N-Acyl-Homoserine Lactone Quorum Sensing Switch from Acidogenesis to Solventogenesis during the Fermentation Process in *Serratia marcescens* MG1^S

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N-acyl-homoserine lactone quorum sensing (AHL-QS) has been shown to regulate many physiological behaviors in *Serratia marcescens* MG1. In the current study, the effects of AHL-QS on the biosynthesis of acid and neutral products by *S. marcescens* MG1 and its isogenic $\Delta swrI$ with or without supplementing exogenous *N*-hexanoyl-L-homoserine lactone (C₆-HSL) were systematically investigated. The results showed that *swrI* disruption resulted in rapid pH drops from 7.0 to 4.8, which could be restored to wild type by supplementing C₆-HSL. Furthermore, fermentation product analysis indicated that $\Delta swrI$ could lead to obvious accumulation for acidogenesis products such as lactic acid and succinic acid, especially excess acetic acid (2.27 g/l) produced at the early stage of fermentation, whereas solventogenesis products by $\Delta swrI$ appeared to noticeably decrease by an approximate 30% for acetoin during 32–48 h and by an approximate 20% for 2,3-butanediol during 24–40 h, when compared to those by wild type. Interestingly, the excess acetic acid produced could be removed in an AHL-QS-independent manner. Subsequently, quantitative real-time PCR was used to determine the mRNA expression levels of genes responsible for acidogenesis and solventogenesis and showed consistent results with those of product synthesis. Finally, by close examination of promoter regions of the analyzed genes, four putative *luxI* box-like motifs were found upstream of genes encoding acetyl-CoA synthase, lactate dehydrogenase, α -acetolactate decarboxylase, and Lys-like regulator. The information from this study provides a novel insight into the roles played by AHL-QS in switching from acidogenesis to solventogenesis in *S. marcescens* MG1.

Keywords: *Serratia marcescens* MG1, N-acyl-homoserine lactone quorum sensing, acidogenesis, solventogenesis, switch

Introduction

Quorum sensing (QS) is a communication system that allows bacteria to monitor their population density through a process of chemical cell-to-cell signaling mediated by diffusible signal molecules [1–3]. Previous studies showed that a considerable number of bacteria employ a QS system to regulate their physiological behaviors, including

bioluminescence, biofilm development, virulence factor secretion, and other cellular processes [4, 5]. *Serratia liquefaciens* MG1 was isolated from a softened cucumber [6] and was later renamed *Serratia marcescens* MG1 in 2005 [7]. *S. marcescens* MG1 can secrete a variety of hydrolytic enzymes with potential applications, including nuclease, lipase, protease, and phospholipase [6, 8]. In addition, *S. marcescens* MG1 also produces the biosurfactant serrawettin

[9], which exhibits antimicrobial, antitumor and plant protecting properties. These hydrolytic enzymes and serrawettin production have been shown to be controlled by QS. The *N*-acyl-homoserine lactone (AHL) QS system in *S. marcescens* MG1 consists of *swrI* and *swrR* genes, which belong to the *luxIR* type of QS system. *swrI*, a homolog of *luxI*, is responsible for directing the synthesis of two AHL signal molecules, *N*-butanoyl-L-homoserine lactone (C_4 -HSL) and *N*-hexanoyl-L-homoserine lactone (C_6 -HSL); *swrR*, a homolog of *luxR*, is a transcriptional factor that senses and interacts with C_4 -HSL and C_6 -HSL autoinducers in the cytoplasm and then binds to its target gene promoters, functioning as either a transcriptional activator or repressor.

