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Quantitative measurement of pH influence on SalR regulated gene expression in *Acinetobacter baylyi* ADP1

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ABSTRACT

The influence of medium pH conditions on regulation of the *sal* operon was quantitatively measured by using a genetically engineered *Acinetobacter baylyi* ADP1 strain ADPWH_*lux*. Live-cell based bioluminescence assays determined that the SalR, a LysR-type transcriptional regulator (LTTR), functioned in a range of medium pH conditions. During exponential growth, SalR regulated bioluminescence production peaked at the neutral pH and tolerated medium pH changes of 5.5–9.0. In the stationary phase, the SalR regulated gene expression reached 20–35% of the maximum productivity during the exponential phase. This report reveals the influence of pH conditions on a LTTR system and demonstrates that the biosensor strain can be a useful tool for understanding transcriptional regulations in different environments.

1. Introduction

The *lysR* gene encodes a positive regulator required for full expression of the *lysA* gene that encodes diaminopimelate decarboxylase in *Escherichia coli* K12 (Stragier et al., 1983). The LysR-type transcriptional regulators (LTTRs) form a large family of bacterial activator proteins, regulating expressions of enzymes that are associated with the β-ketoadipate pathway (Henikoff et al., 1988; Tropel and van der Meer, 2004). They are 270–330 amino acid residues in length and form dimers or tetramers, with an N-terminal DNA binding domain (DBD) and a C-terminal co-factor binding domain. A helix-turn-helix motif in the conserved N-terminal plays a central role in DNA binding. In a non-induced condition, a dimer unit of regulators binds to a repressor binding site (RBS) and another dimer unit binds to an activator binding site (ABS). In the presence of a co-inducer chemical, the LTTR protein changes conformation and shifts binding location on the ABS to upstream. By interacting with the α-subunit domain of RNA polymerase, the LTTR protein directs the RNA polymerase to bind a so-called UP-DNA sequence motif, which is located between the RBS and ABS, resulting in increased binding affinity to the promoter sequence and initiating transcription (Tropel and van der Meer, 2004; Maddocks and Oyston, 2008). Research on LysR family has focused on gene sequence, LTTR structure and the mechanism of DNA binding, but there is little information on the physiological characteristics of gene activity (Maddocks and Oyston, 2008). It has been reported that expressions of some LTTR regulated genes are significantly different between exponential and stationary phases (e.g., Lee et al., 2006; Turlin et al., 2001; Schellhorn, 1995; Renna et al., 1993; Jin and Sonenshein, 1994). Sigma factors have been reported for controlling LTTR regulated gene expression; although the mechanisms have not been clear (Nystrom, 2004; Costanzo and Ades, 2006).

Acinetobacter spp. (family *Moraxellaceae*) is widespread in the natural environment. The non-pathogenic soil bacterium *Acinetobacter baylyi* ADP1 closely relates to *Pseudomonas aeruginosa* and *Pseudomonas putida*. But unlike these bacteria, *A. baylyi* ADP1 is highly competent for natural transformation thus facilitating genetic engineering (Metzgar et al., 2004). It has a 3.6 Mbp genome that encodes 3325 genes of which 20% are associated with catabolic functions (Barbe et al., 2004). Many of them are regulated by LTTRs, e.g., BenM regulating benzoate degradation (Collier et al., 1998); CatM regulating catechol degradation (Romero-Arroyo et al., 1995); OxyR which is a peroxide response regulator (Geissdorfer et al., 1999) and SalR regulating salicylate degradation (Jones et al., 2000).

