of strains JNB5-1, SK8-37, SK8-37/pXW1903, SK8-37/pUCP18, ΔPsrA, ΔPsrA/pXW1903 and ΔPsrA/pUCP18 was by acidified ethanol and absorbance measurement as previously described (10,20). Briefly, after collecting samples at time intervals of 4, 6, 8, 10, 12 and 24 h, the amount of prodigiosin produced by different strains was calculated according to the standard curve: $Y = 1.1936X - 0.001$. (Y indicates the wavelength of samples measured at A_{534} after the fermentation broth was dissolved in acid ethanol at pH 3.0; X indicates the amount of prodigiosin produced by the strains, for which 1 unit equals 10 mg/liter). Relative prodigiosin production of different strains was calculated per cell as $A_{534}/OD_{600} \times 50$, where OD_{600} is the optical density at 600 nm. Experiments were independently replicated three times.

Growth curve assays

To analyze the growth curve of strains JNB5-1, SK8-37 and SK8-37/pXW1803, the exponential-phase cells (OD_{600} of 0.6) of these three strains were inoculated in fresh LB medium at 3% inoculation volume. Optical densities of cultures were measured at 600 nm wavelength at time intervals of 0, 2, 4, 6, 8, 10, 12, 24, 36 and 48 h, and the growth curves were plotted by the values of A_{600} versus the incubation time. Experiments were independently replicated three times.

Real-time quantitative PCR (RT-qPCR) assay

RT-qPCR assay was performed as described previously (10). In brief, to assess the expression levels of prodigiosin synthesis-related genes, cell motility-related genes, biofilm formation-related genes, extracellular polysaccharide production-related genes, serrawettin W1 synthesis-related genes, and T6SS-related genes, 1-ml of the cultures of strains JNB5-1 and SK8-37 were collected after 12 h of shake flask fermentation with an OD_{600} of 6.0. The collected cells were then subjected to total RNA extraction using an RNAprep pure Cell/Bacteria Kit (Tiangen). After treating the total bacterial RNA with DNase I (Promega), the concentration and quantity of the total bacterial RNA

Table 1. Strains and plasmids used in this study

Strains and plasmids	Description	Source
***E. coli* strains**		
DH5α	*hsdR recA lacZYAF80 lacZDM15*	BRL
BL21 (DE3)	*F-dcm ompT hsdS (rB-mB-) gal λ(DE3)*	Laboratory collection
S17-1	F⁻*recA hsdR* $RP_{4\text{-}2}$ (Tc::Mu)(Km::Tn7) lysogenized with λpir phage	Laboratory collection
DH5α/pUCP18/pDN19lacΩ-P*pigA*	*E. coli* DH5α containing plasmid pUCP18 and pDN19lacΩ-P*pigA*	This study
DH5α/pXW1903/pDN19lacΩ-P*pigA*	*E. coli* DH5α containing plasmid pXW1903 and pDN19lacΩ-P*pigA*	This study
DH5α/pUCP18/pDN19lacΩ-$P_{SMWW4\_v1c29280}$	*E. coli* DH5α containing plasmid pUCP18 and pDN19lacΩ-$P_{SMWW4\_v1c29280}$	This study
DH5α/pXW1903/pDN19lacΩ-$P_{SMWW4\_v1c29280}$	*E. coli* DH5α containing plasmid pXW1903 and pDN19lacΩ-$P_{SMWW4\_v1c29280}$	This study
***S. marcescen*s strains**		
JNB5-1	*S. marcescens* wild type strain	(10)
SK8-37	*psrA*::Gm^R mutant of JNB5-1, prodigiosin producing mutant	This study
SK8-37/pXW1903	Mutant SK8-37 containing plasmid pXW1903	This study
SK8-37/pUCP18	Mutant SK8-37 containing empty vector pUCP18	This study
SK8-37/pXW2006	Mutant SK8-37 containing plasmid pXW2006	This study
ΔPsrA	*psrA* deleted mutant of *S. marcescens* JNB5-1	This study
ΔPsrA/pXW1903	Mutant ΔPsrA containing plasmid pXW1903	This study
ΔPsrA/pUCP18	Mutant ΔPsrA containing empty vector pUCP18	This study
ΔTssE	*tssE* deleted mutant of *S. marcescens* JNB5-1	This study
ΔTssE/pXW2002	Mutant ΔTssE containing plasmid pXW2002	This study
JNB5-1/pDN19lacΩ-P*pigA*	*S. marcescens* JNB5-1 containing plasmid pDN19lacΩ-P*pigA*	This study
SK8-37/pDN19lacΩ-P*pigA*	*S. marcescens* SK8-37 containing plasmid pDN19lacΩ-P*pigA*	This study
Plasmids		
pRK2013Tn5G	Tn5G carrying plasmid, $Km^R Gm^R$	(87)
pMD18T	Cloning vector, 2692 bp, Amp^R, *lacZ*	TaKaRa
pET28a	*E. coli* expression vector, Km^R	Laboratory collection
pET28a-PsrA	A derivative of pET-28a, harboring the *psrA* gene, Km^R	This study
pXW1903	*psrA* gene driven by P*lac* promoter cloned in pUCP18, Ap^R	This study
pUCP18	Broad-host-range shuttle vector, Ap^R	(88)
pXW2002	*tssE* gene driven by P*lac* promoter cloned in pUCP18, Ap^R	This study
pXW2006	*rhs2* gene driven by P*lac* promoter cloned in pUCP18, Ap^R	This study
pDN19lacΩ	Promoterless *lacZ* fusion vector, $Sp^R Sm^R Tc^R$	(89)
pDN19lacΩ-p*pigA*	*pig* operon promoter cloned in pDN19lacΩ, $Sp^R Sm^R Tc^R$	This study
pUTKm	Tn5-based delivery plasmid with $Km^R Amp^R$	(90)

were determined using a NanoDrop spectrophotometer (Thermo Scientific), and 0.5 μg of the total bacterial RNA was subjected to reverse transcription using the HiScript II Q RT SuperMix Kit (Vazyme). 0.4 μg of cDNA was mixed with 4 pmol of forward and reverse primers and mixture was subjected to RT-qPCR analysis using the ChamQ Universal SYBR qPCR master mix kit (Vazyme) in a total reaction volume of 20 μl in a CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). No-reverse transcription controls were carried out for each experiment to ensure that there was no chromosomal DNA contamination in each RNA sample. RNA from three biological replicates were analyzed and three technical replicates were performed. Additional details of RT-qPCR procedures are included in the 'MIQE checklist' (Supplementary Table S6) in compliance with the MIQE guidelines (Minimum Information for Publication of Quantitative real-time PCR Experiments) (21). The 16S rRNA protein-encoding gene was used as an internal control as described previously (22,23). To determine the expression differences of prodigiosin synthesis-related genes in strains JNB5-1 and SK8-37 at incubation time intervals of 2, 4, 6, 8, 10, 12 and 24 h, the standard curves of expression levels of *pigA* and *pigC* genes in *S. marcescens* were determined by the methods described previously (24). Then, the expression levels of *pigA* and *pigC* genes in strains JNB5-1 and SK8-37 at different time intervals were calculated according to the standard curve $Y = -1.724\ln(X) + 58.375$ and $Y = -1.654\ln(X) + 56.802$ (as shown in Supplementary Figure S2), respectively [Y indicates that the RT-qPCR Ct values; X (copy/ml) indicates the expression levels of *pigA* or *pigC* gene in strains JNB5-1 and SK8-37 at different time intervals]. The primers used for RT-qPCR analysis are listed in Supplementary Table S1.

Construction of transcriptional reporters

To confirm the relationship between PsrA and the *pig* operon, the DNA fragment P$pigA_{406}$ containing the pro-

moter region of the *pig* operon from positions -368 to + 38 with respect to the transcriptional start site (TSS) of *pig* operon as reported previously (15) was amplified with the primers PigA-F3 and PigA-R3 listed in Supplementary Table S1. The 406-bp-long PCR product was then cloned into *lacZ*-containing plasmid pDN19lacΩ to obtain the recombinant plasmid pDN19lacΩ-P$pigA_{406}$. The resulting recombinant plasmid pDN19lacΩ-P$pigA_{406}$ was then introduced into strains JNB5-1, SK8-37, DH5α/pUCP18 or DH5α/pXW1903 by electroporation. The expression levels of prodigiosin synthesis-related genes were assessed by measuring β-galactosidase activities of the transformants. To identify the DNA sequences necessary for PsrA binding, a transcriptional fusion reporter gene expression of the *pig* operon was constructed by cloning the DNA fragments P$pigA_{281}$ (DNA fragment containing the regulatory binding site (RBS) and activator binding site (ABS) spanning nucleotide positions −243 to + 38 relative to the TSS of the *pig* operon), P$pigA_{281}$ ($A^{-90}A^{-89}A^{-88}$) (carrying mutation in RBS of positions TGT), P$pigA_{281}$ ($A^{-90}A^{-89}$) (carrying mutation in RBS of positions TG), P$pigA_{281}$ ($G^{-76}G^{-75}G^{-74}$) (carrying mutation in RBS of positions ACA), P$pigA_{281}$ ($G^{-75}G^{-74}$) (carrying mutation in RBS of positions CA), P$pigA_{281}$ ($G^{-44}G^{-43}G^{-42}$) (carrying mutation in ABS of positions ACT), P$pigA_{281}$ ($G^{-44}G^{-43}$) (carrying mutation in ABS of positions AC), P$pigA_{281}$ ($C^{-30}C^{-29}C^{-28}$) (carrying mutation in ABS of positions AGT), P$pigA_{281}$ ($C^{-29}C^{-28}$) (carrying mutation in ABS of positions GT), P$pigA_{281}$ ($T^{-96}T^{-95}T^{-94}$) (carrying mutation of positions CAA), and P$pigA_{281}$ ($G^{-47}G^{-46}G^{-45}$) (carrying mutation of positions ATT) upstream of *lacZ*, respectively, with the primers listed in Supplementary Table S1 in the supplemental material, and β-galactosidase activity in the strain JNB5-1 was determined. To analyze the possible effectors of PsrA, different concentrations of L-proline (0, 1.0, 2.0, and 3.0 mM), 2-Octenal (0, 1.0, 2.0, and 3.0 mM), L-serine (0, 1.0, 2.0 and 3.0 mM) and S-adenosylmethionine (0, 1.0, 2.0 and 3.0 mM) were added to LB medium, respectively, and then the β-galactosidase assays were determined in *S. marcescens* JNB5-1 carrying the P$pigA_{281}$-*lacZ*. To determine whether L-proline has a direct effect on PsrA binding to the *pig* operon and the *SMWW4_v1c29280* gene, different concentrations of L-proline (0, 1.0, 2.0 and 3.0 mM) were added to the LB medium, and then the β-galactosidase assays were determined in *E. coli* DH5α/pUCP18/pDN19lacΩ-P*pigA*, DH5α/pXW1903/pDN19lacΩ-P*pigA*, DH5α/pUCP18/pDN19lacΩ-P$_{SMWW4_v1c29280}$, and DH5α/pXW1903/pDN19lacΩ-P$_{SMWW4_v1c29280}$strains, respectively. The β-galactosidase activity was determined by SDS- and chloroform-permeabilized cells using *o*-nitrophenyl-β-D-galactopyranoside as the substrate, and the results were expressed in Miller units with formula β-galactosidase activity = $[1000 \times (A_{420} - 1.75 \times A_{550})]/(V \times T \times A_{600})$ as described previously (25–28) (A_{420} indicates the absorbance of the yellow *o*-nitrophenol; A_{550} indicates the scatter from cell debris, which, when multiplied by 1.75 approximates the scatter observed at 420 nm; T indicates reaction time in minutes; V indicates volume of culture assayed in milliliters; and A_{600} reflects cell density). The experiments were independently replicated three times and Student's t test was used for statistical analysis.

Production and purification of the PsrA protein

For the production and purification of the PsrA protein, the *psrA* gene was amplified with the primer pairs PsrA-F2 and PsrA-R2 listed in Supplementary Table S1. The PCR product was then cloned into the pET28a plasmid to obtain recombinant plasmid pET28a-PsrA and electroporated into *E. coli* BL21(DE3) to construct recombinant strain *E. coli* BL21(DE3)/pET28-PsrA for overexpression of a His-tagged PsrA. The single colonies of *E. coli* BL21(DE3)/pET28-PsrA were grown overnight at 37°C in LB medium with the addition of 50 μg/ml kanamycin. One milliliter of the overnight culture was then inoculated into 100 ml of LB medium and cultured at 37°C until the cell density reached 0.8 at OD_{600}. PsrA expression was induced at 16°C for 10 h by the addition of 0.5 mM isopropyl-D-1-thiogalactopyranoside (IPTG). The cells were harvested by centrifuging at 6000 × g for 10 min at 4°C and washed twice with phosphate-buffered saline (pH 7.4). Pellets were resuspended in phosphate-buffered saline (pH 7.4) and disrupted by sonication to obtain the soluble intracellular proteins. The cellular lysate was centrifuged at 16 000 × g for 30 min at 4°C to remove bacterial debris, and the supernatant was loaded onto a 1-ml HisTrap HP column on an AKTA purifier system (GE Healthcare, Sweden) with binding buffer A (20 mM Tris–HCl buffer and 500 mM sodium chloride, pH 7.4). Proteins were eluted with buffer B (20 mM Tris–HCl buffer, 500 mM sodium chloride, and 700 mM imidazole, pH 7.4), and the eluted fractions were pooled for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. A Bradford protein assay kit was used to determine protein concentration of PsrA.

