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The Essential WalK Histidine Kinase and WalR Regulator Differentially Mediate Autolysis of *Staphylococcus aureus* RN4220

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Abstract

The two-component regulatory system, WalR/WalK is necessary for growth of different gram-positive bacteria, including *Staphylococcus aureus*. In present study, we confirmed the essentiality of both the histidine kinase protein WalK and the response regulator WalR for growth using *S. aureus* RN4220 strain and demonstrated that the histidine kinase protein WalK and the response regulator WalR function differently in regulation of staphylococcal autolysis. The down-regulation of *walR* expression effectively inhibited Triton X-100-induced lysis and had a weak impact on bacterial tolerance to penicillin induced cell lysis. In contrast, the down-regulation of *walK* expression had no influence on either Triton X-100- or penicillin-caused autolysis. Moreover, we determined the effect of WalR and WalK on bacterial hydrolase activity using a zymogram analysis. The results showed that the cell lysate of down-regulated *walR* expression mutant displayed several bands of decreased cell wall hydrolytic activities; however, the down-regulation of WalK had no dramatic impact on the hydrolytic activities. Furthermore, we examined the impact of WalR on the transcription of *cidA* associated with staphylococcal autolysis, and the results showed that the down-regulation of WalR led to decreased transcription of *cidA* in the log phase of growth. Taken together, the above results suggest that the essential WalR response regulator and the essential WalK histidine kinase might differently control bacterial lysis in RN4220 strain.

Keywords

S. aureus; essential WalRK; autolysis; tolerance of penicillin

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Introduction

Staphylococcus aureus is a major animal and human pathogen that causes a wide range of infections [1]. The emergence of multi-drug resistant staphylococcal isolates, especially, methicillin resistant *S. aureus* (MRSA), is generating enormous public health concern and highlights an urgent need for new, alternative agents for treating multi-drug-resistant pathogens. Previous studies demonstrated that a two-component regulatory system, WalRK (also named YycFG or VicRK), is essential for different gram-positive bacteria [2-6], and indicated that WalRK may be a potential target for developing novel antibacterial agents [7-8]. Numerous studies have revealed that daptomycin resistance involves mutation of this essential regulatory system [9-11].

Bacterial autolysins play important roles in cell wall biosynthesis pathway, including cell separation and ongoing peptidoglycan remodeling [12-13]. Autolysins are responsible for hydrolysis of peptidoglycan. Autolysin (Atl) is composed of glucosaminidase (GL) and amidase (AM) domains and contains two extracellular lytic enzymes through extracellular hydrolysis process, including a 51-kDa GL and a 62-kDa AM, which cleaves MurNAc(1-4)GlcNAc and GlcNAc(1-4)MurNAc, respectively [14-15]. Peptidoglycan hydrolases are involved in bacterial autolysis by hydrolyzing either the glycan or the peptide moieties of peptidoglycan of the gram-positive cell wall [16]. The major peptidoglycan hydrolases in staphylococci include N-acetyl muramidase, N-acetyl glucosaminidase, N-acetylmuramyl-L-alanine amidase, transglycosylases, and endopeptidase [17-19]. It has been clearly indicated that these peptidoglycan hydrolases are involved in critical biological processes during cell division and growth, including cell wall biosynthesis, daughter cell separation, and cell wall turnover in gram-positive bacteria [20-21].

The production and activity of peptidoglycan hydrolases must be tightly controlled due to their importance for the maintenance of bacterial cell integrity and growth. Different regulators coordinately regulate the expression of the peptidoglycan hydrolases in *S. aureus*. Negative regulators include the two-component systems, *lytSR* and *arlRS*, which repress the expression of genes involved in peptidoglycan hydrolases activity [12, 22], and transcriptional regulators such as *sar* [23] and *rat* (also known as mgr) [24]. Both LytSR and ArlRS mediate the expression of *lrgA* and *lrgB*, encoding murein hydrolase transporter proteins (known as holins) that are able to inhibit murein hydrolases [12, 25, 26]. On the other hand, the Agr two-component regulator [23], CidAB [27], and Gcp [28] positively affect the activity of peptidoglycan hydrolases.