In *A. baylyi* ADP1, salicylate is hydroxylated into catechol which enters the β-ketoadipate pathway, and is then converted to acetyl CoA and succinyl CoA which enter the tricarboxylic acid cycle. The *sal* operon is composed of four open reading frames: *salA*, *salR*, *salE*, and *salD*. SalA is a salicylate-inducible hydroxylase. SalR regulates the expression of *salA* but not *salE*. SalE hydrolyzes ethyl salicylate to

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ethanol and salicylic acid. SalD is proposed to be a membrane protein involved in the transport of hydrocarbons. SalA and SalR closely resemble two *Pseudomonas* proteins, NahG and NahR, respectively. The regulator protein SalR has been identified as a member of LTTR, with the family signature motif of a helix-turn-helix at residues 17 to 47 (Jones et al., 2000). A salicylate biosensor strain ADPWH_*lux* has been constructed (Huang et al., 2005) to contain a chromosomally located fusion of the *salA* gene at the *A. baylyi* ADP1 *sal* operon with the promotorless bioluminescence gene cassette *luxCDABE* from *Photorhabdus luminescens*. The bacterium produces bioluminescence specifically in response to salicylate induction and can be used quantitatively over a wide range of concentrations (Huang et al., 2005; Huang et al., 2006). Although the biosensor strain has been successfully used to measure salicylate concentrations in contaminated soil samples (Huang et al., 2005) and plant samples infected by pathogens (Huang et al., 2006; Zhang and Mou, 2009), a practical concern remains whether or not the live-cell biosensor is robust when mixed with samples in different chemical conditions, e.g., pH conditions, that are often variable among soil extracts and plant sap.

Environmental pH is an important factor for the survival of bacterial communities. In this report, we assessed SalR regulated protein expression of *A. baylyi* ADP1 in Luria-Bertani medium at differing pH, by using live-cell bioluminescence assays. This report demonstrates an unknown feature of LTTR regulated gene expression, i.e., tolerance to medium pH changes. The determination on working pH range of the *A. baylyi* ADPWH_*lux* strain showed that the biosensor can be a sensitive tool for understanding SalR regulation in different environments.

Table 1
The final pH values of bacteria culture mixed (1:1, v/v) with LB media of 27 pH values.

pH set	Mixture pH	
	Exponential phase culture	Stationary phase culture
1.0	1.45	1.53
1.5	2.40	2.63
2.0	3.15	3.61
2.5	3.69	4.27
3.0	4.11	5.01
3.5	4.53	5.79
4.0	5.00	6.50
4.5	5.54	6.91
5.0	6.10	7.21
5.5	6.53	7.51
6.0	6.79	7.66
6.5	6.95	7.75
7.0	7.16	7.92
7.5	7.41	8.12
8.0	7.68	8.35
8.5	7.94	8.57
9.0	8.23	8.78
9.5	8.63	9.05
10.0	9.05	9.33
10.5	9.44	9.62
11.0	9.70	9.79
11.5	9.87	9.95
12.0	9.98	10.09
12.5	10.25	10.28
13.0	10.96	11.11
13.5	12.98	13.01
14.0	13.80	13.76

2. Materials and methods

2.1. Cultivation of bacteria in exponential phase

A single colony of *A. baylyi* ADPWH_*lux* was inoculated into 5 ml of Luria-Bertani (LB, Axgen Co.) medium and incubated overnight (12 h) at 30 °C, with shaking at 220 rpm. 0.5 ml of the overnight culture was diluted into 50 ml LB medium, and incubated at 30 °C, 220 rpm for 2 h. The culture was halved and salicylate (final concentration 1 mM in water) was added to one half of the culture (induced, CK + SA), an equal volume of water was added to the other half of the culture (blank, CK, negative control).

2.2. Cultivation of bacteria in stationary phase

A single colony of *A. baylyi* ADPWH_*lux* was inoculated into 50 ml of LB medium and incubated at 30 °C, 220 rpm for 48 h. The induced (CK + SA) and blank (negative control, CK) cultures were set up as previously described for the exponential phase culture (Section 2.1).