To determine the predominant oligomeric state of PsrA protein in solution, the native molecular weight of PsrA protein was determined using a gel filtration column (Superdex 200 10/300 GL; GE Healthcare) as described previously (29). The eluent buffer used for determination of PsrA data was buffer C (20 mM sodium phosphate and 500 mM sodium chloride, pH 7.4), and the flow rate was 0.5 ml/min.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (30). To prove whether PsrA directly binds to the *pig* operon, a 406-bp-long DNA fragments P$pigA_{406}$ containing the promoter region of the *pig* operon from positions −368 to + 38 with respect to the TSS of *pig* operon was amplified with the primers PigA-F3 and PigA-R3 listed in Supplementary Table S1. For the analysis of PsrA binding sites in the promoter region of the *pig* operon, a set of truncated DNA fragments P$pigA_{281}$ (281-bp-long DNA fragment containing the RBS and ABS of PsrA spanning nucleotide positions −243 to + 38 relative to the TSS of the *pig* operon), P$pigA_{134}$ (134-bp-long DNA fragment containing the RBS and ABS of PsrA spanning nucleotide positions −96 to + 38 relative to the TSS of the *pig* operon), P$pigA_{62}$ (62-bp-long DNA fragment without the RBS and ABS of PsrA spanning nucleotide positions −24 to + 38 relative to the TSS of

the *pig* operon), P*pigA*$_{73}$ (73-bp-long DNA fragment containing the RBS and ABS of PsrA spanning nucleotide positions −96 to −24 relative to the TSS of the *pig* operon), P*pigA*$_{170}$ (170-bp-long DNA fragment containing the RBS of PsrA spanning nucleotide positions −243 to −74 relative to the TSS of the *pig* operon), P*pigA*$_{169}$ (169-bp-long DNA fragment without the RBS and ABS of PsrA spanning nucleotide positions −243 to −75 relative to the TSS of the *pig* operon), P*pigA*$_{82}$ (82-bp-long DNA fragment containing the ABS of PsrA spanning nucleotide positions −44 to + 38 relative to the TSS of the *pig* operon), and P*pigA*$_{81}$ (81-bp-long DNA fragment without the RBS and ABS of PsrA spanning nucleotide positions −43 to + 38 relative to the TSS of the *pig* operon) were amplified by the primer pairs listed in Supplementary Table S1 in the supplemental material. To verify the PsrA-binding sites in the genome of *S. marcescens* WW4 (NC_020211.1), different DNA fragments containing the promoter regions of the *cytR*, *SMWW4_v1c21000*, *phoH*, *SMWW4_v1c48120*, *hemN*, *btuB*, *SMWW4_v1c09530*, *cueR*, *flhDC*, *swrW* and *SMWW4_v1c29280* genes were amplified by PCR method with the primers listed in Supplementary Table S1. Then, the purified DNA fragments were incubated with serial dilutions of the PsrA proteins in 20-μl reaction mixtures containing 10-μl EMSA binding buffer (40 mM Tris–HCl, 4 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, 2 mM DTT, 0.2 mg/ml BSA and 1 mM EDTA) at room temperature for 30 min. Electrophoresis was carried out on the 5% native PAGE gels, and the gels were stained with ethidium bromide to visualize DNA bands.

Isothermal titration calorimetry

ITC experiments were performed on a MicroCal PEAQ-ITC microcalorimeter (Malvern, UK) as described previously (31). For determination of PsrA binding isotherms for the effectors, 30 μM PsrA solution was titrated with 0.3 mM L-proline, L-serine or *S*-adenosylmethionine in 50 mM PBS (pH 7.4). The potential effector (2 μl) was injected 19 times into the 0.2-ml cell with stirring at 350 rpm at 25°C. Both protein and effectors solutions were centrifuged for 5 min at 5000 × g. The binding isotherm was calculated from raw data and fitted to a one-site binding model utilizing MicroCal PEAQ-ITC analysis software. The data were derived from three parallel assays.

Transcriptome sequencing and data analysis

To analyze the effect of PsrA on the cellular processes in *S. marcescens*, three individuals of JNB5-1 and SK8-37 cells were grown to OD_{600} of 5.0 before collecting. One milliliter of the collected cells was quickly frozen in liquid nitrogen, treated with RNAprep pure kit (Tiangen) to extract total bacterial RNA, and delivered to Genewiz (Genewiz, Suzhou, China) in dry ice for transcriptome resequencing analysis. The integrity, concentration and quantity of the bacterial RNA were determined using a NanoDrop spectrophotometer (Thermo Scientific), a Bioanalyzer 2100 system (Agilent) and a 1% agarose gel. 1 μg total RNA with RIN value above 6.5 was used for following library preparation. With Ribo-Zero rRNA Removal Kit (Illumina, San Diego, CA, USA), the total bacterial RNA was subjected to rRNA removing to obtain mRNA. The ribosomal depleted RNA was then fragmented and reverse-transcribed. ProtoScript II Reverse Transcriptase with random primers and Actinomycin D were used to synthesize the first strand cDNA. The second-strand cDNA was synthesized using Second Strand Synthesis Enzyme Mix (includes dACGTP/dUTP). The purified double-stranded cDNA by beads was then treated with End Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of Adaptor-ligated DNA was then performed using beads, and fragments of about 420 bp (with the approximate insert size of 300 bp) were recovered. The dUTP-marked second strand was digested with Uracil-Specific Excision Reagent enzyme. Each sample was then amplified by PCR for 13 cycles using P5 and P7 primers, with both primers carrying sequences, which can anneal with flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using beads, validated using a Qsep100 (Bioptic, Taiwan, China), and quantified by Qubit3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Then sequencing was carried out using a 2 × 150 paired-end (PE) configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. The sequences were processed and analyzed by GENEWIZ (Genewiz, Suzhou, China).

For annotation, the genome of *S. marcescens* WW4 (NC_020211.1) was used as reference. The significant differentially expressed genes were determined between strains SK8-37 and JNB5-1 using the DESeq software, with the standards of *P*-value ≤0.05, and fold change |$\log_2$Ratio| ≥1. KEGG_B_class database was used to classify the genes with significantly differential expression based on their functions using the tool Omicshare (https://www.omicshare.com/tools/Home/Soft/pathwaygsea).

Motility assays

For swimming and swarming motility assays, one microliter of the exponential-phase cells (OD_{600} of 0.6) of JNB5-1, SK8-37, ΔPsrA and SK8-37/pXW1903 strains was spotted onto 0.3% and 0.5% semi-solid LB medium, respectively. After incubation at 30°C for 24 h, diameter of the swimming and swarming zones of these four strains were measured. Experiments were independently replicated three times.

Biofilm formation assay

Biofilm formation assay was performed as described previously (24,32). Briefly, 20 μl exponential culture (OD_{600} = 0.6) was transferred into a 96-well microtiter plate (Corning, NY, USA) containing 200 μl fresh 3-fold diluted LB medium. Then, the cultures in the microplates were removed and gently washed three times with water after incubation at 30°C for 48 h without shaking. The biofilm in the plates was stained with 0.1% crystal violet solution for 15 min at room temperature. 95% ethanol was used to extract the crystal violet and the biofilm amounts were measured by the optical densities at 590 nm wavelength using a

BioTek Epoch2 microplate reader. Experiments were independently replicated three times.

Extracellular polysaccharide quantitation

Extracellular polysaccharide produced by strains JNB5-1, SK8-37, ΔPsrA and SK8-37/pXW1903 was determined by a method described previously with slight modification (8). In brief, bacterial cultures of the indicated strains were incubated in LB medium for 18 h, and then centrifuged at 12 000 rpm for 15 min to harvest the bacterial cells. The pelleted cells were resuspended in PBS solution (30 ml) and 1% Zwittergent 3–14 citric acid solution (100 mM, pH 2.0, 6 ml) and incubated at 50°C for 20 min. The supernatant cells were transferred into 50-ml centrifugal bottles after centrifugation at 12 000 rpm for 30 min, and four volumes of cold ethanol (−20°C) were added to each sample and placed at −20°C overnight. After overnight precipitation, the samples were centrifuged at 14 000 rpm and 4°C for 45 min and the supernatants were discarded. The exopolysaccharides in the sediment of each sample were air-dried in a chemical fume hood, and the exopolysaccharides weighed. Experiments were independently replicated three times.

Haemolytic activity assay

Haemolytic activity assays were performed as previously described with slight modification (33,34). Briefly, 5 μl exponential culture (OD_{600} = 0.6) of strains JNB5-1, SK8-37, ΔPsrA and SK8-37/pXW1903 were plated on blood agar and the hemolysis zones visualized. Experiments were independently replicated three times.

Antibacterial competition assay

Antibacterial competition assays were performed as described previously (35,36). Briefly, the attacker strain *S. marcescens* and the target strain *E. coli* MC4100 or *E. cloacae* ATCC13047 were grown overnight, normalized to an OD_{600} of 0.5, and mixed at a ratio of attacker/target of 5:1 (the control mixture contained a 5:1 ratio of sterile LB to target). 25 μl of this mixture was then spotted onto a pre-warmed agar plate and incubated for 4 h at 37°C (for *E. coli* used as the target strain) or 30°C (for *E. cloacae* used as the target strain). After cells were recovered from the spot and resuspended in 1 ml LB broth, the surviving target cells were enumerated by serial dilution (10-fold) and viable counts on streptomycin-supplemented media. The recovery of viable cells is reported as the total number recovered per coculture spot. Control group consisted of sterile LB mixed with target bacteria at a ratio of 5:1. Experiments were independently replicated four times.

Identification of PsrA-binding sites

PsrA-binding sites were identified by searching DNA motif in the genome sequence of *S. marcescens* WW4 (https://www.ncbi.nlm.nih.gov/nuccore/NC_020211.1). The palindromic nucleotide sequences 5′-TGTN$_{11}$ACA-3′ (RBS) and 5′-ACTN$_{11}$AGT-3′ (ABS) were used as query for sequence similarity searching against the genome of WW4 (NC_020211.1) using an online tool (http://meme-suite.org/tools/mast).

Phylogenetic tree construction of *psrA*-like genes and PsrA-like proteins

The homologs of *psrA* gene in *S. marcescens* were analyzed by searching database Nucleotide collection (nr/nt) using Megablast. PSI-BLAST in NCBI was used to search non-redundant protein sequence (nr) database and analyze the distribution of PsrA homologues in other bacteria. The phylogenetic trees of the *psrA*-like genes and PsrA-like proteins were generated using BLAST pairwise alignments in NCBI and online software iTOL (http://itol.embl.de/) was used to modify the phylogenetic trees.

Statistical analysis

Student's test or one-way ANOVA was used for comparing statistical difference between the groups of experimental data. Experiments were independently replicated at least three times.

RESULTS

Identification of a prodigiosin synthesis activator PsrA

A Tn5G transposon insertion library using *E. coli*/pRK2013 Tn5G as the donor strain and *S. marcescens* JNB5-1 as the recipient strain was constructed to identify genes that positively regulate prodigiosin production in strain JNB5-1, and a mutant SK8-37 with severely reduced prodigiosin synthesis was isolated (Figure 1A). Shake flask fermentation analysis showed that the mutant SK8-37 could synthesize 2.72 mg/L of prodigiosin after 24 h of fermentation, which was only 0.05-times that of the wild-type strain JNB5-1 (50.42 mg/l, $P < 0.001$, Figure 1B). Furthermore, the ability for single-cell synthesis of prodigiosin in the mutant SK8-37 was significantly weaker compared to strain JNB5-1 ($P < 0.001$, Figure 1C).

Inverse PCR and sequencing identified that the transposon Tn5G was inserted between 153 bp and 154 bp in the coding region of the *BVG90_12635* gene, which encoded a predicted regulator of 299 amino acids in the mutant SK8-37 (Figure 1D). Further bioinformatics analysis showed that the regulator BVG90_12635 contained a helix-turn-helix (HTH) DNA binding domain ranging from 7 aa to 66 aa and an effector binding domain ranging from 95 aa to 295 aa (Figure 1D). In the complementation assay, introduction of the intact *BVG90_12635* gene into the mutant SK8-37 restored its ability to synthesize prodigiosin (Figure 1B and C). These results suggest that the regulator encoded by the *BVG90_12635* gene might function as a positive prodigiosin synthesis regulator A (PsrA). To further confirm the function of the *psrA* gene, a mutant strain (ΔPsrA) completely deleted of the *psrA* gene was generated, and prodigiosin synthesis of this strain was analyzed. As shown in Figure 1B and C, compared with the strain JNB5-1, the ability of the *psrA* deleted mutant ΔPsrA to synthesize prodigiosin was significantly decreased, demonstrating that PsrA positively regulates prodigiosin synthesis in strain JNB5-1. Taken together, these results suggest that PsrA probably is a prodigiosin synthesis activator in strain JNB5-1.