It has been demonstrated that the essential WalRK system is involved in modulation of staphylococcal autolytic activity and controls cell wall metabolism through regulation of the expression of autolysins (AtlA and AtlM) in *S. aureus* [29]. However, it is unclear whether both the essential histidine kinase WalK and WalR (WalR) are involved in modulation of staphylococcal autolysis. In this study, we further defined the essentiality of the histidine kinase WalR and the corresponding regulator WalR and determined their roles in controlling autolysis of *S. aureus* RN4220 laboratory strain.

Materials and Methods

Bacterial strains and growth media

Both the *Pspac*-regulated *walR* and *Pspac*-regulated *walK* expression mutants were created using *S. aureus* RN4220 laboratory strain in this study. The bacterial cells were incubated in Trypticase soy broth (TSB) with appropriate antibiotics at 37°C with shaking unless otherwise stated. *E. coli* cells were grown in Lysogeny Broth (LB) medium.

Construction of *Pspac*-regulated *walR* and *Pspac*-regulated *walK* mutant strains

In order to examine the effect of both WalR and WalK on autolysis of *S. aureus*, a *Pspac*-regulated *walR* expression mutant and *a Pspac*-regulated *walK* expression mutant were created as described [30]. Briefly, a 0.5 kb fragment from the 5′ end of *walR* or *walK*, including a putative ribosome binding site was obtained by PCR. The PCR fragment was digested with *Eco*RI and *Bam*HI and cloned into pSMUTery integration vector ere amplified by PCR and cloned into the downstream of the IPTG-inducible *Pspac* promoter region of pSMUTery vector (a derivative of pMUTIN4 without the *lacZ* gene, a gift from Simon Foster, University of Sheffield, UK), respectively. The resulting recombinant plasmid was then electroporated into *S. aureus* competent cells, and integrations of the plasmid through a single crossover event were selected with erythromycin. The *Pspac*-regulated *walR* and *Pspac*-regulated *walK* expression mutants were confirmed by Southern blot analysis (data not shown) and designated as RNPspac-WalR or RNPspac-WalK.

Titration of bacterial growth

S. aureus growth curves were obtained using an automated microtiter plate format. *S. aureus* strains were incubated at 37°C overnight in TSB with appropriate antibiotics. The cultures were diluted to ~10^4 CFU/ml with TSB containing appropriate antibiotics and IPTG at concentrations of 0, 2.5, 5, 10, 25, 50, 100, 1000μM. Cell growth was monitored at 37°C by measuring OD600nm every 15 min with 1 min mixing before each reading.

Triton X-100-incuced autolysis assays

Autolysis assays were performed as previously described [28]. Both the RNPspac-WalR and RNPspac-WalK cells were grown in TSB containing 1 mM IPTG and appropriate antibiotics at 37°C, with shaking, to an optical density at 600 nm (OD600) of 1.2 to 1.3. The bacterial cultures were then diluted 1:100 with fresh TSB containing 1 M NaCl, with or without inducer (1 mM IPTG), and incubated to an OD580 of 0.6 to 0.8 at 37°C. The bacterial cells were harvested by centrifugation at 4,000× *g* and were suspended in the same volume of buffer containing 50 mM Tris-HCl (pH 7.5) and 0.1% Triton X-100. The bacterial cells were then incubated at 30°C with shaking, and the changes in OD580 were measured. Results were normalized to the OD580 at time zero (OD0), i.e., percent lysis at time *t*= [(OD0- OD at time *t*)/OD0] × 100. All experiments were repeated at least three times.