Another important property of *S. marcescens* MG1 is that it is capable of producing a large number of neutral compounds (instead of mixed acids), such as acetoin and 2,3-butanediol (two important biobased bulk chemicals [10, 11]), when cultured under fermentation conditions using carbohydrates as a carbon source. To date, the physiological function of 2,3-butanediol has not been clarified. In many cases, *Enterobacteriaceae* members utilize sugar to produce organic acids, mainly including acetate, lactate, succinate, and formate, resulting in excessive acidification and growth inhibition during the culture process. However, some microorganisms, such as *Klebsiella* sp., *Clostridium acetobutylicum*, and *Serratia* sp., seem to possess the capability of switching their metabolism to the production of neutral products such as acetone, butanol, alcohols, 1,3-propanediol, or 2,3-butanediol [12–15]. It was reported

that in *Aerobacter aerogenes*, acetate, among all of the acid and neutral products tested, was found to be the most effective inducer for α -acetolactate synthase, α -acetolactate decarboxylase, and butanediol dehydrogenase activities, all of which are involved in the conversion of pyruvate to 2,3-butanediol [16]. In *S. marcescens* MG1, acetate at 40 mM dramatically promotes the mRNA expression levels of Lys-like regulator (*slaR*), α -acetolactate decarboxylase (*slaA*), and α -acetolactate synthase (*slaB*), but not butanediol dehydrogenase (*slaC*) [14]. Moreover, fermentation media supplemented with various amounts of sodium acetate increased the productivity of acetoin, and acetate boosted the α -acetolactate synthase gene expression in the tested *Serratia* sp. [17]. Therefore, *Serratia* sp. and perhaps those microorganisms that have developed defensive strategies against excessive acidification in the culture medium might employ acetate to switch the fermentation process from acidogenesis to solventogenesis.

Interestingly, acetoin and 2,3-butanediol production regulated by the AHL-QS system were uncovered by Rob *et al.* in the *S. plymuthica* RVH1 and *S. marcescens* MG1 strains [18]. Inactivation of *spII* (a homolog of *luxI*) in the AHL-QS system showed a sharp decrease in pH and less acetoin and 2,3-butanediol production, which could be restored by supplementation with exogenous 3-oxo- C_6 -HSL [18]. Moreover, *spIR* (a homolog of *luxR*) was found to be a negative regulator of acetoin production [18]. In addition, *swrR* negatively regulated *slaR* mRNA expression in *S. marcescens* MG1; however, $\Delta swrR$ produced a

Fig. 1. Fermentation pathways in *S. marcescens* MG1.

Abbreviations: LDH, lactate dehydrogenase; ACS, acetyl-CoA synthase; CS, citrate synthase; SDH, succinate dehydrogenase; ALS, α -acetolactate synthase; ALDC, α -acetolactate decarboxylase; BDH, 2,3-butanediol dehydrogenase; ADH, alcohol dehydrogenase.

comparable amount of 2,3-butanediol as the wild-type strain did [14]. These findings clearly demonstrated that the AHL-QS system is associated with the mixed-acid fermentation pathway and the 2,3-butanediol synthesis pathway. However, how the AHL-QS system in *S. marcescens* MG1 exerts effects on these pathways remains to be elucidated. A previous finding indicated that regulation of a total of 28 genes was dependent on the C₄-HSL-mediated QS system [19], but only *swrA*, *lipB*, *bsmA*, and *bamB* gene functions were uncovered, the gene products of which are responsible for swarming motility, protein transport and biofilm development in *S. marcescens* MG1, respectively [9, 20, 21]. Functions of the remaining 24 genes remain unknown. In other words, these genes might be involved in biological processes other than those mentioned above. In light of AHL-QS associated with both the pH value change and the 2,3-butanediol synthesis pathway during *S. marcescens* MG1 fermentation, we speculated that it might be involved in switching from acidogenesis to solventogenesis. Therefore, we systematically determined the main acid product and neutral product contents generated by *S. marcescens* MG1 during the fermentation process (Fig. 1). Moreover, by applying a quantitative real-time PCR approach, the mRNA expression levels of genes responsible for producing corresponding acids and solvents were analyzed.