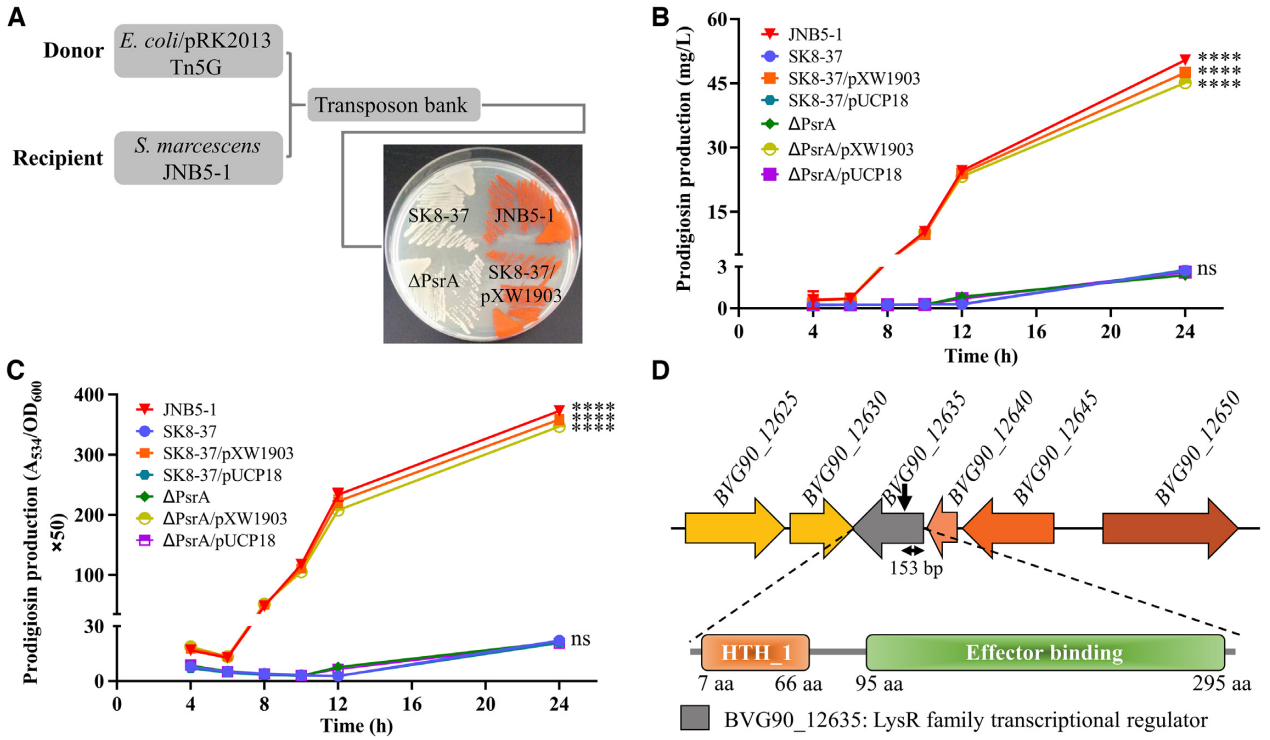

Figure 1. Regulator PsrA positively regulates prodigiosin production in *S. marcescens*. (**A**) A prodigiosin producing mutant SK8-37 was identified by Tn5G transposon insertion mutation. (**B**) Prodigiosin production analysis of strains JNB5-1, SK8-37, SK8-37/pXW1903, SK8-37/pUCP18, ΔPsrA, ΔPsrA/pXW1903 and SK8-37/pUCP18. JNB5-1 is a wild-type *S. marcescens*, SK8-37 is a *psrA* disrupted mutant, ΔPsrA is a *psrA* deleted mutant, SK8-37/pXW1903 and ΔPsrA/pXW1903 are *psrA* complemented strains, and SK8-37/pUCP18 and ΔPsrA/pUCP18 are recombinant strains with empty vector pUCP18. (**C**) Analysis of prodigiosin levels in JNB5-1, SK8-37, SK8-37/pXW1903, SK8-37/pUCP18, ΔPsrA, ΔPsrA/pXW1903, and SK8-37/pUCP18 strains. (**D**) The genetic loci identified in mutant SK8-37. The upper panel indicates the genetic map of the disrupted gene *BVG90_12635* (*psrA*) and its surrounding genes. Tn5G insertion site is denoted by black arrow points. The middle panel represents the domain organization of the BVG90_12635 protein. The lower panel indicates that BVG90_12635 is a LysR family transcriptional regulator. For B and C, the experiments were independently replicated three times. Error bars indicate standard deviations. One-way analysis of variance (ANOVA) was used to examine the mean differences between the data groups. ****$P < 0.001$; ns, no significance difference.

PsrA controls prodigiosin production *via* direct transcriptional regulation of the *pigA-pigN* operon

To investigate how PsrA activates prodigiosin production in *S. marcescens*, the cell growth of strains JNB5-1 (wild-type strain), SK8-37 (*psrA* disrupted mutant), and SK8-37/pXW1903 (complemented strain) were determined, and results showed that there was no significant difference in cell growth between these three strains (Figure 2A). This result suggests that PsrA activation of prodigiosin production in *S. marcescens* was probably not related to biomass and the significantly decreased production of prodigiosin in mutant SK8-37 was possibly due to the lower expression levels of the prodigiosin related *pig* gene cluster.

The enzymes for prodigiosin production in *S. marcescens* are encoded by genes *pigABCDEFGHIJKLMN*, a total of 14 genes (Figure 2B, and Supplementary Figure S1). Among them, *pigB*, *pigD* and *pigE* genes use 2-octenal as the substrate to synthesize 2-methyl-3-n-amyl-pyrrole (MAP), while *pigA*, *pigF*, *pigG*, *pigH*, *pigI*, *pigJ*, *pigM* and *pigN* genes use the L-proline as the substrate to synthesize 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC). Finally, the *pigC* gene encodes terminal condensing enzyme that condenses both MAP and MBC to prodigiosin (Supplementary Figure S1) (1). Hence, to reveal how PsrA regulates prodigiosin synthesis in *S. marcescens*, Real-time quantitative PCR (RT-qPCR) was carried out to analyze the expression levels of *pigA* and *pigC* genes in strains JNB5-1 and SK8-37 at incubation time intervals of 2, 4, 6, 8, 10, 12 and 24 h. These two genes displayed significantly lower expression levels in the seven incubation time intervals in the *psrA* disrupted strain SK8-37 than in the parent strain JNB5-1 (Figure 2B, and Supplementary Figure S3). Further, RT-qPCR was used to analyze the expression levels of *pigA-BCDEFGHIJKLMN* genes in strains JNB5-1 and SK8-37 at the stationary phase (OD_{600} of 6.0) at which prodigiosin began to be produced in large quantities. As shown in Figure 2C, the expression levels of these 14 genes were down-regulated by 14.69-fold to 51.18-fold in mutant SK8-37, suggesting that PsrA positively regulates prodigiosin synthesis related genes in *S. marcescens*. This conclusion was further verified by a transcriptional *lacZ* reporter fusion by cloning the 406-bp-long promoter region of the *pig* operon upstream of the *lacZ* gene, and the expression of the *pig* gene cluster was determined by measurement of cellular β-galactosidase activity. Results showed that the transcription of the *pig* operon exhibited significantly lower expression levels in the absence of PsrA with the P values lower than 0.005 (Figure 2D, $P < 0.005$).

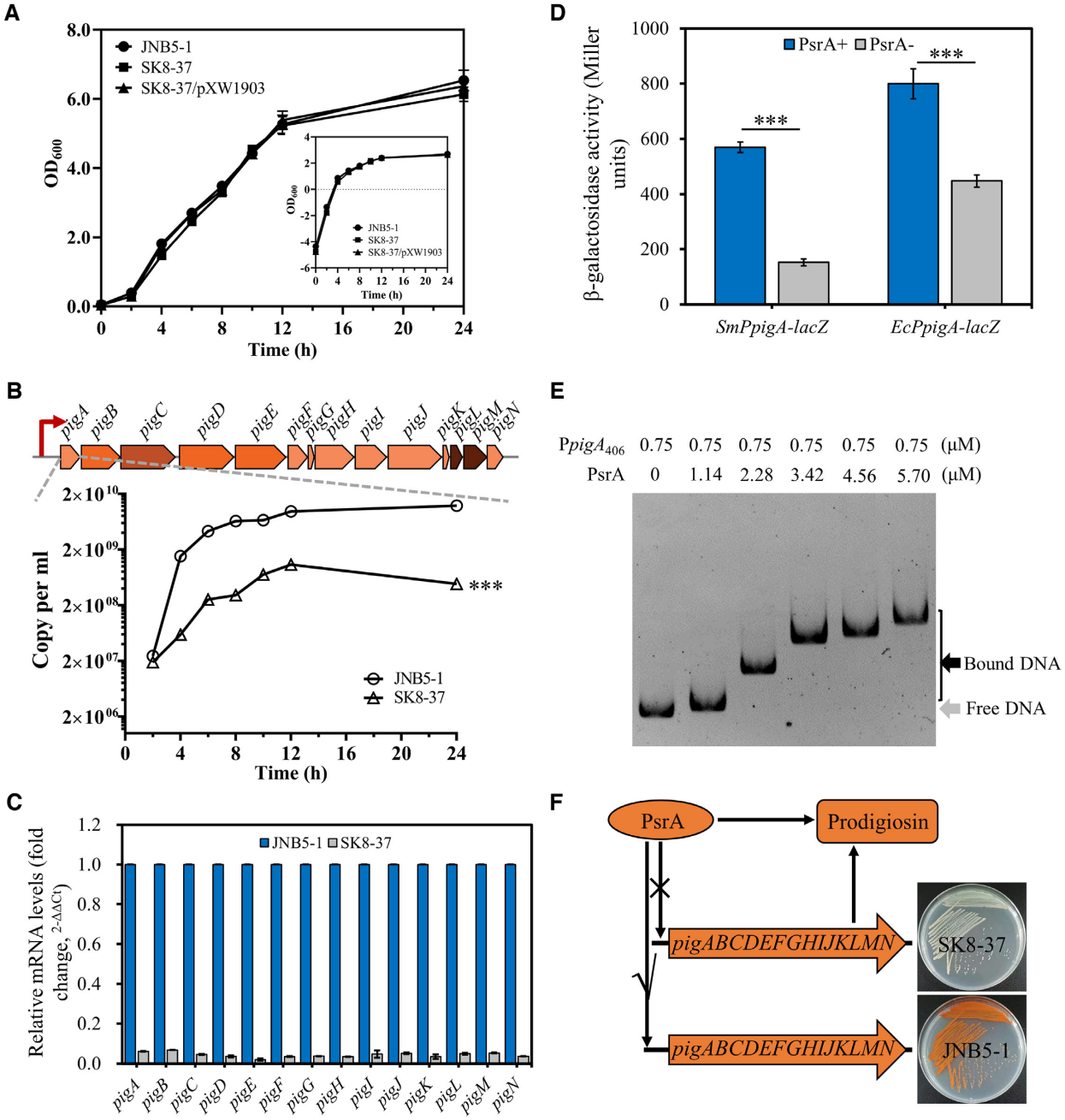

Figure 2. PsrA regulates the prodigiosin synthesis genes directly. (**A**) Growth curves of JNB5-1, SK8-37, and SK8-37/pXW1903 strains. (**B**) RT-qPCR analysis of the expression level of the *pigA* gene in the strains SK8-37 and JNB5-1 at time intervals of 2, 4, 6, 8, 10, 12 and 24 h. (**C**) RT-qPCR analysis of the expression level of *pigABCDEFGHIJKLMN* genes in the strains SK8-37 and JNB5-1 at an OD_{600} of 6.0. (**D**) Analysis of β-galactosidase activity of strains JNB5-1 (PsrA+), SK68 (PsrA-), DH5α/pXW1903 (PsrA+) and DH5α/pUCP18 (PsrA-) harboring the P*pigA-lacZ* reporter fusion at an OD_{600} of 5.0. (**E**) EMSA for PsrA protein binding to the promoter region of the *pig* operon. (**F**) The regulatory network of PsrA controls prodigiosin synthesis in *S. marcescens*. For A–D, the experiments were independently replicated three times. Error bars indicated standard deviations. Student's t test was used to examine the mean differences between the data groups. ***$P < 0.005$.