2.3. Kinetics and quantitative analyses of bioluminescence production under different pH conditions

A set of 27 standard LB media samples was set up, with pH values ranging from 1.0 to 14.0, at intervals of 0.5. The pH of LB media was adjusted with HCl or NaOH, and measured by an Orion 3 star pH benchtop (Thermo). These pH standards were mixed (1:1, v/v) with the induced culture (CK + SA) or the blank culture (negative control, CK). The final pH values of the mixtures were determined (Table 1) and 200 µl of each mixture was immediately transferred into a 96-well optical microplate. Bioluminescence production was measured by a Synergy HT Multi-Detection microplate reader at 30 °C (emission filter 460/40). Kinetic curves were obtained by reading the plate every 2.5 min for 2 h. The OD_{600} values at the beginning and the end of the 2 h period were also determined. Relative bioluminescence (bioluminescence $cell^{-1}$) was calculated for the end point, after 2 h (bioluminescence divided by the OD_{600} value). Twelve replicates were made for every sample. The mean and standard error (SE) was calculated and plotted as a scatter plot. A Bezier curve (Piegl and Tiller, 1997) was used to construct a series of estimations of the relative bioluminescence at pH conditions of 2.0–13.0 by using an Excel macro downloaded from the internet (http://www.xlrotor.com/Smooth_curve_bezier_example_file.zip). Ratios of SalR regulated protein expression between stationary and exponential growth were estimated using the constructed dataset.

2.4. RNA extraction and reverse transcription-PCR

Total RNA of each sample was extracted from the samples after they had been assessed for relative bioluminescence using TRIzol (Invitrogen Co) followed by DNaseI treatment. About 10 ng of the total RNA was reverse transcribed to cDNA using MMLV Reverse Transcriptase (RNase H^-) (Takara) with primers of salAr (5′-TTAAATCGTTGCAACA GGTTGTATTG-3′) and 16Sr (5′-CCTCAGCGTCAGTGTTAG-3′) according to the manufacture's recommendation. PCR amplifications of cDNA were performed in a T1 thermo cycler (Biometra), for the *salA* and 16 S-rRNA regions by using two primer pairs salAr and salAf (5′-AAATTAGTATAGCCATTATTGGTGGTGGTATT-3′) and 16Sr and 16Sf (5′-AGCGGCGGACGGGTGAGTA-3′). The reactions were set up for 20 µl containing 1 µl of the total cDNA, 10 pM of each primer and 10 µl of 2 × Taq PCR mix (Takara). The reaction mixtures were heated to 94 °C for 4 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 54 °C, and 1 min 30 s at 72 °C, then finished with 1 cycle of 72 °C for 10 min.

2.5. DNA extraction and PCR

Cells from 1 ml of culture were harvested by centrifugation and resuspended in 0.5 ml sterilized water. Cell lysates were produced by heating in boiling water for 5 min followed by centrifugation at 12,000 rpm for 2 min. The aqueous phase was stored at −20 °C before being used for PCR amplifications of SalR and 16 S-rRNA encoding fragments in the bacterial genome. The PCR primers and program were as previously described for RT-PCR post the reverse transcription step.

2.6. Repeats and replicates

The entire bioluminescence experiment was repeated three times and more tests were performed for pH 4.0–6.0 and pH 9.0–11.0. Twelve replicates (12 wells in the optical microplate) were made for each sample in each test. The relative bioluminescence was calculated for each well first and then was represented as the mean ± SE for each sample. The experiments of RT-PCR and PCR were repeated once without sample duplication. Data of the last repeat experiments was represented in this report.

3. Results

Conventional RT-PCR showed that transcription of the *salA* gene (~1200 bp band, Fig. 1A) was induced in the presence of SA (CK + SA). No *salA* transcript was amplified in the absence of SA (negative control, CK), indicating no detectable DNA contamination in the RNA extracts. The PCR products of cell lysate DNA showed that the primers were specific to the target fragments (Fig. 1A). The *salA* transcription was detected at pH 5.54–9.05 in the exponential growth (Fig. 1B), and at pH 5.79–9.79 in the stationary phase (Fig. 1C). Quantitative measurement was not carried out using the gel pictures.