Penicillin tolerance assay

To assess the sensitivity of the RNPspac-WalR and RNPspac-WalK conditional mutants to penicillin, the mutants were incubated in TSB containing 1 mM IPTG and appropriate

antibiotics at 37°C, with shaking, to an OD600 of 1.2 to 1.3. The bacterial cultures were then inoculated at 1% with fresh TSB in the absence or presence of 1 mM IPTG inducer and grown at 37°C, with shaking, to reach exponential phase (OD600 of 0.5). Penicillin G was added to a final concentration of 8 μg/ml (20 × MIC). Cultures were incubated continuously, and the OD600 values for cultures were measured every hour for 8 h.

Zymographic analysis

To examine the effect of WalR and WalK on the activity of murein hydrolases, we conducted zymographic analyses as described previously [28]. Briefly, Both the RNPspac-WalR and RNPspac-WalK bacterial cells were grown in TSB, with or without inducer IPTG, for 16 h at 37°C with shaking. The extracellular murein hydrolases were isolated from the cultures by centrifugation at 10,000 ×*g* for 15 min at 4°C. The supernatants were collected, filter sterilized, and concentrated 100-fold by ethanol precipitation overnight at 4°C. The concentration of total proteins in each sample was determined by using the Bradford assay (Pierce Biotech) according to the manufacturer's instructions. A total of 10 mg of proteins from each sample was resolved in a 10% SDS-PAGE gel containing 0.2% autoclaved and lyophilized *S. aureus* RN4220 wet cells. After electrophoresis, gels were washed with water and incubated overnight in 25 mM Tris-Cl, pH 7.0, containing 1% Triton X-100 at 37°C to allow hydrolysis of the embedded bacterial cells. After incubation, gels were scanned (HP Scanjet 4570c). The zones of hydrolysis appeared as white bands in the gels, but black bands indicate regions of murein hydrolase activity in the figure.

Construction of promoter-*lux* reporter fusion system

To determine the effect of WalR on *cidA* transcription, we created a *cidA* promoter-*lux* reporter fusion system by using pFF40 vector [31] as described previously [28]. The amplified 6kb fragments of *luxABCDE* were digested with *EcoR*I and ligated into the *EcoR*I site of pFF40 vector, which resulted in plasmid plux-FF40. The promoter region of *cidA* was amplified by PCR, digested with FseII, and ligated into the FseI site of plux-FF40, resulting in plasmid p2329pr-lux-FF40 as described [28]. The reformed plasmid was electroporated into *S. aureus* RNPspac-WalR generating RNF2329 strain, and *lux* expression was monitored with a Chiron luminometer.

Data analysis

The results were statistically analyzed using Student t-test and $P < 0.05$ was considered significant difference.

Results

Both the WalR response regulator and the WalK histidine kinase are necessary for growth of *S. aureus* RN4220

Using a temperature sensitive mutagenesis, it has been revealed that WalRK is required for survival of *S. aureus* [3]. It has been well known that RN4220 strain possesses numerous mutations [32]. In order to elucidate whether these mutations have any impact of the essentiality of WalRK and confirm the essentiality of WalR and WalK, we employed an alternative approach to examine the dependence of growth on the expression of WalR or

WalK using RN4220 strain. A *Pspac*-promoter regulated *walR* or *walK* mutant was constructed in *S. aureus* and confirmed by Southern hybridization as described [30]. To ensure sufficient repressor levels, the LacI-expression vector pFF40 [31] was introduced in the strain RNPspac-WalR or RNPspac-WalK and resulted in RNPspac-WalR/pFF40 or RNPspac-WalK/pFF40.

The IPTG dependent growth was detected by measuring the cell density (OD_{590nm}) every 15 min (Fig.1). Without IPTG, neither the *Pspac*-regulated w*alR* expression mutant (Fig. 1A) nor the *Pspac*-regulated w*alK* expression mutant (Fig. 1B) was capable of growth; in contrast, addition of IPTG to the culture medium of mutant strains restored their ability to grow to normal levels in a dose-dependent manner. These results demonstrated that both WalR and WalK are indispensible for growth of *S. aureus* RN4220 strain.