To further explore whether PsrA directly controls the expression of the *pig* gene cluster, then influence prodigiosin production in strain JNB5-1, heterogeneous expression regulation was investigated in *E. coli*. Results showed that the transcription fusion P*pigA-lacZ* showed significantly higher expression level in the presence of PsrA, suggesting that PsrA probably directly controls the expression of the *pigA-BCDEFGHIJKLMN* genes (Figure 2D, $P < 0.005$). This data was consistent with the result of EMSA test that PsrA could directly bind to the promoter region of *pig* operon, a 406-bp-long DNA fragment (P$pigA_{406}$) between positions -368 and + 38 relative to the TSS of *pig* operon, which was amplified using primers PigA-F3 and PigA-R3 (Figure 2E). Taken together, our study supports model that PsrA prob-

ably controls prodigiosin production *via* direct transcriptional regulation of the *pigA-pigN* operon (Figure 2F).

Identification of two regulatory elements for PsrA binding in the promoter region of *pig* operon

To further analyze PsrA binding sites in the promoter region of the *pig* operon, the purified His-tagged PsrA protein was incubated with a set of truncated DNA fragments P$pigA_{406}$, P$pigA_{281}$ (281-bp-long DNA fragment located between positions −243 and + 38 relative to the TSS of the *pig* operon), P$pigA_{134}$ (134-bp-long DNA fragment located between positions −96 and + 38 relative to the TSS of the *pig* operon), P$pigA_{62}$ (62-bp-long DNA fragment located between positions -24 and + 38 relative to the TSS of the *pig* operon), and P$pigA_{73}$ (73-bp-long DNA fragment located between positions −96 and −24 relative to the TSS of the *pig* operon) and subjected to EMSA experiments (Figure 3A). The gel mobility shift patterns showed that PsrA could bind to the DNA fragments P$pigA_{406}$, P$pigA_{281}$, P$pigA_{134}$, and P$pigA_{73}$, but could not bind to the DNA fragment P$pigA_{62}$ (Figures 2E and 3A and Supplementary Figure S4). These results narrowed down the PsrA binding sites between positions −96 and −24 relative to the TSS of the *pig* operon.

Usually, the interaction of LysR-type transcriptional regulators (LTTRs) with their target genes occurs at two sites of the promoter regions (37–40). First, the regulatory binding site (RBS) contains the LTTR consensus binding motif (5′-T-N_{11}-A-3′), and the second, the activator binding site (ABS) usually overlaps the -35 box of the target gene. Analysis of the sequences of DNA fragment P$pigA_{73}$ showed that there was a potential RBS of PsrA with sequences 5′-TGTAATAATAGACCACA-3′ (located between positions −90 and −74 relative to the TSS of the *pig* operon) and a potential ABS of PsrA with sequences 5′-ACTTTGCCTGTTTGAGT-3′ (located between positions −44 and −28 relative to the TSS of the *pig* operon) in this DNA fragment (Figure 3B). To further verify the relationship between PsrA and the two potential binding sites, purified PsrA protein was incubated with DNA fragments P$pigA_{170}$ (170-bp-long DNA fragment located between positions −243 and −74 relative to the TSS of the *pig* operon), P$pigA_{169}$ (169-bp-long DNA fragment located between positions −243 and −75 relative to the TSS of the *pig* operon), P$pigA_{82}$ (82-bp-long DNA fragment located between positions −44 and +38 relative to the TSS of the *pig* operon), and P$pigA_{81}$ (81-bp-long DNA fragment located between positions −43 and +38 relative to the TSS the *pig* operon) and subjected to EMSA experiment. The gel mobility shift patterns showed that PsrA could bind to the DNA fragments P$pigA_{170}$, and P$pigA_{82}$, but could not bind to the DNA fragments P$pigA_{169}$, and P$pigA_{81}$ (Figure 3A and Supplementary Figure S5). These results suggest that the binding site between positions −90 and −74 upstream of the TSS of *pig* operon was probably an RBS of PsrA, and the binding site between positions −44 and −28 upstream of the TSS of *pig* operon was probably an ABS of PsrA (Figure 3B).

Next, to analyze *in vivo* the *S. marcescens pigABCDEFGHIJKLMN* PsrA binding sites, transcriptional reporter gene fusion of the *pig* operon was constructed by cloning the DNA fragment P$pigA_{281}$ (DNA fragment containing the binding sites RBS and ABS spanning nucleotide positions −243 to +38 relative to the TSS of the *pig* operon), P$pigA_{281}$ ($A^{-90}A^{-89}A^{-88}$) (carrying mutation in RBS of positions TGT), P$pigA_{281}$ ($A^{-90}A^{-89}$) (carrying mutation in RBS of positions TG), P$pigA_{281}$ ($G^{-76}G^{-75}G^{-74}$) (carrying mutation in RBS of positions ACA), P$pigA_{281}$ ($G^{-75}G^{-74}$) (carrying mutation in RBS of positions CA), P$pigA_{281}$ ($G^{-44}G^{-43}G^{-42}$) (carrying mutation in ABS of positions ACT), P$pigA_{281}$ ($G^{-44}G^{-43}$) (carrying mutation in ABS of positions AC), P$pigA_{281}$ ($C^{-30}C^{-29}C^{-28}$) (carrying mutation in ABS of positions AGT), P$pigA_{281}$ ($C^{-29}C^{-28}$) (carrying mutation in ABS of positions GT), P$pigA_{281}$ ($T^{-96}T^{-95}T^{-94}$) (carrying mutation of positions CAA), and P$pigA_{281}$ ($G^{-47}G^{-46}G^{-45}$) (carrying mutation of positions ATT) upstream of *lacZ*, and β-galactosidase activity in strain JNB5-1 was determined (Figure 3C). Results showed that transcription of the *pig* operon was significantly lower for the DNA fragments when the potential RBS and ABS was mutated, whereas the mutation of other sites had no significant effect on *pig* operon expression levels (Figure 3C). Together, these results further demonstrate that the sequence located between −90 and −74 bp upstream of the TSS of *pig* operon was probably RBS of PsrA, which contains a palindromic structure (5′-TGTAATAATAGACCACA-3′), and the sequence located between −44 and −28 bp upstream of the TSS of *pig* operon was probably ABS of PsrA, which contains a palindromic structure (5′-ACTTTGCCTGTTTGAGT-3′) (Figure 3B). This result was consistent with the report that LTTRs recognize a DNA fragment of ∼60 bp in the promoter region of target gene (41).

PsrA is tetrameric and L-proline is a potential effector of PsrA

LTTRs typically form a homo-tetramer in solution (42–44). To reveal the predominant oligomeric state of PsrA in solution, the PsrA protein was purified *via* a His tag, and the deduced size of the monomer, 36.87 kDa, was confirmed by SDS-PAGE (Supplementary Figure S6). Further, by analytical gel filtration, the heterologously produced PsrA-His6 was eluted as a single peak corresponding to about 150 kDa (Supplementary Figure S7). These results suggest that PsrA is probably a tetramer in solution.

The binding activity of LTTRs is generally influenced by one or more effectors (29,45–48), and the effectors of the LTTRs is usually the substrate or the intermediate in the metabolism of the products (29,48). To identify the possible effectors of PsrA, different concentrations of L-proline (0, 1.0, 2.0 and 3.0 mM), 2-Octenal (0, 1.0, 2.0 and 3.0 mM), L-serine (0, 1.0, 2.0 and 3.0 mM) and *S*-adenosylmethionine (0, 1.0, 2.0 and 3.0 mM) were added to LB medium, respectively, and the β-galactosidase assays were determined with *S. marcescens* JNB5-1 carrying the P$pigA_{281}$-*lacZ*. Results showed that the expression of promoter activities was significantly increased in strain JNB5-1 harboring P$pigA_{281}$-*lacZ* in the presence of L-proline, while 2-octenal, L-serine and *S*-adenosylmethionine conferred no significant effect on the expression of promoter activities (Supplementary Figure