Materials and Methods

Strains, Plasmids, and Culture Conditions

The strains and plasmids used in this study are listed in Table 1. All strains were routinely maintained in Luria-Bertani (LB) media at 30°C for *S. marcescens* MG1, *ΔswrI*, and *Chromobacterium violaceum* CV026, at 37°C for *E. coli* DH5α and *E. coli* S17-1 λpir. Antibiotics were added in the following amounts: 50 μg/ml kanamycin for *E. coli* S17-1 λpir, 20 μg/ml kanamycin for *Chromobacterium violaceum* CV026, and 100 μg/ml kanamycin for *ΔswrI*. For flask fermentation experiments, seed culture was prepared in LB media. After overnight cultivation, 5% seed culture of *S. marcescens* MG1

and *ΔswrI* were inoculated into the fermentation medium (50 ml in a 250 ml shake flask). Fermentation medium for *S. marcescens* MG1 and *ΔswrI* consisted of 7% glucose, 1.5% yeast extract, 0.1% NH₄H₂PO₄, 0.03% MgSO₄, and 0.01% MnSO₄, pH 7.0. For the rescue experiments, in parallel with the wild-type strain fermentation, a final concentration of 10 μM C₆-HSL (Sigma, USA) was added to the *ΔswrI* medium at the initial stage of fermentation. Samples were collected at 8 h intervals and were used for the analysis of the culture pH values, dry cell weight, residual sugar, acid products, neutral products, and target gene mRNA expression levels. All flask experiments were performed in triplicate.

Construction of the *ΔswrI* Mutant Strain

swrI-KO-S and *swrI*-KO-AS primers were designed according to the released *swrI* gene sequence in GenBank (GenBank Accession No. AY168877.1) and were subsequently used to amplify partial *swrI* fragments using the *S. marcescens* MG1 genome as a PCR template. All primers used in this study are provided in Table S1. The amplified DNA fragment was digested with *KpnI* and *ScaI*, purified, and ligated to the suicide vector pUTkm1, which was treated with the same enzymes as the *swrI* fragment. Thus, the marker-exchange plasmid pUT-*swrI* vector was created. The pUT-*swrI* vector insertion was verified by double enzyme digestion and commercial DNA sequencing. Then, the pUT-*swrI* vector was transformed into *E. coli* S17-1 λpir for mating with *S. marcescens* MG1. Transconjugants were screened on LB agar plates supplemented with 100 μg/ml ampicillin and 100 μg/ml kanamycin. A PCR-based approach was used to confirm disruption of the *swrI* locus with KR-S and KR-AS primers, which were designed to complement the Km^R cassette and the endogenous *swrI* locus outside of the insert in pUT-*swrI* (data not shown).

Analytic Methods

The biomass and glucose concentrations in the collected samples were determined by the methods described previously in our laboratory [22]. Gas chromatography (Agilent 7890A, Waldblum, Germany) was used to analyze and quantify ethanol, acetoin, and 2,3-butanediol as described previously [22]. The method for acid product analysis was described as follows: Samples were centrifuged at 10,000 g for 10 min to remove strain pellets, and then, acidic ethanol (80 ml ethanol plus 20 ml

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Characteristics	Reference or source
<i>S. marcescens</i> MG1	Wild Type, Tc ^r Ap ^r	CICC 25567 and laboratory stock
<i>ΔswrI</i>	<i>S. marcescens</i> MG1 harboring <i>swrI</i> deletion mutant, Tc ^R Ap ^R Km ^R	This study
<i>E. coli</i> S17-1 λpir	<i>recA pro hsdR</i> RP4-2-Tc::Mu-Km::Tn7	Laboratory stock and [32]
<i>C. violaceum</i> CV026	a mutant strain deficient in production of autoinducers used as quorum sensing reporter organism	Laboratory stock and [33]
pUTKm1	Ap ^R Km ^R <i>oriR6K oriTRP4</i>	Laboratory stock and [32]
pUT- <i>swrI</i>	pUTKm1 harboring <i>swrI</i> fragment, Km ^R	This study