Down-regulation of *walR* expression decreases Triton X-100-induced autolysis

Although it has been reported that the WalRK system controls the bacterial autolysis [29], it is unclear whether both the response regulator WalR and its corresponding histidine kinase WalK are involved in modulation of autolysis. To decipher their functions, we firstly examined the effect of WalR on susceptibility to cell lysis induced by a nonionic detergent, Triton X-100, using the *Pspac*-regulated *walR* mutant strain. In the presence of 1 mM IPTG, 70% of the bacterial cells harvested from a mid-exponential-phase culture lysed within 3 h at 30°C in the presence of 0.1% Triton X-100 (Fig.2A). In contrast, fewer than 10% of the down-regulated *walR* mutant cells lysed within 3 h in the absence of IPTG (Fig. 2A).

However, the down-regulation of the *walK* expression had no remarkable effect on Triton X-100-induced autolysis comparing to the control with 1 mM of IPTG (Fig. 2B).

Down-regulation of *walR* expression has a weak effect on bacterial tolerance to penicillin-induced autolysis

Penicillin induces bacterial lysis through β-lactam moiety's binding to the transpeptidase and inhibiting the transpeptidation reaction and the pentaglycine bridge formation between two peptidoglycan chains [33]. We examined the effect of both WalR and WalK on penicillin-induced cell lysis in the presence of 8 μg/ml of penicillin (20 x MIC), using the *Pspac*-regulated *walR* or *walK* expression mutant as described (Zheng *et al.* 2007). In the presence of the inducer IPTG (1 mM), penicillin-induced cell lysis significantly increased from 0 to 40% within 8 hours period (Fig. 3A). In contrast, in the absence of IPTG, the *Pspac*-regulated *walR* mutant cells tolerated to penicillin-induced lysis (Fig. 3A). However, for the *Pspac*-regulated *walK* expression mutant, the addition 1 mM of inducer IPTG had no influence on penicillin-induced cell lysis compared to the absence of IPTG (Fig. 3B).

Down-regulation of *walR* expression inhibits the autolysins activity

In order to explore the reason why WalR and WalK differentially modulate of both detergent X-100 and penicillin induced cell lysis, we performed zymographic assays and determined the effect of both WalR and WalK on the hydrolytic activity described previously [24, 28]. No significant difference of zymographic patterns was observed in the exported hydrolases that were isolated from the supernatants of the stationary cultures of

either the *Pspac*-regulated walK expression mutant with or without the inducer IPTG (Fig. 4, lane 1 to 2). In contrast, using the exported hydrolases isolated from the supernatants of stationary cultures of the *Pspac*-regulated w*alR* expression mutant in the absence of the inducer IPTG, the zymogram displayed several bands of reduced murein hydrolytic activity, especially to hydrolases of between 52 and 93 kDa and those under 30KD (Fig. 4, lane 3).

Down-regulation of WalR has different effects on the expression of *cidA*

It has been revealed that the *cidAB* operon is positively associated with autolysis of *S. aureus* [27]. It was reported that WalR is able to medicate bacterial autolysis through transcriptional regulation of autolysins (AtlA) and peptidoglycan hydrolase (LytM) by binding to the promoter regions of *atlA* and *lytM* genes [29]. In order to explore whether WalR may regulate autolysis through the modulation of *cidA*, we constructed a transcriptional *cidA* promoter-*lux* reporter system using the *Pspac*-regulated *walR* expression vector. Bioluminescence activity was detected using a luminometer. No light signal was detected in the *Pspac*-regulated *walR* expression control strain carrying a promoterless plux-FF40 (data not shown). In the absence of inducer IPTG, the luciferase activity significantly decreased in the log phase cultures compared to those in the presence of IPTG in the *Pspac*-regulated *walR* expression strain carrying *cidA* promoter-*lux* reporter (Fig. 5). This suggests that WalR may control the transcription of *cidA* in the log phase of growth. To examine whether WalR directly or indirectly regulates the transcription of *cidA*, we performed gel shift assay and found that WalR was unable to bind the *cidA* promoter region (data not shown), whereas WalR bound to the promoter region of *lytM* (data not shown), indicating that WalR probably indirectly mediates the *cidA* transcription in *S. aureus*.