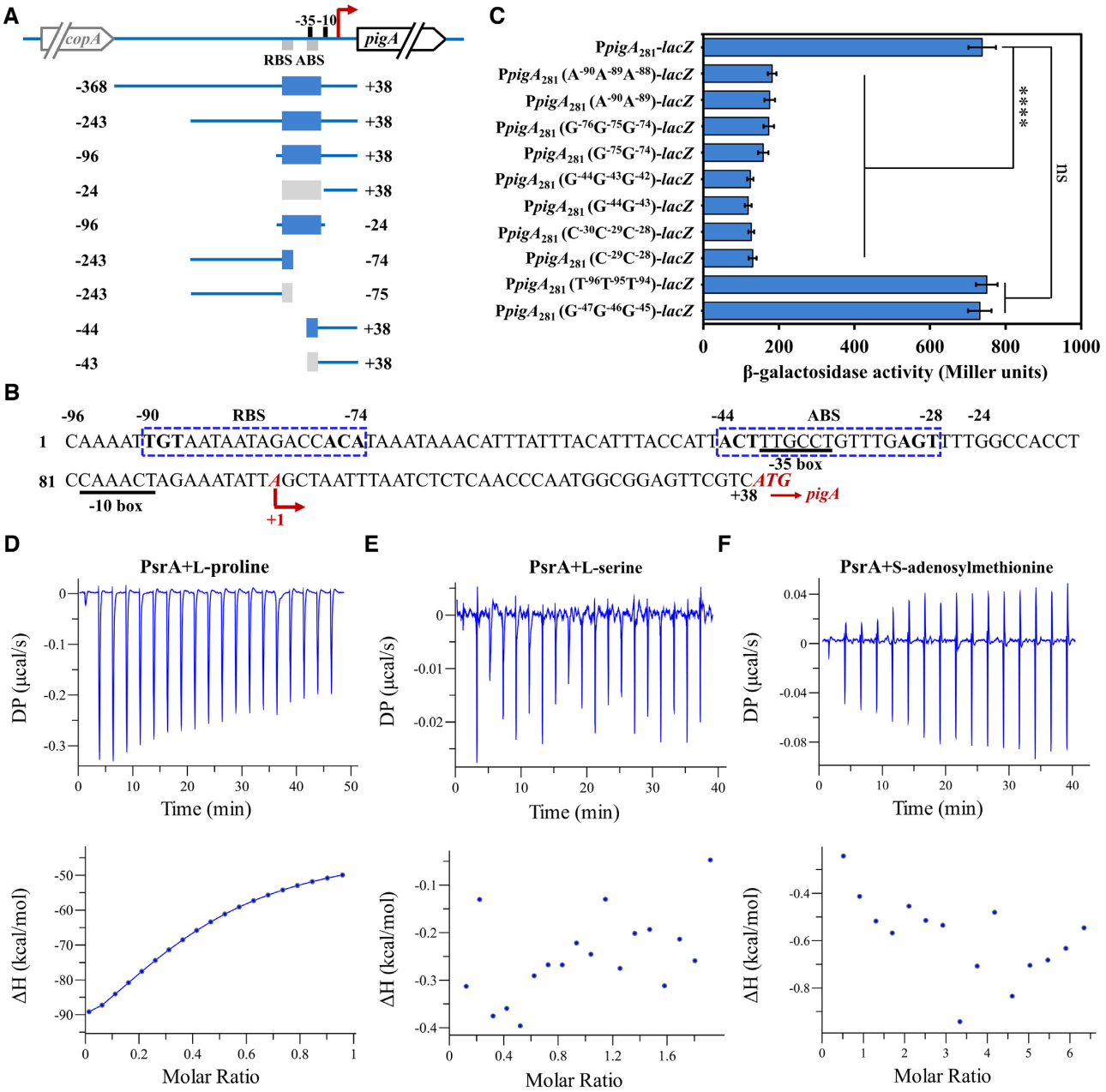

Figure 3. Identification of the binding motif and effector of PsrA. (**A**) Mapping of the PsrA-binding sequences by EMSA assay. DNA fragments with the PsrA-regulatory binding site (RBS) or PsrA-activating binding site (ABS) showed positive bindings (blue color). DNA fragments with the defective PsrA-RBS and PsrA-ABS showed no bindings (gray color). Arabic numerals represent the distances (bp) upstream from the TSS of the *pig* operon. (**B**) The organization of the upstream region of the *pig* operon is shown. The −10 box and −35 box of the *pig* operon are underlined. The start codon ATG and TSS of *pig* operon are in red, italic and bold. TSS is denoted by bent arrows and + 1, and the direction of the arrow indicates the direction of gene transcription. The putative PsrA-RBS with sequences 5′-TGTAATAATAGACCACA-3′ and putative PsrA-ABS with sequences 5′-ACTTTGCCTGTTTGAGT-3′ are boxed, and the palindromes are in bold. (**C**) In *vivo* analysis of the PsrA RBS and ABS motifs using site-directed mutagenesis of the promoter region of *pig* operon. The transcription fusions P*pigA*281-*lacZ*, P*pigA*281 ($A^{-90}A^{-89}A^{-88}$)-*lacZ*, P*pigA*281 ($A^{-90}A^{-89}$)-*lacZ*, P*pigA*281 ($G^{-76}G^{-75}G^{-74}$)-*lacZ*, P*pigA*281 ($G^{-75}G^{-74}$)-*lacZ*, P*pigA*281 ($G^{-44}G^{-43}G^{-42}$)-*lacZ*, P*pigA*281 ($G^{-44}G^{-43}$)-*lacZ*, P*pigA*281 ($C^{-30}C^{-29}C^{-28}$)-*lacZ*, P*pigA*281 ($C^{-29}C^{-28}$)-*lacZ*, P*pigA*281 ($T^{-96}T^{-95}T^{-94}$)-*lacZ* and P*pigA*281 ($G^{-47}G^{-46}G^{-45}$)-*lacZ* were used in the *S. marcescens* strain JNB5-1. P*pigA*281: DNA fragment containing PsrA-RBS and ABS. P*pigA*281 ($A^{-90}A^{-89}A^{-88}$): P*pigA*281 carrying mutation in positions TGT. P*pigA*281 ($A^{-90}A^{-89}$): P*pigA*281 carrying mutation in positions TG. P*pigA*281 ($G^{-76}G^{-75}G^{-74}$): P*pigA*281 carrying mutation in positions ACA. P*pigA*281 ($G^{-75}G^{-74}$): P*pigA*281 carrying mutation in positions CA. P*pigA*281 ($G^{-44}G^{-43}G^{-42}$): P*pigA*281 carrying mutation in positions ACT. P*pigA*281 ($G^{-44}G^{-43}$): P*pigA*281 carrying mutation in positions AC. P*pigA*281 ($C^{-30}C^{-29}C^{-28}$): P*pigA*281 carrying mutation in positions AGT. P*pigA*281 ($C^{-29}C^{-28}$): P*pigA*281 carrying mutation in positions GT. P*pigA*281 ($T^{-96}T^{-95}T^{-94}$): P*pigA*281 carrying mutation in positions CAA. P*pigA*281 ($G^{-47}G^{-46}G^{-45}$): P*pigA*281 carrying mutation in positions ATT. (**D**) Direct binding of L-proline to the soluble PsrA protein was measured by isothermal titration microcalorimetry (ITC). L-proline exhibited binding affinity of $K_D = 1.36 \times 10^{-5}$ M to PsrA. (**E** and **F**) ITC analysis revealed that PsrA could not directly binding to the L-serine (E) and S-adenosylmethionine (F). ITC experiments were performed on a MicroCal PEAQ-ITC microcalorimeter. For C, the experiment was independently replicated three times. Error bars indicated standard deviations. Student's *t* test was used to examine the mean differences between the data groups. $^{****}P < 0.001$; ns: no significant difference.

S8). This result indicates that L-proline is a possible effector of PsrA.

To further confirm that the effector of PsrA is the L-proline, the binding of possible effectors with PsrA was tested using isothermal titration calorimetry (ITC). As shown in Figure 3D–F, the ITC assays revealed an active interaction between PsrA and L-proline but not with L-serine and S-adenosylmethionine. Furthermore, to determine whether L-proline affects the DNA binding ability of PsrA, we performed EMSA assay with the promoter region of the *pig* operon. Results showed that supplementation of 0.20 μM L-proline to PsrA protein exhibited an increased promoter binding ability (Supplementary Figure S4A and B). This data was consistent with the result that L-proline could activate transcription of the *pig* operon which was confirmed by determination of β-galactosidase activities derived from the P*pigA-lacZ* reporter gene fusion in DH5α/pXW1903/pDN19lacΩ-P*pigA* strain (Supplementary Figure S4C, $P < 0.01$). Also, the direct effect of L-proline on PsrA binding to the promoter region of the *SMWW4_v1c29280* gene, containing the PsrA-RBS was assessed by EMSAs and β-galactosidase activity assay. Results showed that supplementation of 0.20 μM L-proline to 5.30 μM PsrA protein resulted in undetectable free DNA fragment, whereas in the absence of L-proline, free DNA fragment remained with the same amount of PsrA (Supplementary Figure S9). This result was further supported by the fact that L-proline could repress transcription of the *SMWW4_v1c29280* gene in the DH5α/pXW1903/pDN19lacΩ-$P_{SMWW4\_v1c29280}$ strain carrying the intact *psrA* gene (Supplementary Figure S9, $P < 0.05$). Collectively, these data suggest that L-proline is an effector of PsrA.

Transcriptome analysis reveals PsrA as a pleiotropic regulator

More and more regulators have now been experimentally proved to play important roles in various bacterial metabolic pathways (24,49–52). As a transcription regulator of LysR family encoded in bacteria, PsrA may also play important regulatory role in the cellular metabolism. To analyze the effect of PsrA in the cellular processes in *S. marcescens*, RNAseq analysis was performed to evaluate gene expression differences between wild-type strain JNB5-1 and *psrA* disrupted mutant SK8-37. Comparative transcriptome data showed that the expression levels of 60 genes were significantly upregulated ($\log_2FC \geq 1$, P-value < 0.05), while 558 genes were significantly downregulated ($\log_2FC \leq -1$, P-value < 0.05) when comparing the *psrA* disrupted mutant strain SK8-37 to its parent strain JNB5-1 (Figure 4A, Supplementary Table S2, S3 and S7). According to the annotation of KEGG_B_Class, these 618 significantly upregulated or downregulated genes were classified into 20 major cellular processes, including global and overview maps, membrane transport, and amino acid metabolism (Figure 4B). Further, these 618 genes could be grouped into several major metabolic pathways, such as regulator proteins, flagellar assembly, two-component system, prodigiosin synthesis, biofilm formation, and ribosome proteins (Figure 4C). Altogether, these results suggest that regulator PsrA possibly functions as a key regulator controlling versatile cellular functions in strain JNB5-1.

PsrA positively controls swarming motility

Swimming and swarming are two modes of motilities found in *S. marcescens*, and it is reported that multiple cellular systems were involved in these two motilities, especially the presence of functional flagellar and type IV pili (10,53). Transcriptome analysis showed that the transcription of *flgB*, *flgC*, *flgD*, *flgE*, *flgF*, *flgG*, *flgH*, *flgI*, *flgJ*, *flhA*, *fliA*, *fliE*, *fliF*, *fliG*, *fliH*, *fliI*, *fliJ*, *fliN*, *flip* and *fliR* genes involved in functional flagellar synthesis was significantly decreased in the *psrA* disrupted mutant SK8-37 ($\log_2FC \leq -1$, P-value < 0.05, Figure 5A, Supplementary Table S3 and S7). This data was further confirmed by RT-qPCR analysis, with the *flgB*, *flgD*, *flgJ*, *fliH* and *fliR* genes showed significantly decreased levels of expression in the mutant SK8-37 than that of the parental strain JNB5-1 (Figure 5B), suggesting that PsrA probably positively regulates cell motility in *S. marcescens*.

To further explore the role of PsrA in cell motility in *S. marcescens*, swarming and swimming motility assay was conducted in JNB5-1, SK8-37, ΔPsrA and SK8-37/pXW1903 strains. Results showed that when testing for swimming motility with 0.3% semi-solid agarose plates, no significant difference was observed among JNB5-1, SK8-37, ΔPsrA and SK8-37/pXW1903, suggesting PsrA probably had no effect on bacterial swimming motility (Figure 5C and D). For swarming test, absence of PsrA (strains SK8-37 and ΔPsrA) resulted in a smaller swarming zone compared to parental strain JNB5-1 and complementary strain SK8-37/pXW1903, indicating that PsrA probably positively influences the swarming motility (Figure 5C, and E).

PsrA regulates biofilm formation, extracellular polysaccharide production, and serrawettin W1 biosynthesis

Biofilm formation, extracellular polysaccharide production, and serrawettin W1 biosynthesis are other three important cellular processes in *S. marcescens* and they are regulated by multiple genes. Transcriptome data highlighted that the transcription of biofilm formation-related genes *pgaA*, *pgaB*, *pgaC*, and *fliA*, extracellular polysaccharide production-related genes *SMWW4_v1c28880*, *SMWW4_v1c28890*, *SMWW4_v1c28900*, *SMWW4_v1c28910*, *SMWW4_v1c28920*, *wzc*, *wza*, *wecA*, *SMWW4_v1c32850*, *SMWW4_v1c41530* and *SMWW4_v1c47610*, and serrawettin W1-related gene *swrW* were dramatically downregulated in the *psrA* disrupted mutant SK8-37 ($\log_2FC \leq -1$, P-value < 0.05, Figure 6A, Supplementary Tables S3 and S7). To further verify the transcriptome data, the expression patterns of *pgaA*, *wzc*, *wza*, *wecA* and *swrW* genes in strains JNB5-1 and SK8-37 were validated by RT-qPCR. The transcription levels of these five genes were reduced by 7.22-times to 44.05-times in the mutant SK8-37 compared to the wild-type strain JNB5-1 (Figure 6B). Taken together, these data suggest that PsrA probably plays an important role in biofilm formation, extracellular polysaccharide production, and serrawettin W1 biosynthesis in *S. marcescens*.

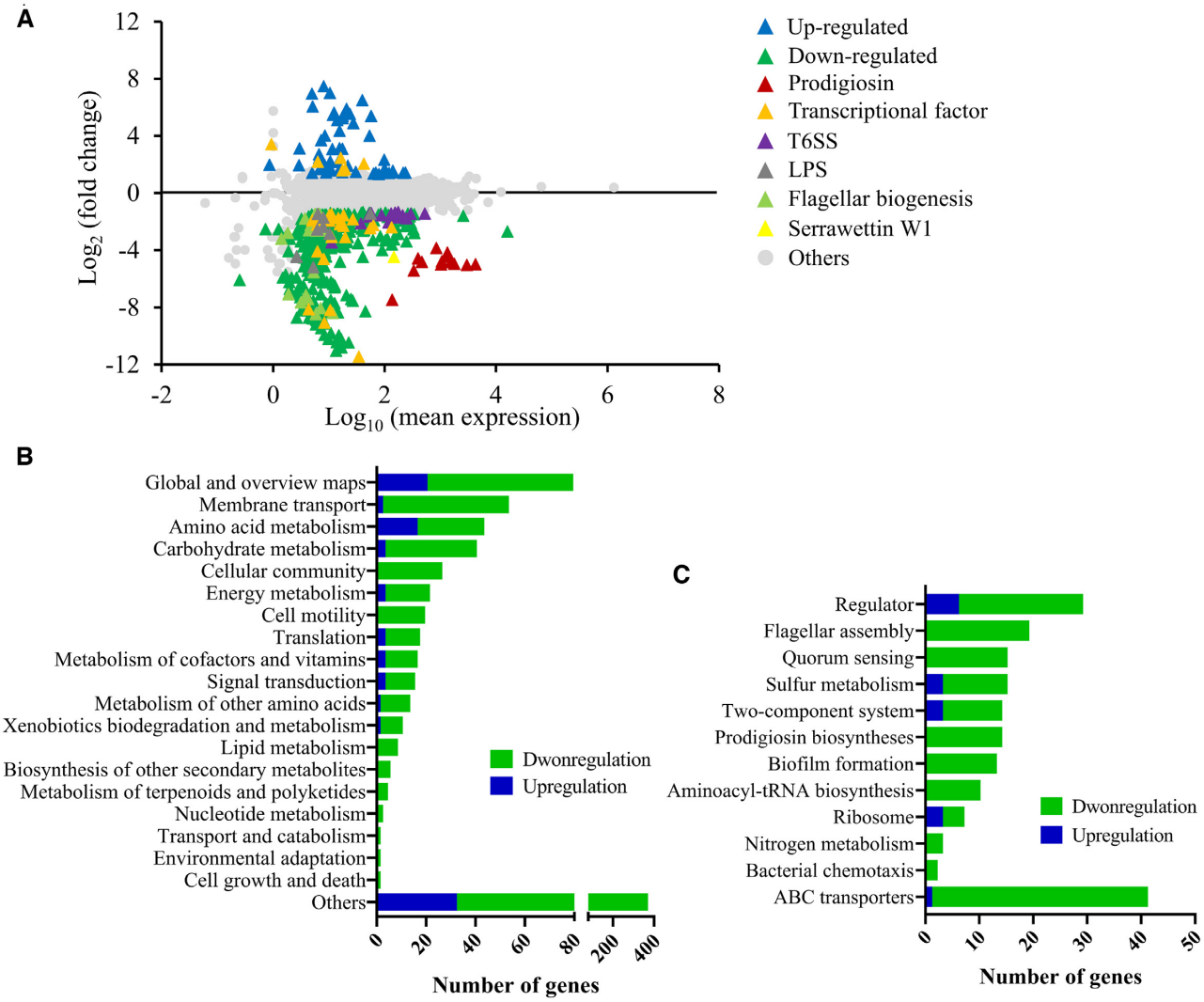

Figure 4. Transcriptome analysis of strains JNB5-1 and *psrA* disrupted mutant SK8-37. (**A**) Genome-wide analysis of gene expression differences between *psrA* disrupted mutant SK8-37 and wild-type strain JNB5-1 at an OD_{600} of 6.0. *X-axis* represents the logarithmic transformation value of gene expression levels in strain JNB5-1. *Y-axis* represents log_2-transformed value of gene expression change folds between strains SK8-37 and JNB5-1. Genes belonging to different pathways are represented by different colored shapes as indicated. Others represent genes not belonging to the indicated pathways. (**B**) Based on the KEGG_B_class, significant differentially expressed genes between strains SK8-37 and JNB5-1 were classified into different cellular processes. (**C**) Expression profiles of the genes belonging to the indicated metabolic pathways.