deionized water, adjusted to pH 3.0 by H_3PO_4) was added to the supernatant, mixed, and then centrifuged again to remove any residual proteins. The obtained supernatant was filtered through a 0.22 μm filter membrane (Millipore, Germany). Finally, the lactic acid, acetic acid, citric acid, and succinic acid contents were determined by a high-performance liquid chromatography system (Agilent 1100, Germany) equipped with a Diode Array Detector (DAD). Gradient elution was conducted using a mobile phase starting with 95% 0.01 M KH_2PO_4 (pH 2.05) and 5% organic (100% methanol) solvent on an SB-Aq C18 column at 30°C. The flow rate was set at 0.6 ml/min, with a total run time of 30 min. The signal was monitored at 210 nm. Data were collected and calculated according to the strand curve generated by the corresponding standard samples.

Quantitative Real-Time PCR Analysis

Total RNA from the samples collected was extracted using Trizol reagent (Invitrogen, USA) individually, according to the manufacturer's protocols. cDNA was synthesized from the extracted total RNA with a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, China). All real-time primers with the exception of *slaC* were designed with Beacon Designer 7 software,

based on the available *S. marcescens* WW4 genomic sequence data. *slaC* real-time primers were used as designed by Rao *et al.* [14]. Relevant gene expression profiles were determined by StepOne Real-Time PCR (Applied Biosystems, Singapore) with TB Green Premix Ex Taq (Tli RNase H Plus) (Takara). The relative target gene RNA expression levels were analyzed. Data were normalized to 16S RNA expression levels and calculated as $2^{-\Delta\Delta\text{CT}}$. Experiments were performed in triplicate.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software, Inc., USA). The results were expressed as the mean values with SDs and were conducted with two-way ANOVA. A *p*-value of <0.05 was considered to be significant.

Results and Discussion

Effect of *swrI* Disruption on the Biomass Concentration, pH Profile, and Sugar Consumption of *S. marcescens* MG1

To test whether *N*-acyl-homoserine lactone quorum sensing (AHL-QS) functions in switching from acidogenesis

Fig. 2. Effects of *swrI* disruption on biomass concentration, pH profile, and sugar consumption of *S. marcescens* MG1.

Construction of *S. marcescens* MG1 *swrI* disruption mutant (A). Samples were collected at the indicated fermentation time points and biomass concentration (B), pH profile (C), and sugar consumption (D) determination assays for *S. marcescens* MG1 WT and $\Delta swrI$ complemented with or without $C_6\text{-HSL}$ were performed. Values are means, and bars indicate standard deviations (SDs) ($n = 3$).

to solventogenesis, we first used a marker-exchange strategy to construct a *swrI* disruption mutant. As shown in Fig. 2A, the wild-type strain (WT) efficiently elicited the indicative strain *C. violaceum* CV026 to produce violacein, which could not be induced by $\Delta swrI$ under the same tested conditions. This demonstrates that $\Delta swrI$ lost the capability to synthesize AHL autoinducers and indicates that the endogenous *swrI* locus was destroyed completely in this mutant strain. Subsequently, disruption of the *swrI* locus was confirmed by PCR-based analysis (data not shown). The cell growth profile, culture pH, and sugar consumption of WT and $\Delta swrI$ were evaluated and compared. The cell growth profiles determined by dry cell weight assays showed that WT produced more biomass than $\Delta swrI$, but slightly less than $\Delta swrI$ complemented with exogenous C₆-HSL, during the whole fermentation process (Fig. 2B). The data presented in Fig. 2B demonstrate that the AHL-QS system positively contributes to the growth rate of *S. marcescens* MG1 to a certain degree, but it is dispensable