To determine the strength of SA induced expression, the bioluminescence signal was recorded at 2.5 min intervals for 2 h. In the exponential phase, the bioluminescence background remained low (<200 Arbitrary Unit, AU) in the absence of SA (negative control, CK) under different pH conditions (Fig. 2A). Higher backgrounds were generated in alkaline environments than in acidic environments (Fig. 2A). When SA was added to the medium to a final concentration of 1 mM, induced bioluminescence production was observed (Fig. 2B, CK + SA). The expression appeared a simple kinetics during the 2 h period. Differences in the strength of bioluminescence signal were evident at the different pH, although the bioluminescence curves were still rising at the end of the observation (Fig. 2B).

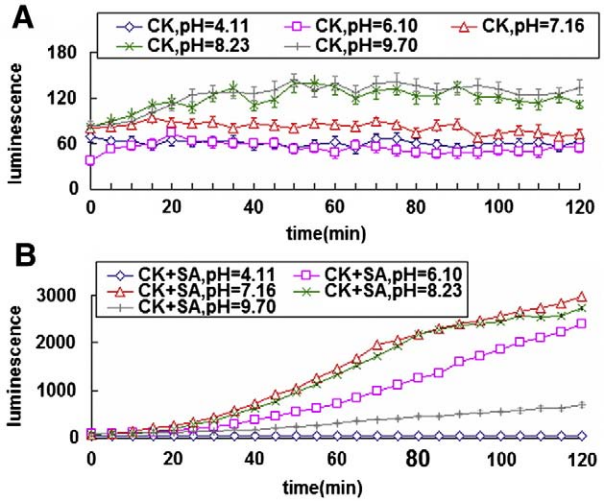

Fig. 2. Kinetic curves of *Acinetobacter* ADPWH_*lux* grew in LB medium under pH 4.11/6.10/7.16/8.23/9.70 in the absent (A) and present (B) of salicylate in the exponential phase. Error bar represents one standard deviation of the mean ($n = 12$). Data is represented at 5 min reading intervals for 2 h, although data was collected at 2.5 min intervals during the experiment.