The effect of PsrA on biofilm formation and extracellular polysaccharide production was evaluated further, and the results indeed showed that the *psrA* mutants SK8-37 (*psrA* disrupted mutant) and ΔPsrA (*psrA* deleted mutant) produced much less biofilm and extracellular polysaccharide than strain JNB5-1 or SK8-37/pXW1903 (Figure 6C and D), indicating that PsrA positively influences the biofilm formation and extracellular polysaccharide production in *S. marcescens*. Hemolysis, an indirect measurement of serrawettin W1 production, was used in serrawettin W1 biosynthesis analysis in strains JNB5-1, SK8-37, ΔPsrA and SK8-37/pXW1903, and results showed that the *psrA* mutants SK8-37 and ΔPsrA were defective in hemolysis (Figure 6E), suggesting that PsrA positively regulates the serrawettin W1 biosynthesis in *S. marcescens*.

PsrA required for T6SS-mediated antibacterial activity

The type VI secretion system (T6SS) is widely distributed in Gram-negative bacteria and bacteria can use them as weapons against prokaryotic or eukaryotic target cells with diverse effectors (54–57). Transcriptome data showed that lots of the T6SS-related genes, including *tssABCEFGHKLM*, *tagF*, *hcp*, *fha*, *pppA*, *tagJ*, *ppkA*, *vgrG2* and *rhs2* genes were significantly downregulated in the *psrA* disrupted mutant SK8-37 compared to wild-type strain JNB5-1 ($log_2FC \leq -1$, P-value < 0.05, Figure 7A, Supplementary Tables S3 and S7). This result was further verified by RT-qPCR analysis, with the expression levels of the *tssE*, *hcp*, *vgrG2*, *tssB* and *tssC* genes reduced by 2.65-times to 14.38-times in the mutant SK8-37 compared to the strain JNB5-1 (Figure 7B). These results suggest that PsrA might affect T6SS-mediated antibacterial activity in strain JNB5-1.

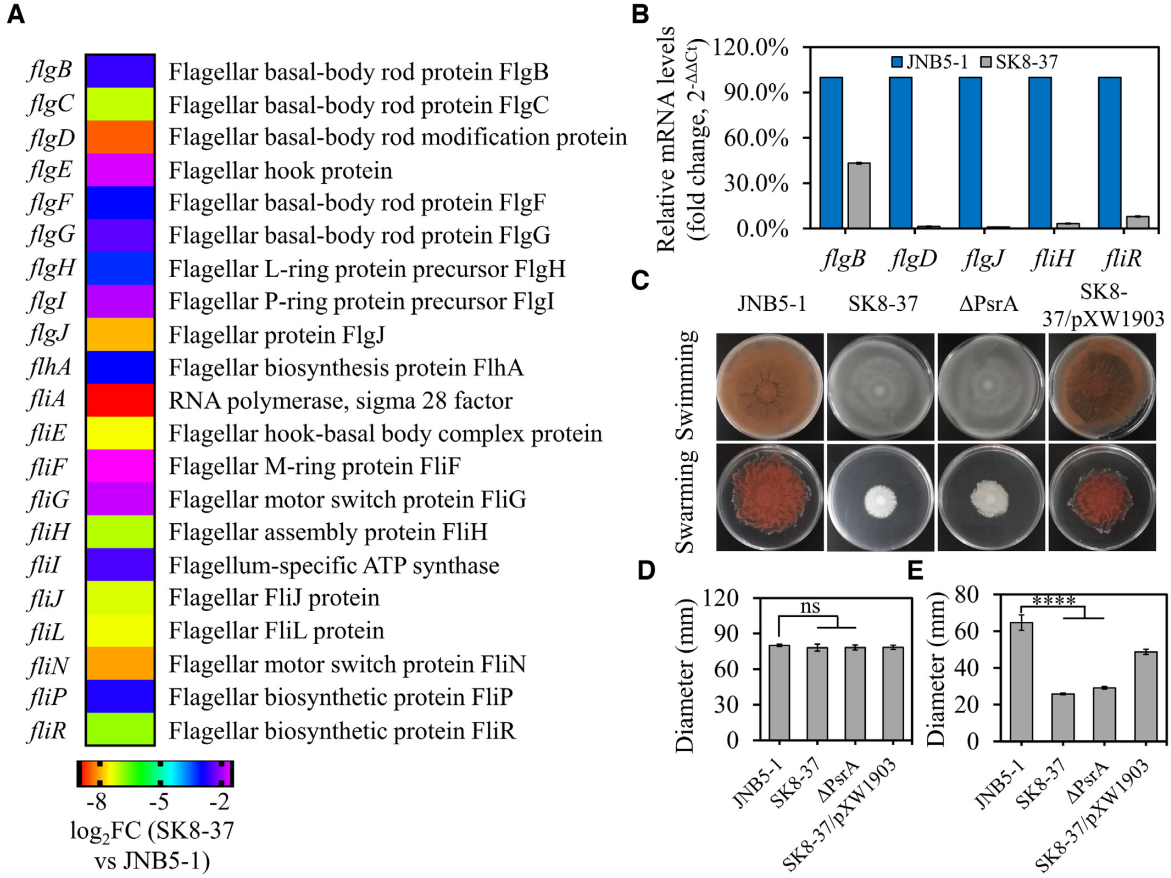

Figure 5. PsrA positively controls flagellar gene expression and swarming motility in *S. marcescens*. (**A**) Comparative transcriptomics data showed that the expression level of flagellum synthesis genes was significantly decreased in *psrA* disrupted mutant SK8-37. (**B**) RT-qPCR analysis of the expression levels of the *flgB*, *flgD*, *flgJ*, *fliH* and *fliR* genes in the strains JNB5-1 and SK8-37. (**C**) Swimming and swarming motility tests of JNB5-1, SK8-37, ΔPsrA and SK8-37/pXW1903 strains. Upper panel: swimming motility assay. Lower panel: swarming motility assay. (**D**) Colony diameter of strains JNB5-1, SK8-37, ΔPsrA and SK8-37/pXW1903 in swimming assay. (**E**) Colony diameter of strains JNB5-1, SK8-37, ΔPsrA and SK8-37/pXW1903 in swarming assay. The experiments were independently replicated three times. Error bars indicated standard deviations (B, D and E). One-way analysis of variance (ANOVA) was used to examine the mean differences between the data groups (D and E). ****$P < 0.001$; ns, no significant difference.

To verify the hypothesis that PsrA affects the T6SS-mediated antibacterial activity in *S. marcescens*, strains JNB5-1, SK8-37, ΔPsrA and ΔTssE (*tssE* deleted mutant) were co-cultured with *E. coli* MC4100 and *E. cloacae* ATCC13047 to test their antibacterial activities. As shown in Figure 7C and D, coculture of *E. coli* and *E. cloacae* with wild-type strain JNB5-1 caused a significant drop in the number of viable cells recovered, and *psrA* mutant SK8-37 and ΔPsrA, and *tssE* mutant ΔTssE showed a modest decrease in antibacterial activity compared to strain JNB5-1 (Figure 7C and D). This killing deficiency was restored to wild-type levels when the *psrA* mutant SK8-37 and *tssE* mutant ΔTssE was complemented by the expression of intact *psrA* gene and *tssE* gene, respectively (Figure 7C and D). These results suggest that PsrA positively regulates the expression levels of the T6SS related genes and hence probably influences the T6SS-mediated antibacterial activity in strain JNB5-1.

The T6SS is a bacterial nanomachine that bacterial pathogens deploy to compete against rival bacterial cells by injecting multiple antibacterial toxins into target cells (55,58,59). Among them, Rhs proteins, rather than cargo effectors, have been confirmed as the primary determinants of intraspecies competition for T6SS-mediated antibacterial activity between *S. marcescens* and closely related organisms (60). Given that several hundred genes have altered expression in the *psrA* mutant (Figure 4), it is a clear possibility that the increment in number of viable cells recovered from co-culture experiments between *psrA* mutants (SK8-37 and ΔPsrA) and its competitor organisms compared to strain JNB5-1 and its competitor organisms is not only due to a reduced antibacterial activity (specifically T6SS activity), but also due to the reduced ability of strains to compete for resources. To determine whether the increment in number of viable cells recovered from co-culture experiments between *psrA* mutants (SK8-37 and ΔPsrA) and its competitor organisms *E. coli* MC4100 and *E. cloacae* ATCC13047 was dependent on T6SS-mediated antibacterial activity, a *rhs2* overexpressed strain SK8-37/pXW2006 and a recombinant strain SK8-37/pUCP18 containing the empty vector pUCP18 were constructed, and viable cells recovered from different co-cultures were determined. As shown in Figure 7C and D, the killing deficiency of SK8-37 was restored to wild-type levels when the *psrA* mutant SK8-37 was comple-

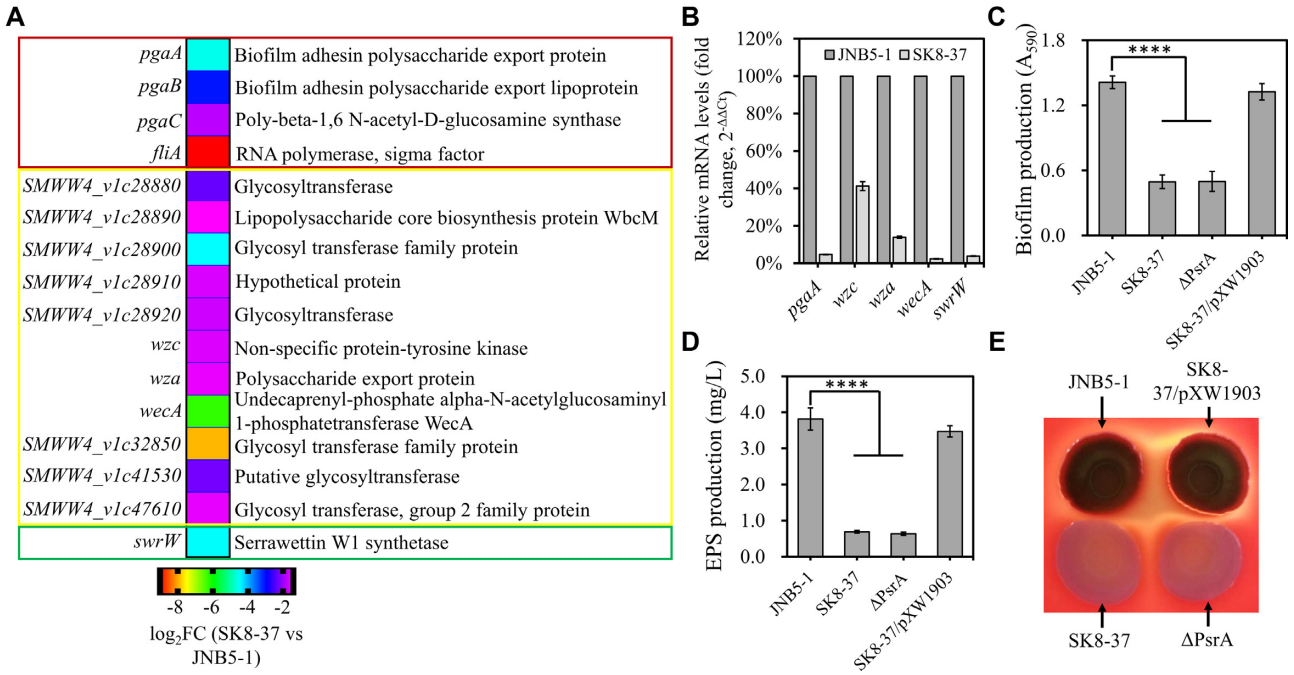

Figure 6. PsrA contributes to extracellular polysaccharide production, biofilm formation, and serrawettin W1 biosynthesis in *S. marcescens*. (**A**) PsrA positively regulates extracellular polysaccharide production-related genes, biofilm formation-related genes, and serrawettin W1 biosynthesis-related genes expression level in strain JNB5-1. (**B**) RT-qPCR analysis of the expression levels of the *pgaA*, *wzc*, *wza*, *wecA*, and *swrW* genes in the strains JNB5-1 and SK8-37. (**C**) PsrA positively regulates biofilm formation in strain JNB5-1. (**D**) PsrA positively regulates extracellular polysaccharide production in strain JNB5-1. (**E**) PsrA positively regulates serrawettin W1 biosynthesis in strain JNB5-1. The experiments were independently replicated three times. Error bars indicated standard deviations (B to D). One-way analysis of variance (ANOVA) was used to examine the mean differences between the data groups (C and D). $****P < 0.001$.

mented by the expression of intact *rhs2* gene, and the killing deficiency of strain SK8-37/pUCP18 was similar to that of *psrA* mutants SK8-37 and ΔPsrA. Collectively, these results suggest that PsrA regulates the T6SS-mediated antibacterial activity in strain JNB5-1, hence controlled the number of viable cells recovered from co-culture experiments between strain JNB5-1 and closely related organisms.