for its growth. Our finding is consistent with results discovered by Rob *et al.* [18] but is in contrast to the data found from *Aeromonas hydrophila* AH-1N and *V. fischeri* [23, 24], both of which showed that AHL-QS and AinS (specifically for synthesizing autoinducer-2) QS systems are essential for their growth. In agreement with the strain growth profiles, $\Delta swrI$ exhibited the lowest sugar consumption kinetics, but the defective phenotype of $\Delta swrI$ in sugar consumption could be rescued by the addition of exogenous C₆-HSL (Fig. 2D). After analyzing the data generated by examining the culture pH values at different fermentation time points, the corresponding results showed that the pH values recorded for WT and $\Delta swrI$ supplemented with or without exogenous C₆-HSL represented inversely typical parabolic curves that reached the baseline at 24 h for WT and $\Delta swrI$ supplemented with exogenous C₆-HSL and at 32 h for $\Delta swrI$ (Fig. 2C). The lowest pH value for WT was approximately 5.5, while that for $\Delta swrI$ was 4.8 during the whole fermentation process. As culture acidifiers

Fig. 3. Effect of *swrI* disruption on acidogenesis in *S. marcescens* MG1.

Samples were collected at the indicated fermentation time points and lactic acid content (A), acetic acid content (B), citric acid content (C), and succinic acid content (D) produced by *S. marcescens* MG1 WT and $\Delta swrI$ complemented with or without C₆-HSL were determined. Values are means, and bars indicate standard deviations (SDs) (n = 3).

gradually accumulate, the growth of the fermentation strain gradually ceases [13]. In the case of *S. marcescens* MG1, the higher amount of culture acidifiers accumulated in the spent medium might account for the lower biomass.

Effect of *swrI* Disruption on Acidogenesis in *S. marcescens* MG1

The pH values of $\Delta swrI$ culture were dramatically lower compared with those of WT culture during the fermentation process (Fig. 2C) ($p < 0.05$, data not shown). To explore the possible factor(s) contributing to this phenomenon, we systematically analyzed the contents of the mixed acid products (Fig. 1), including lactic acid, acetic acid, citric acid and succinic acid, which are the main *S. marcescens* MG1 fermentation acid products secreted to the medium, according to our preliminary experiment results (data not shown). As the fermentation began, WT quickly produced lactic acid and succinic acid. Subsequently, lactic acid contents were maintained at an approximate 2 g/l during the whole fermentation process (Fig. 3A), while succinic acid contents gradually decreased after 8 h fermenting (Fig. 3D), implying that succinic acid must be reused in some way. In contrast, WT produced a slight amount of citric acid and did not produce acetic acid at all during the fermentation period of 0–24 h. After that period, these two acids were concomitantly accumulated. The kinetics of lactic acid, citric acid and succinic acid produced by $\Delta swrI$ exhibited a similar pattern to those of WT or $\Delta swrI$ complemented with exogenous C_6 -HSL (Figs. 3A, 3C, and 3D). However, $\Delta swrI$ produced large amounts of acetic acid as early as the fermentation period of 0–8 h which was then gradually removed from the media (Fig. 3B). It was reported that *V. fischeri* cells initially excrete acetic acid, and the excess acetic acid can be removed from the media in an AHL-QS-dependent manner [24]. However, our data clearly demonstrate that $\Delta swrI$ can remove excess acetic acid in the medium with an unknown strategy separate from the AHL-QS system, although it was also obvious that AHL-QS is involved in the process of acetic acid production (Fig. 3B). According to the conclusion addressed by Sarah *et al.*, adding the same amount of exogenous acetic acid as generated by the AinS strain to uninoculated medium is sufficient to account for the pH drop [24]. The large amount of acetic acid produced by $\Delta swrI$ during the fermentation period of 0–24 h might account for the sharp pH drop (Figs. 2C and 3B). Since WT generated more citric acid than $\Delta swrI$ (Fig. 3C), the combined contribution of the other three acids analyzed in the culture to acidification

Fig. 4. Effect of *swrI* disruption on solventogenesis in *S. marcescens* MG1.

Samples were collected at the indicated fermentation time points. Ethanol content (A), acetoin content (B), and 2,3-butanediol content (C) produced by *S. marcescens* WT and $\Delta swrI$ complemented with or without C_6 -HSL were determined. Values are means, and bars indicate standard deviations (SDs) ($n = 3$).

possibly leads to the phenomenon whereby the pH values of $\Delta swrI$ culture were accordingly lower than those of WT culture during the whole fermentation process (Figs. 3 and 2C).