Discussion

In this study, we defined that both WalR and WalK are indispensible for growth of *S. aureus* RN4220 during *in vitro* culture. This is consistent with previous findings using an alternative approach [3] and a similar strategy [6]. Our data also indicate that the identified numerous mutations in RN4220 chromosomal DNA [32] have no effect on the requirement of WalRK. Moreover, our data are the first to suggest that the essential histidine kinase WalK may not play an important role in regulation of staphylococcal autolysis, whereas the response regulator WalR positively controls the autolysis.

Different regulatory mechanisms are involved in regulation of autolysis such as modifying cell wall peptidoglycan (the substrate of murein hydrolases) and regulating the expression of genes associated with cell wall hydrolases activities [25, 27, 28]. Our results clearly indicate that WalR is associated with the modification of the cell wall peptidoglycan synthesis, because the consequences of down-regulating *walR* expression include increased bacterial resistance to detergent- and penicillin-induced lysis. Our data show that the essential WalRK system effectively regulates bacterial autolysis induced by nonionic detergent, Triton X-100, is consistent with the previous report [29]; but, WalR seems play a moderate role in controlling penicillin-induced autolysis. On the other hand, our data show that WalR, but not WalK, also partially contributes to the positive regulation of different murein hydrolases

activities. Taken together, our studies suggest that WalR and WalK may play different roles in the modulation of staphylococcal autolysis. However, we cannot rule out the possibility of polar effect due to the integration of the *Pspac*-regulatory cassette into *walR* and *walk* genes. In addition, we observed that the down-regulation of *walR* differently affected hydrolyses activities, including decreased hydrolases activities under 93 kDa, but increased hydrolases activities above 95 kDa, with unknown mechanism (Figure 5).

The *cidA* promoter-*lux* reporter studies clearly indicate that WalR mediates the transcription of the *cidAB* operon, which encodes the holin-like counterpart of the *lrgAB* operon and is a positive regulator of cell autolysis in *S. aureus* [27]. Gel-shift analysis showed that WalR could not bind the *cidA* promoter region (data not shown). Taken together, these suggest that besides directly controlling the autolysins activities WalR probably indirectly involves in the modulation of autolysis via positive controlling the production of CidA. Our results are partially consistent with Dubrac et al (2007) report that WalRK positively controls the transcription of *lytM*, *atlA*, *isaA*, *sceD*, and *ssaA* genes. It has been demonstrated that WalR directly medicates bacterial autolysis through transcriptional regulation of autolysins (AtlA) and peptidoglycan hydrolase (LytM) by binding to the promoter regions of *lytM* genes [29]. In this study, we also confirmed that WalR is able to regulate *lytM* gene in *S. aureus* RN4220 (data not shown).

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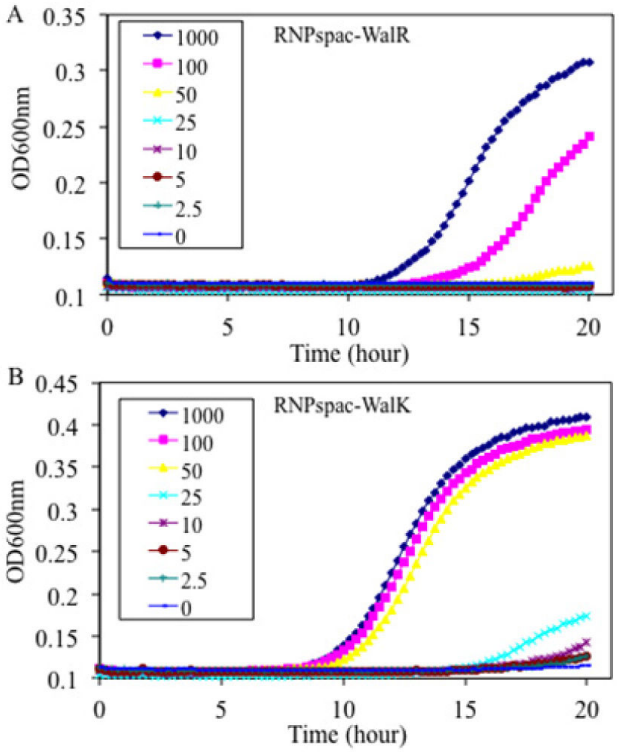