Identification of PsrA-binding sites in bacterial genome

PsrA can bind to RBS and ABS in the promoter of the *pig* operon. The sequence of RBS and its derivative sequences (5′-**TGT**N_{11}**ACA**-3′), and the sequence of ABS and its derivative sequences (5′-**ACT**N_{11}**AGT**-3′) were further searched across the genome sequence of strain WW4 (https://www.ncbi.nlm.nih.gov/nuccore/NC_020211.1) and 235 potential PsrA-RBS and 209 potential PsrA-ABS were identified. Among them, 51 RBS were found in the regions proximal to the promoters of various genes, including six genes encoding regulators (SMWW4_v1c21000, CytR, SMWW4_v1c48120, SMWW4_v1c01230, SMWW4_v1c25790 and SMWW4_v1c09220), 37 genes encoding metabolic enzymes and structural proteins, such as HemN, PhoH, and ManA, and 8 genes encoding putative proteins or hypothetical proteins (Figure 8, and Supplementary Table S4). Also, 29 ABS were found in the regions proximal to the promoters of various genes, including 3 genes encoding regulators (SMWW4_v1c09530, SMWW4_v1c15090 and CueR), and 26 genes encoding metabolic enzymes, structural proteins and hypothetical proteins, such as BtuB, FolA and HutU (Supplementary Figure S10, Supplementary Table S5). Further, to verify the PsrA-RBS binding sites and PsrA-ABS binding sites, the promoter regions of *cytR*, *phoH*, *hemN*, *SMWW4_v1c48120* and *SMWW4_v1c21000* genes containing potential RBS of PsrA, and *btuB*, *SMWW4_v1c09530* and *cueR* genes containing potential ABS of PsrA were amplified and the PCR products were subjected to EMSA test. The promoter region of *flhDC* and *swrW* genes that lack PsrA-RBS and PsrA-ABS were used as negative controls. As the results shown in Supplementary Figure S11 and S12, the sequences that contain the potential PsrA-RBS and PsrA-ABS can all be bound by the PsrA protein. Additionally, the results showed that PsrA could not bind to the promoter regions of *flhDC* and *swrW* genes (Supplementary Figure S11 and S12), which lack PsrA-RBS and PsrA-ABS suggesting that an unknown regulator may exist that controls other PsrA-mediated cellular processes in strain JNB5-1. More experiments are required to clarify the factors influencing the interaction between PsrA and different cellular processes in the future.

PsrA-like proteins are widely distributed among bacteria

As identified in our study, PsrA controls prodigiosin synthesis, swarming motility, biofilm formation, extracellular polysaccharide production, serrawettin W1 biosynthesis, and T6SS-mediated antibacterial activity in *S. marcescens*. Hence, further research on PsrA and similar proteins is warranted if they are widely present in other bacteria.

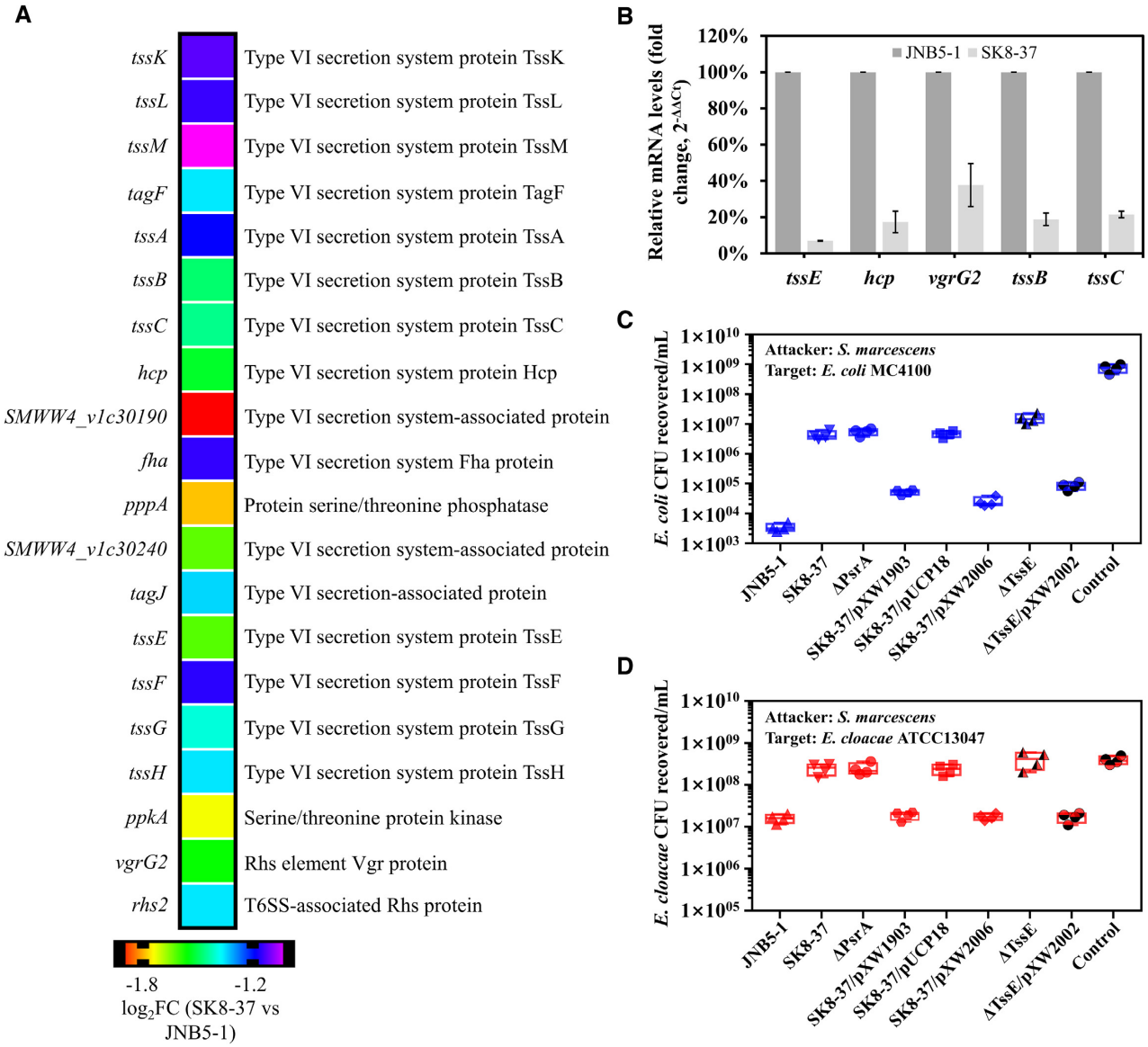

Figure 7. PsrA required for T6SS-mediated antibacterial activity in *S. marcescens*. (**A**) PsrA positively regulates T6SS-related genes expression level in strain JNB5-1. (**B**) RT-qPCR analysis of the expression levels of the *tssE*, *hcp*, *vgrG2*, *tssB* and *tssC* genes in the strains JNB5-1 and SK8-37. (**C**) Recovery of viable *E. coli* MC4100 cells after coculture with the indicated *S. marcescens* strains for 4 h at 37°C, with an initial ratio of 5:1 (attacker/target). (**D**) Recovery of viable *E. cloacae* ATCC13047 cells after coculture with the indicated *S. marcescens* strains for 4 h at 30°C, with an initial ratio of 5:1 (attacker/target). For C and D, the control mixture contained a 5:1 ratio of sterile LB to target. JNB5-1 is a wild-type *S. marcescens*, SK8-37 is a *psrA* disrupted mutant, ΔPsrA is a *psrA* deleted mutant, SK8-37/pXW1903 is a *psrA* complemented strain, SK8-37/pUCP18 is a recombinant strain with empty vector pUCP18, SK8-37/pXW2006 is a recombinant strain with plasmid pXW2006, ΔTssE is a *tssE* deleted mutant, and ΔTssE/pXW2002 is a recombinant strain with plasmid pXW2002. Plasmid pXW1903, pXW2002, and pXW2006 carries intact *psrA*, *tssE* and *rhs2* gene, respectively. The experiments were independently replicated four times. Error bars indicated standard deviations (B–D).

Homologs of *psrA* gene were searched in *S. marcescens*, and found that the *psrA* gene was highly conserved in *S. marcescens* with similarities ranging 92.33–99.56% and the strains containing *psrA* gene were found worldwide (Figure 9A). Also, homologs of PsrA protein were searched with an E-values lower than 1E-121, and result showed that the top 1000 proteins show similarities ranging 59.86–100% and are widely found in the groups of *Serratia* sp., *Klebsiella* sp., *Cronobacter* sp., *Pantoea* sp., *Erwinia* sp., *Pseudomonas* sp. and *Enterobacter* sp. (Figure 9B). The fact that the PsrA-like proteins are highly homologous suggests that PsrA or PsrA-like proteins in *S. marcescens* and other bacteria probably control similar cellular processes as in strain JNB5-1.

DISCUSSION

S. marcescens, a part of the *Enterobacteriaceae* family of eubacteria, is found in a wide range of ecological niches and can produce many high-value secondary metabolites like prodigiosin (1), althiomycin (2), serratamolide (3). Besides the well-studied bacterial genes involved in the metabolic pathway of the secondary metabolites, a number of tran-

Figure 8. Identification of PsrA-RBS in *S. marcescens* genome. The PsrA-RBS binding sequences (5′-TGT-N_{11}-ACA-3′) were searched across the genome sequence of strain WW4 (https://www.ncbi.nlm.nih.gov/nuccore/NC_020211.1). Different colors indicate the $\log_2$-transformed value of expression change folds in mutant SK8-37 compared to strain JNB5-1of the indicated genes.

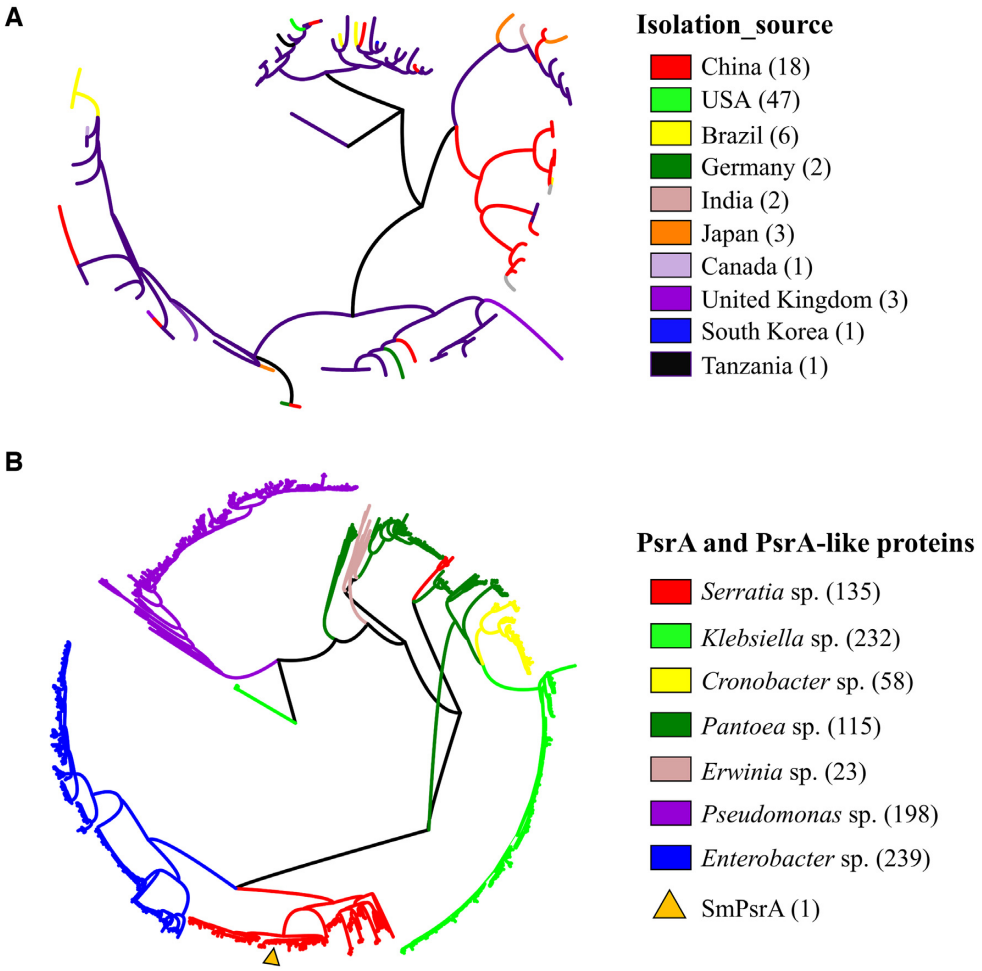

Figure 9. PsrA-like proteins are widely distributed among bacteria. (**A**) Phylogenetic tree of the *psrA*-like genes of *S. marcescens* with similarities ranging from 92.33–99.56%. (**B**) Phylogenetic tree of the top 1000 homologous proteins of PsrA with the E-values lower than 1E−121. Orange triangle stands for the PsrA protein of strain JNB5-1. The scale bar represents the number of substitutions per site. Colored rectangles stand for different species as indicated.

scriptional regulator-encoding genes that play important roles in secondary metabolites synthesis in *S. marcescens* have also been investigated, such as regulator MetR (10), SpnR (11), CopA (12), CRP (13), HexS (14), RssB (15), RcsB (16,17), SmaR (18), EepR (6), PigP (7), GumB (8), RbsR (9), and RpoS (61) mediates prodigiosin production and regulator GumB (8), EepR (6,34), PigP (62) and HexS (7) regulation of serratamolide synthesis. However, our understanding of the regulatory mechanisms behind secondary metabolites synthesis in *S. marcescens* is still limited. In this study, an uncharacterized LysR family regulator PsrA was confirmed to function as a prodigiosin activator for the first time by directly binding to the promoter region of the prodigiosin synthesis related *pig* operon for transcriptional activation (Figures 1 and 2).