Effect of *swrI* Disruption on Solventogenesis in *S. marcescens* MG1

S. marcescens MG1 produced a small amount of ethanol, and the maximal records for WT, $\Delta swrI$, and $\Delta swrI$ complemented with exogenous C₆-HSL were approximately 1.2, 3, and 1.3 g/l, respectively. $\Delta swrI$ produced more ethanol content than WT (Fig. 4A). The QS regulatory system involved in playing a positive role in acetoin synthesis has been well documented [18, 23]. Our results collected from acetoin content determination assays are in agreement with those previous findings. $\Delta swrI$ produced fewer amounts of acetoin compared with WT at the end of fermentation (Fig. 4B). For 2,3-butanediol production, in the fermentation period of 0–40 h, WT produced more 2,3-butanediol content than $\Delta swrI$, which correlates well with previous findings described by Rob *et al.* [18]. However, at the end of fermentation, all strains tested here showed comparable 2,3-butanediol production (Fig. 4C). According to our data, AHL-QS in *S. marcescens* MG1 negatively contributes to ethanol production but positively promotes acetoin and 2,3-butanediol generation. In addition, *S. marcescens* MG1 seems to reuse its neutral products, with the exception of acetoin [25].

Metabolic Flux Analysis

As shown in Table 2, $\Delta swrI$ generated more acid products than WT during the fermentation period of 0–8 h. To be more specific, the amounts of lactic acid, acetic acid, and succinic acid generated by $\Delta swrI$ were increased by up to

5.1%, 18.0%, and 8.0% compared with that produced by WT, respectively. As the fermentation process progressed, both WT and $\Delta swrI$ reduced the generation of their acid products but increased the generation of their neutral products. However, the acid products generated by $\Delta swrI$ accordingly were much more than those produced by WT. Importantly, by the addition of exogenous C₆-HSL to the $\Delta swrI$ medium, the fermentation products generated by $\Delta swrI$ could be restored to the levels produced by WT. The metabolic flux analysis results showed that AHL-QS exerts a role in the switch from acidogenesis to solventogenesis in *S. marcescens* MG1 during the fermentation process.

Effect of *swrI* Disruption on Relevant Enzyme Gene Expression in *S. marcescens* MG1

In light of the AHL-QS involved in the switch from acidogenesis to solventogenesis, it is intriguing for us to test the hypothesis that AHL-QS might directly exert effects on the expression of relevant enzyme genes, which are responsible for synthesizing the acid and neutral products we determined. It has been demonstrated that exogenous autoinducer turnover was approximately 20 h when added to the media [26]. Glucose was completely exhausted at approximately 32 h during the *S. marcescens* MG1 fermentation process (Fig. 2D). Therefore, samples used for gene expression analysis were prepared at fermentation time points of 8, 16, and 24 h. The mRNA expression levels of *ldh* encoding lactate dehydrogenase in all tested strains exhibited the same pattern at the analyzed fermentation time points, which showed that the mRNA levels of *ldh* expressed by $\Delta swrI$ were approximately four times those generated by WT; the overexpressed *ldh* mRNA levels in $\Delta swrI$ could be restored by the addition of exogenous C₆-HSL (Figs. 5A–5C). It was reported that *acs*,

Table 2. Metabolic flux analysis for the products generated by WT and $\Delta swrI$ with or without exogenous C₆-HSL.