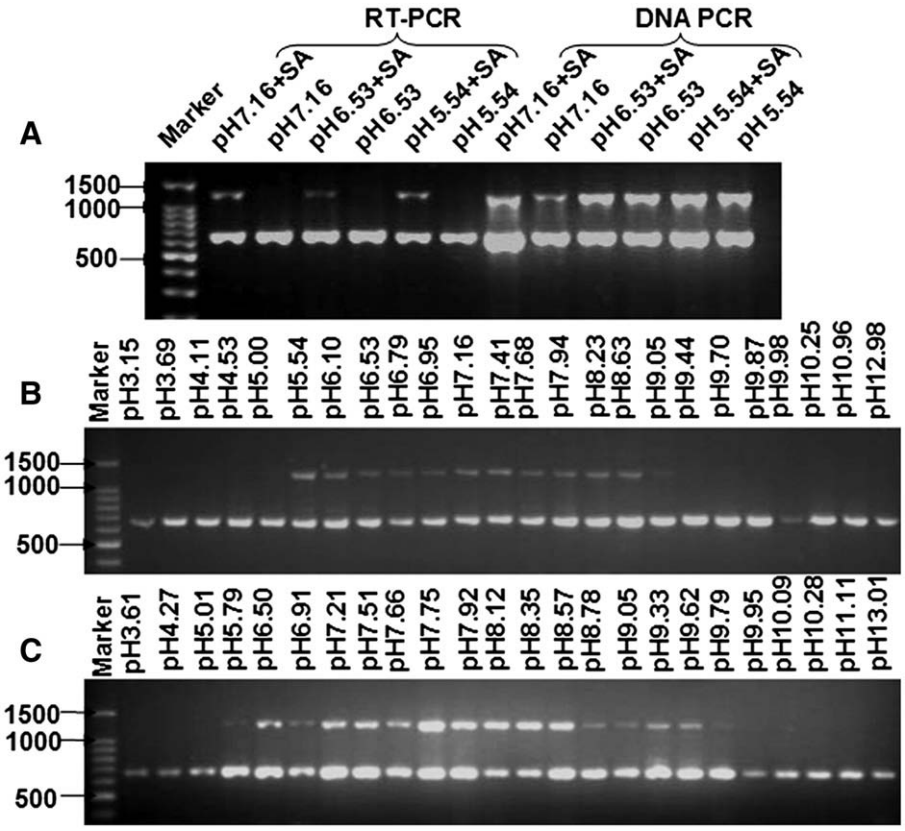

Fig. 1. Agarose gels of RT-PCR/PCR products amplified from *Acinetobacter* ADPWH_*lux* grown in LB medium. (A) Gel picture shows RT-PCR (left half) and PCR (right half) for samples of pH 7.16/6.53/5.54 with and without salicylate in the exponential phase. The sizes of the major molecular markers (100 bp–1500 bp) are indicated on the right. The upper band (~1200 bp) represents the *salA* product, and the lower band (~650 bp) shows the *16 S-rRNA* product. (B) 24 pH conditions with salicylate in the exponential phase. (C) 24 pH conditions with salicylate in the stationary phase. No *salA* product was detected in negative controls (samples without salicylate, data not shown).

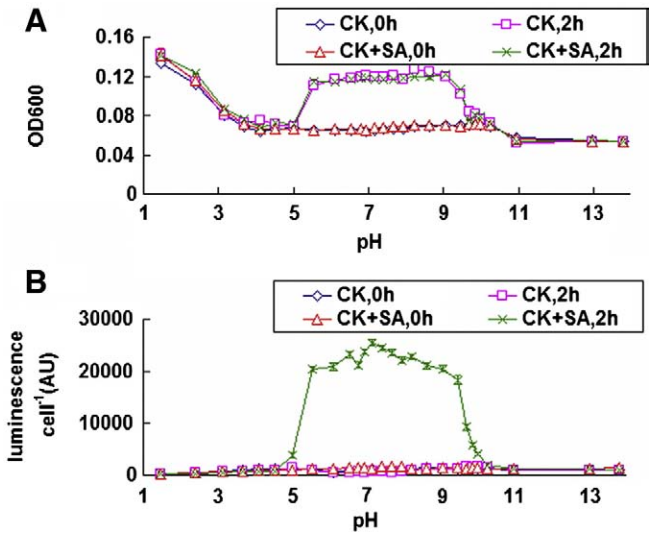

Fig. 3. Scatter diagram for OD_{600} (A) and relative bioluminescence (B) of *Acinetobacter* ADPWH_*lux* in LB medium under different pH conditions in the absence (CK) and presence (CK + SA) of salicylate for 2 h in the exponential growth. Error bars represent one standard deviation of the mean ($n = 12$).

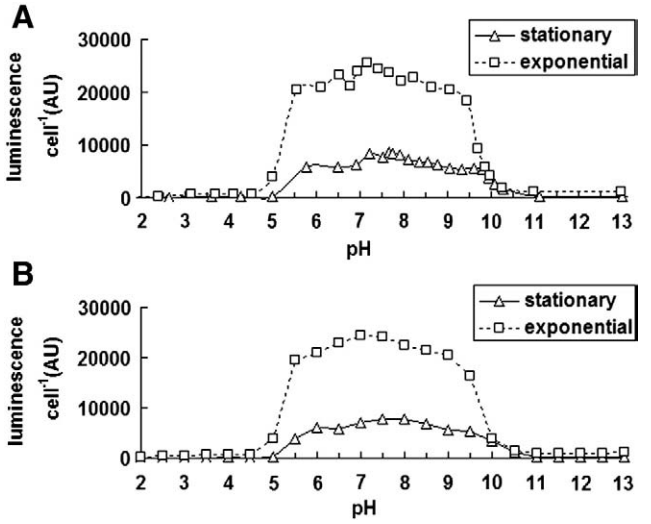

Fig. 5. Scatter diagram (A) for relative bioluminescence of *Acinetobacter* ADPWH_*lux* in LB medium under pH 2.0–13.0 with salicylate in the exponential and stationary phase, and line chart (B) of reconstructed data based on Bezier curve estimation (Table 2).