Figure 1.

IPTG dependent growth of the *Pspac*-regulated *walR* mutant (A) and the *Pspac*-regulated *walK* mutant (B). The *Pspac*-regulated *walR* mutant (RNSpac-WalR) and the *Pspac*-regulated *walK* mutant (RNPspac-WalK) were incubated overnight in the TSB in the presence of 1mM IPTG. The bacteria were diluted in fresh TSB and incubated in TSB with different concentrations of IPTG (μM) at 37°C with shaking. The optical density was measured every 15 min for 20 hours at OD600nm.

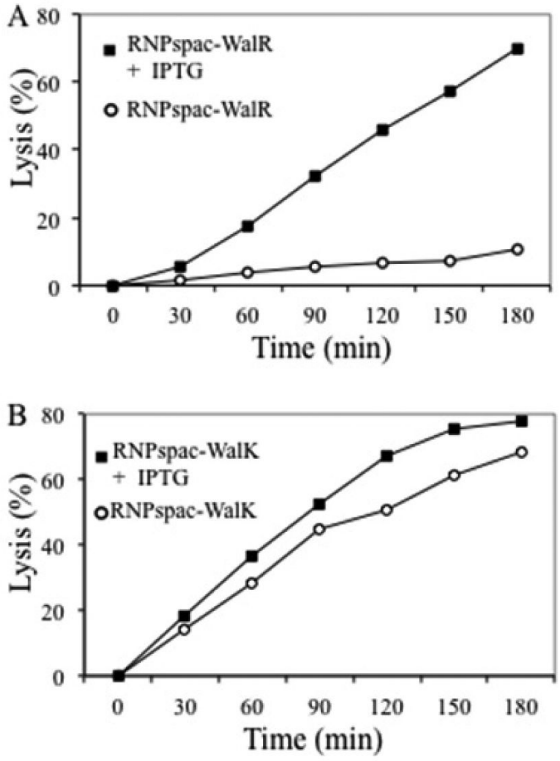

Figure 2.

Triton X-100-induced autolysis of the conditional w*alR and walK* mutants. The *Pspac*-regulated *walR* mutant, RNPspac-WalR (A) and the *Pspac*-regulated *walK* mutant, RNPspac-WalK (B) were grown in TSB in the presence of different concentrations of IPTG (solid square:1 mM; open circle: 10 μM) or in the absence of IPTG to an OD_{580} of 0.6-0.8. The bacterial cells were harvested by centrifugation and resuspended in the same volume buffer containing 50 mM Tris-HCl (pH 7.5) and 0.1% Triton X-100. The cells were incubated at 30°C with shaking, and the changes of optical densities (OD_{580}) were measured. Results were normalized to OD_{580} at time zero (OD_0). The percent lysis at time *t* = $[(OD_0\text{-}OD_t)/OD_0] \times 100$. The experiments were repeated at least three times. Each figure represents the results of one experiment.