<i>S. marcescens</i> MG1	WT ^a	$\Delta swrI$ ^b	$\Delta swrI$ + C ₆ -HSL ^a	WT ^b	$\Delta swrI$ ^b	$\Delta swrI$ + C ₆ -HSL ^b	WT ^c	$\Delta swrI$ ^c	$\Delta swrI$ + C ₆ -HSL ^c	WT ^d	$\Delta swrI$ ^d	$\Delta swrI$ + C ₆ -HSL ^d
Biomass	9.82	9.38	10.45	7.19	6.37	7.23	2.35	7.00	2.34	6.47	1.58	9.88
Lactic acid	9.25	14.32	10.52	0.13	0.36	0.11	1.48	5.44	1.53	-3.54	-1.91	-7.84
Acetic acid	0.00	18.03	0.00	0.00	-15.24	0.00	0.00	-2.70	0.00	3.83	-2.26	4.45
Succinic acid	34.02	42.06	33.76	-3.11	-0.04	-4.79	-3.96	5.04	-2.95	-4.04	0.18	-8.80
Citric acid	1.83	0.94	1.64	-2.09	0.73	-1.68	-0.14	0.39	0.01	9.49	2.74	11.94
Ethanol	7.64	5.24	9.28	4.80	7.09	3.83	-4.48	14.58	-0.73	2.85	8.52	0.60
Acetoin	2.63	0.68	2.47	-3.21	7.09	-2.67	0.37	2.30	0.64	45.96	11.27	59.72
2,3-butanediol	34.82	9.35	31.88	96.30	93.64	97.96	104.38	67.95	99.16	38.98	79.88	30.04

Note: Metabolic flux was calculated based on the assumption that acid and neutral end products were generated by bacteria consuming 100 g glucose. Superscript letters ^a, ^b, ^c, and ^d represent the fermentation periods of 0–8 h, 8–16 h, 16–24 h, and 24–32 h, respectively.

Fig. 5. Effect of *swrI* disruption on relevant enzyme gene expression in *S. marcescens* MG1.

Quantitative real-time PCR was used to determine mRNA expression levels of samples collected at fermentation time points of 8 h (A), 16 h (B), and 24 h (C). Relative quantification is reported as fold expression against the WT sample in each set of data. Values are means, and bars indicate standard deviations (SDs) ($n = 3$). Single and double asterisks represent $p < 0.05$ and $p < 0.01$, respectively. The consensus motif was created by aligning *V. fischeri* *luxI* with: *Pseudomonas aeruginosa* *lasI* and *rhII*; *Ralstonia solanacearum* *soli*; *Acidithiobacillus ferrooxidans* *afeI*; *Burkholderia cepacia* *cepI*; *S. marcescens* SS-1 *spnR* and *recA*; *Pantoea stewartii* *esaR*, *luxIQS6-1*, *luxIQS10-1*, and *luxRQS10-2* as described elsewhere previously [30, 31]. The consensus sequence is shown below the alignment, N=A, T, C or G; V=A, C, or G; R=A or G; H=A, T, or C; D=A, T, or G; Y=C or T; B=T, C, or G (D). Four putative *luxI* box-like motifs existing upstream of *S. marcescens* MG1 *slaR*, *slaA*, *acs*, and *ldh* (E).

encoding acetyl-CoA synthase, was under the control of the AinS-QS regulatory system in *V. fischeri* [24]. Our data clearly demonstrate that the AHL-QS regulatory system

negatively regulated *acs* mRNA expression levels. However, AHL-QS seems to be unrelated to regulating the mRNA expression profiles of *adh*, *cs*, and *sdh*, which encodes