Medium pH did not affect growth significantly in the range of pH 5.54–9.05. However, harsher conditions did inhibit bacterial growth, and caused morphological changes that affected OD_{600} when pH < 5.00 and pH > 10.96 (Fig. 3A). In CK + SA samples when the bioluminescence was divided by the OD_{600} value, representing the relative bioluminescence production (AU cell unit^{-1}) at the end of observation, the strongest signal was 25,523 (AU cell unit^{-1}) at pH 7.16. Except at the neutral pH, the relative bioluminescence remained above 20,000 AU cell unit^{-1} (80% of the maximum signal) between the pH range 5.54–9.05 (Fig. 3B). Outside this range, growth was inhibited (Fig. 3A), and bioluminescence was also reduced (Fig. 3B). Bioluminescence was not induced at a pH > 4.53 and < 10.96.

In the stationary phase, no significant bacterial growth was observed in the 2 h period (Fig. 4A). A plateau about 8000 AU cell unit^{-1} was observed at pH 7.21–7.92 for the CK + SA samples (Fig. 4B). In acidic conditions bioluminescence production declined to 6000 AU cell unit^{-1} at pH 5.79, then dropped to the background level at pH 5.01. In alkaline conditions, bioluminescence production gradually declined to 5400 AU cell unit^{-1} at pH 9.79, then sharply decreased to the background level at pH 10.28 (Fig. 4B).

Bezier curves (Piegl and Tiller, 1997) were constructed to smooth the observed bioluminescence curves and to determine the trends in the scatter plot (Fig. 5). Using this method, the dataset (Fig. 5A) was reconstructed (Table 2) and represented as smooth curves for the exponential phase and stationary phase (Fig. 5B). Also based on the reconstructed dataset (Table 2), the relative bioluminescence generated at the stationary phase was estimated at 20–35% of that of the exponential growth.

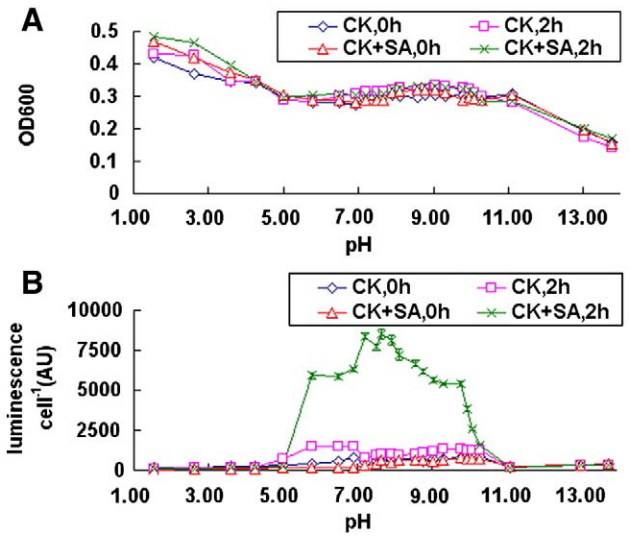

Fig. 4. Scatter diagram for OD_{600} (A) and relative bioluminescence (B) of *Acinetobacter* ADPWH_*lux* in LB medium under different pH conditions in the absence (CK) and presence (CK + SA) of salicylate for 2 h in stationary phase. Error bars represent one standard deviation of the mean ($n = 12$). Data of pH 7.75, 8.35, and 9.62 was in line with their adjacent points but is not shown to improve presentation.

Table 2
Relative bioluminescence estimated by Bezier curve.