Usually, the interaction between LysR-type transcriptional regulators (LTTRs) and their target genes occurs at the regulatory binding site (RBS) and the activator binding site (ABS). The RBS is generally centered at position −65 relative to the TSS of the activated promoter, containing the LTTR consensus binding motif (5′-T-N_{11}-A-3′). The ABS usually overlap the −35 box of the target gene and often possesses no conserved sequence motif (42,43,63). Moreover, active complex formation of a LTTR protein is a complex process, which includes the following steps: (i) perception signal *via* the effector binding domain at the C-terminal of LTTRs, (ii) oligomerization *via* the DNA binding and amino acids involved in dimerization, (iii) binding to the promoter region of the target DNA *via* the helix-turn-helix motif at the N-terminal of LTTRs, (iv) DNA bending during higher ordered complex formation and (v) transcriptional activation of the target gene by the interaction of the formed complex with the RNA polymerase (42). In this study, we demonstrated that PsrA binds to the promoter region of the *pig* operon including both RBS and ABS (Figures 3A–C, Supplementary Figure S4, and S5). The sequences located between −90 and −74 bp upstream of the TSS of *pig* operon was probably RBS of PsrA, which contains a palindromic structure (5′-TGTAATAATAGACCACA-3′). The sequences containing a palindromic structure (5′-ACTTTGCCTGTTTGAGT-3′) located between −44 and −28 bp upstream of the TSS of *pig* operon was probably ABS of PsrA (Figure 3B). Interestingly, unlike the regulator AlsR of *B. subtilis* (26), the

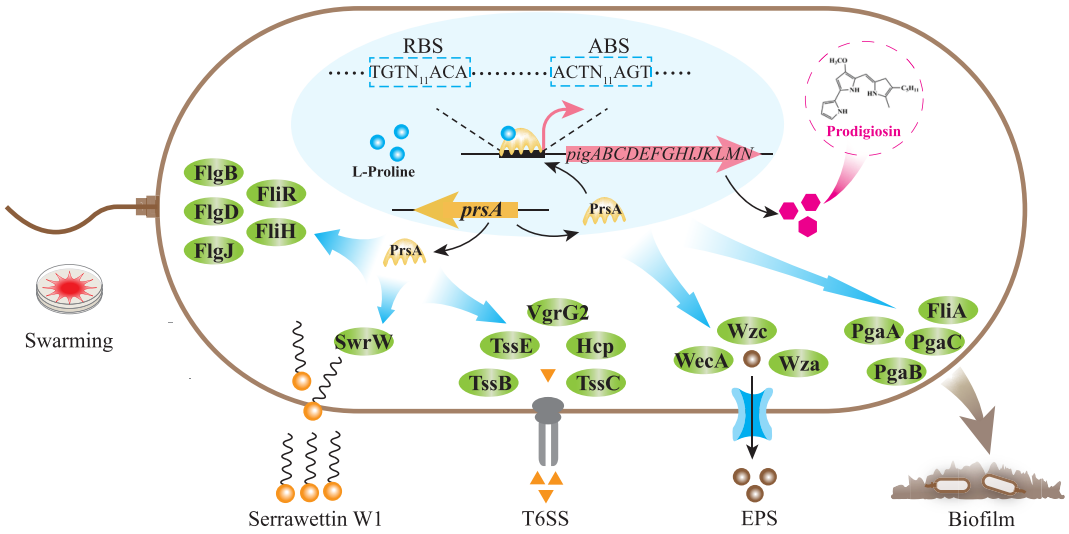

Figure 10. Proposed model of PsrA mediated regulatory pathways in strain JNB5-1. The regulatory pathways include the cellular processes and the genes directly or indirectly influenced by the PsrA regulator. PsrA is an important regulator controls many cellular processes including prodigiosin synthesis, serrawettin W1 biosynthesis, extracellular polysaccharide (EPS) production, biofilm formation, swarming motility and T6SS-mediated antibacterial activity in strain JNB5-1. Black arrows represent direct regulatory relationships between PsrA and the target genes. Blue arrows represent indirect regulatory relationships between PsrA and the target genes. PsrA controls prodigiosin synthesis of strain JNB5-1 by directly binding to the PsrA-RBS and PsrA-ABS in the promoter region of the *pig* operon. L-proline is the effector of PsrA. PsrA controls other cellular processes of strain JNB5-1 by indirectly influence the expression levels of genes involve in different cellular processes. FlgB, FlgD, FlgJ, FliR and FliH are key enzymes in flagellum synthesis and swarming motility. SwrW is a key enzyme in serrawettin W1 biosynthesis. VgrG2, TssE, TssB, Hcp and TssC are key enzymes in T6SS-mediated antibacterial activity. PgaA, PgaB, PgaC and FliA are key enzymes in biofilm formation. Wza, Wzc and WecA are key enzymes in extracellular polysaccharide production.

regulator CysB of *Salmonella enterica* serovar *Typhimurium* (64), and the regulator LigR of *Sphingobium* sp. strain SYK-6 (47) binding to target DNA which resulted in the formation of three retarded complexes, PsrA binding results in the concentration-dependent generation of complexes with different migration velocities (Supplementary Figures S2E and S4). There are several possible explanations for this, such as multiple binding sites, different oligomeric states of PsrA and DNA deformations. More experiments are needed to further understand the underlying mechanisms.

To efficiently activate the transcription of corresponding target genes and hence influence cellular processes in bacterial, the binding activity of LTTRs to its target gene is generally influenced by one or more effectors. For example, acetate is the effector of AlsR and activates the transcription of the *alsSD* operon for acetoin formation in *Bacillus subtilis* (26,38), glutarate is the effector of GcdR and controls glutarate catabolism in *Pseudomonas putida* KT2440 (29), hydroxyhydroquinone or possibly 2,3,5-trihydroxybenzoate is the effector of DbdR and influences expression of the entire 3,5-DHB anaerobic degradation pathway in *Thauera aromatica* (48), benzoate and cis,cis-muconate are the effectors of BenM and control aromatic compound degradation in *Acinetobacter* sp. ADP1 (45), 1-Naphthol is the effector of McbG to activate *mcbBCDEF* cluster transcription and involved in the upstream pathway of carbaryl degradation in *Pseudomonas* sp. XWY-1 (65), and shikimate, the effector of QuiR induces transcriptional activation of protocatechuate biosynthesis genes and modulates the conversion of quinate and shikimate metabolites to protocatechuate (66). In this study, through β-galactosidase activity analysis, isothermal titration calorimetry (ITC), and EMSAs, we found that L-proline is the effector of PsrA (Figure 3D, Supplementary Figures S4A–C, S8 and S9). Interaction with L-proline molecules increases the binding of PsrA to its target DNA, resulting in upregulation of the expression of the prodigiosin synthesis related *pigABCDEFGHIJKLMN* genes in strain JNB5-1 (Supplementary Figure S8).

LTTRs are the large family of transcriptional regulators in bacteria, and a number of this family regulators are involved in the control of a series of core cellular processes in different microorganisms, such as the synthesis of virulence factors, the control of the central carbon catabolism, the influence of expression of antibiotic resistance, and the induction of aromatic compound metabolization (42,43,63,67–69). In this study, using transcriptomics and further experiments, PsrA, was demonstrated to be a global regulator, which influences prodigiosin synthesis (Figure 1A–C), swarming motility (Figure 5C and E), biofilm formation (Figure 6C), extracellular polysaccharide production (Figure 6D), serrawettin W1 biosynthesis (Figure 6E), and T6SS-mediated antibacterial activity (Figure 7) in *S. marcescens* for the first time. Additionally, the results showed that there were no PsrA-binding sites in the promoter region of these cellular processes control genes (Figure 8, and Supplementary Figure S10, and Supplementary Tables S4 and S5), and PsrA could not bind to the promoter regions of *flhDC* and *swrW* genes (Supplementary Figure S11 and S12) suggesting that PsrA may indirectly controls the cellular processes of swarming motility, extracellular polysaccharide production, biofilm formation, serrawettin W1 biosynthesis, and T6SS-mediated antibacterial activity in strain JNB5-1 (Figure 10).

The presence of functional flagellar contributes to swarming motility in bacteria. Using transcriptomics and RT-qPCR analysis, we found that PsrA positively regulated the transcription of flagellar synthesis *flgBCDEFGHIJ*,

flhA and *fliAEFGHIJNPR* genes in strain JNB5-1 (Figure 5A and B). Additionally, in this work, data showed that PsrA positively regulates swarming motility in strain JNB5-1 (Figure 5C and E). Taken together, this work shows that PsrA plays an important role in swarming motility in *S. marcescens*, probably by controlling the transcription of flagellar synthesis-related genes (Figure 10).

A variety of transcription regulators have been identified playing important roles in biofilm synthesis in different microorganisms, such as the transcription regulator SrpA (24), BsrA (30), PyeR (70), AmzR (71), BswR (72) and FleQ (73) in *Pseudomonas aeruginosa*, the regulator RydC (74) and LrhA (75) in *E. coli*, the regulator SinR (76) in *B. subtilis*, the regulator CytR (77) in *Vibrio cholerae*, and the regulator Fur (78) in *Yersinia pestis*. In *S. marcescens*, a few regulators have been reported to be involved in biofilm formation, including positive regulators MetR (10), OxyR (79) and RpoS (61), and negative regulators GumB (8) and CpxR (80). In this study, PsrA was identified as a novel regulator which positively regulates biofilm formation in *S. marcescens*. When *psrA* was disrupted, the ability of *S. marcescens* to synthesize biofilm was significantly decreased (Figure 6D). Transcriptomics, RT-qPCR analysis and bioinformatics analysis showed that the way PsrA affected the formation of biofilm was likely by indirectly regulating the expression of the biofilm formation related genes (Figures 6A, B and 10).

Type VI secretion systems (T6SSs) are nanomachines widely used by Gram-negative bacteria to deliver toxic effector proteins into neighbouring cells and provide a competitive advantage in mixed bacterial populations. Numbers of transcription regulators have been identified to play important roles in T6SS-meditated antibacterial activity in different bacteria, such as the regulators SrpA (24), SuhB (52), CueR (81), AnvM (82) and AmrZ (83) in *P. aeruginosa*, the regulators BaeSR (84) and HpaR (85) in *E. coli*, and the regulator QstR (86) in *V. cholerae*. In *S. marcescens*, only regulator RcsB (36) has been identified to be important for T6SS-mediated antibacterial activity. In this work, using transcriptomics, RT-qPCR analysis and further experiments, we show that PsrA, indirectly regulates T6SS-meditated antibacterial activity in *S. marcescens* (Figures 7 and 10).

In summary, this work describes a novel regulator PsrA, which regulates prodigiosin synthesis, swarming motility, biofilm formation, extracellular polysaccharide production, serrawettin W1 biosynthesis, and T6SS-mediated antibacterial activity in *S. marcescens* (Figure 10). Further research is needed to reveal the roles played by other PsrA or PsrA-like proteins, which are widely present in bacteria.

DATA AVAILABILITY

The raw data of RNA-seq has been deposited in the GEO database of NCBI with the accession number GSE158829.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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