alcohol dehydrogenase, citrate synthase, and succinate dehydrogenase, respectively (Figs. 5A–5C). Unexpectedly, *sdh* mRNA levels were dramatically reduced to baseline levels in $\Delta swrI$ supplemented with or without exogenous C₆-HSL compared to WT. Although the succinic acid contents produced by WT and $\Delta swrI$ supplemented with or without exogenous C₆-HSL were comparable during the fermentation time period of 0–32 h (Fig. 3D), the exact cause for the resulting conflicting results is currently unknown. The genes encoding α -acetolactate synthase, α -acetolactate decarboxylase, and 2,3-butanediol dehydrogenase in some microorganisms such as *Klebsiella terrigena*, *Enterobacter aerogenes*, and *Vibrio cholera* cluster together to form an operon [27, 28]. However, in *S. marcescens* MG1, *slaA* and *slaB* were found in one operon apart from *slaC*, and *slaR* was divergently transcribed with *slaA* [14]. QS was found to exert a positive effect on the expression of *slaR* and *slaA* [14, 29]. Our data presented here clearly demonstrate that *slaA*, *slaB*, and *slaR* expression levels were positively influenced by AHL-QS (Figs. 5A–5C). It is of note that AHL-QS seems to exert more profound effects on the *slaA* and *slaB* rather than *slaR* expression levels, especially at the later fermentation stage (Fig. 5C). This implies that *slaR* expression might also be controlled by other regulatory circuits. Unexpectedly, *slaC* expression levels in $\Delta swrI$, complemented with or without exogenous C₆-HSL, were accordingly higher than in WT (Figs. 5B and 5C), which is in contrast to the conclusion drawn by Rao *et al.* [14]. The different fermentation media used for assays might account for these conflicting results.

To further uncover how the AHL-QS exerts its effects on the expression of the genes analyzed above, we attempted to determine whether these gene promoter regions contain *luxI* box-like elements by *in silico* analysis. We cloned and sequenced the promoter regions (approximately -350 to the translation start site) of the genes we analyzed. All primers used to amplify the target promoter regions are provided in Table S1, and the corresponding sequences are provided in Table S2. Based on the known *luxI* box-like elements [30, 31], we aligned these elements and created a consensus motif (Fig. 5D). Four bases are conserved in all elements, including C3, T4, A17, and G18, implying that these four bases might be very important for *luxI* box-like elements recognized and bound by LuxR-type protein. We scanned target gene promoter regions using the created consensus motif with two criteria: 1) with the best similarity only, and 2) the candidate motif including C3, T4, A17, and G18 bases. Scanning results showed that there are four

candidate motifs localized on the *slaR*, *slaA*, *acs*, and *ldh* promoter regions (Fig. 5E), implying that these four genes are the true targets of AHL-QS in *S. marcescens* MG1, although it requires further experiments to validate.

Based on our results, we propose a SwrR working model during the *S. marcescens* MG1 fermentation process that, in the absence of autoinducers, SwrR directly binds to the *acs*, *ldh*, *slaR* and *slaA* promoter and activates *acs* and *ldh* but represses *slaR* and *slaA*. In the presence of autoinducers, SwrR interacts with autoinducers and then detaches from the target gene promoters, resulting in the repression of *acs* and *ldh* and removing the repression of *slaR* and *slaA*. This model allows *S. marcescens* MG1 to produce more acid products at low cell density, but to generate more neutral products at high cell density. However, this model seems to be a contradiction where SwrR exerts two opposite functions when binding to its different targets. However, it is reminiscent to us of the fact that SpnR represses its target genes but activates its own expression [30] and that Lys-like regulator activates α -acetolactate decarboxylase gene expression but represses its own expression [29].

In conclusion, according to the data presented in this study, three conclusions can be drawn. First, AHL-QS is required, but not essential, for *S. marcescens* MG1 growth. Second, AHL-QS facilitates *S. marcescens* MG1 to produce acid products during the early phase of fermentation and is susceptible to generating more neutral products during the later phase of fermentation. However, the excess acetic acid is removed in an AHL-QS-independent manner. Third, AHL-QS in *S. marcescens* MG1 exerts its negative effects on regulating the expression of genes responsible for acidogenesis and plays an active role in promoting the expression of genes involved in solventogenesis. By close examination of these analyzed gene promoter regions, *slaR*, *slaA*, *acs*, and *ldh* promoter regions contain the putative *luxI* box-like motifs, implying that these four genes are the true targets of AHL-QS in *S. marcescens* MG1. However, it should be noted that there may be some unknown regulatory circuits involved in the switch from acidogenesis to solventogenesis, other than the AHL-QS regulatory system, in *S. marcescens* MG1.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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