Bezier curve estimation (Lux/cell)			
pH	Stationary	Exponential	Ratio[a]
2.00	72.10	283.29	0.25
2.50	80.58	371.59	0.22
3.00	96.89	549.92	0.18
3.50	119.00	618.06	0.19
4.00	140.32	726.10	0.19
4.50	85.81	771.99	0.11
5.00	203.47	3914.97	0.05
5.50[b]	3978.10	19,584.47	0.20
6.00[b]	6029.06	20,860.13	0.29
6.50[b]	5840.77	22,994.12	0.25
7.00[b]	6999.41	24,342.51	0.29
7.50[b]	7706.46	24,073.10	0.32
8.00[b]	7708.49	22,317.87	0.35
8.50[b]	6714.23	21,542.82	0.31
9.00[b]	5683.10	20,512.03	0.28
9.50	5473.22	16,249.86	0.34
10.00	3355.64	3916.35	0.86
10.50	1146.37	1424.68	0.80
11.00	356.28	1091.79	0.33
11.50	150.24	1042.03	0.14
12.00	181.23	1060.89	0.17
12.50	232.07	1095.13	0.21
13.00	283.38	1115.01	0.25
13.50	290.57	1080.02	0.27

[a] Ratios were calculated for Stationary/Exponential.
[b] indicates working range of the biosensor.

4. Discussion

In the SA biosensor strain *A. baylyi* ADPWH_*lux*, a promotorless *luxCDABE* cassette is fused with *salA* and the expression is regulated by SalR (Huang et al., 2005). The five *lux* genes encode the components of a self-contained light emitting system that does not require exogenous substrate (Winson et al., 1998). The bioluminescence method is quantitative, and it showed an increased sensitivity for detecting SA induced expressions in a wider pH range (Figs. 3 and 4), compared with RT-PCR amplification of the *salA* transcript (Fig. 1). It is assuring that the two methods produced results largely conforming to each other for both exponential and stationary growth. In general, SalR regulated protein expression was fairly reliable in pH 5.54–9.05 in the exponential growth and pH 5.79–9.79 in the stationary growth (Figs. 3B and 4B). Bioluminescence production maintained at >80% (exponential growth) and >60% (stationary growth) levels within these two ranges, respectively. This indicated that the LuxCDABE activity *in vivo* was not drastically affected by the medium pH, although damages on the lux activity could not be ruled out outside of this pH range. However, as RT-PCR was negative in all the pH conditions in which bioluminescence signal declined, it is more likely that the reduction of bioluminescence signal was mainly due to reduced *luxCDABE* transcription rather than damaged LuxCDABE activities.

Fig. 3B showed that the SalR regulated bioluminescence production peaked at medium pH 7.16, indicating that SalR regulation requires an optimum environment to perform the maximum promotion of its regulated gene expression in the exponential growth. Because growth was not deleteriously affected during pH 5.54–9.05 (Fig. 3A), generic physiological and biochemical slow down was not in evidence. Therefore, the reduced (up to 20%) expression was locally in the *sal* operon rather than globally. In the stationary phase, no growth was observed at the neutral pH (Fig. 4A) and the *sal* operon could only be promoted to achieve about 30% of the maximum production level of the exponential growth (Fig. 5, Table 2), indicating that it was subject to a global control (Gerischer, 2002; Marques et al., 2006). The ratios of relative bioluminescence signals (stationary/exponential) were stable at 20–35% between pH 5.5–9.0 (Fig. 5B, Table 2), indicating that the global repression on *sal* operon was similar throughout this pH range in which the exponential growth was not affected (Fig. 3A).

By using the *A. baylyi* ADPWH_*lux* biosensor strain, we conclude that the SalR regulating gene expression is fairly stable at a pH range of 5.5–9.0, demonstrating that a LTTR tolerance in environment change. As some other LTTRs, *salR* operon is subject to controls by global factor (s). The global repression can be up to 80% for the induced expression. The induced *sal* expression suffers in both acidic and alkaline conditions, in which growth is inhibited in the exponential phase. This work determined the pH range of the biosensor strain. Additionally, as for the pH conditions, this strain may be useful to assess *salR* operon performance under many other environmental conditions, e.g. ionic strength, nutrition, and temperature, etc.

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