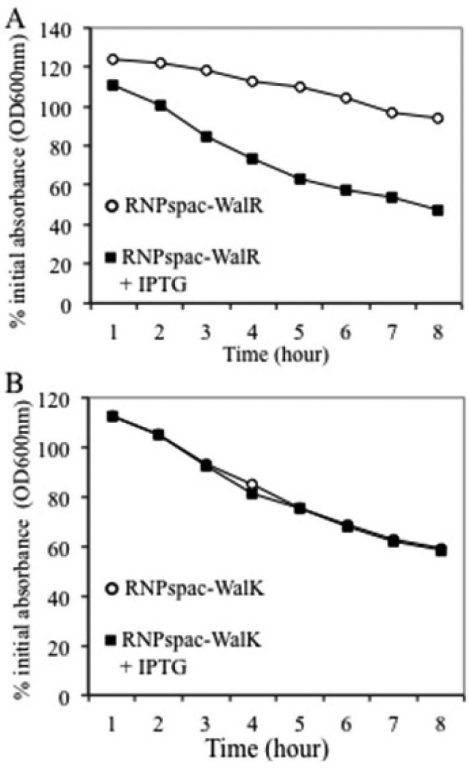

Figure 3.

Effect of WalR and WalK on penicillin-induced bacterial autolysis. The *Pspac*-regulated *walR* mutant, RNPspac-WalR (A) and the *Pspac*-regulated *walK* mutant, RNPspac-WalK (B) were grown in TSB in the presence of different concentrations of IPTG (solid square:1 mM; open circle: 10 μM) at 37°C with shaking to reach the exponential phase (OD_{600} ~0.5). Penicillin (20 × MIC) was added to the exponential growth cultures at a final concentration of 8μg/ml. The bacterial cultures were continuously incubated, and the optical densities of the cultures were measured at OD_{600} every hour for 8 hours.

Figure 4.

Zymogram analysis of the *Pspac*-regulated w*alR* and *walK* mutants. Equal amounts (10μg) of proteins prepared from the supernatants of the *Pspac*-regulated *walR* and *walK* mutants were loaded and separated on 10% SDS-PAGE containing *S. aureus* RN4220 cells (0.2% wet weight of heat-killed cells). Following electrophoresis, gels were soaked in water for 30 min at room temperature under gentle agitation, then transferred to a renaturing buffer (25mM Tris-HCl, pH 7.0 containing 1% Triton X-100) and incubate at 37°C with gentle agitation for 1-4 h. Lytic bands appeared as clear zones on an opaque background, but showed dark zones after scanning. Lane 1, RNPspac-WalK without IPTG; Lane 2, RNPspac-WalK with IPTG; Lane 3, RNPspac-WalR without IPTG; Lane 4, RNPspac-WalR with IPTG.

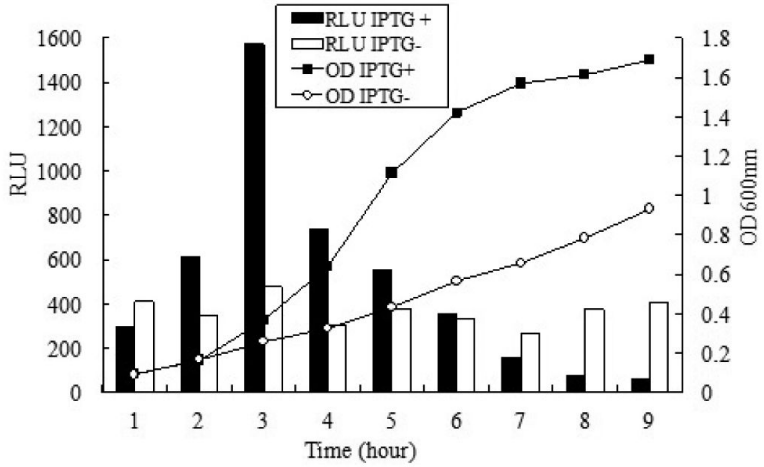

Figure 5.

The effect of the down-regulating w*alR* on the *cidA* promoter activity using *cidA* promoter-*lux* reporter fusion. The *Pspac*-regulated *walR* mutant carrying *cidA* promoter-*lux* reporter was grown in TSB in the presence of different concentrations of IPTG (solid square: 1 mM; open circle: 10 μM) at 37°C with shaking. Both bioluminescence signals and cell growth were monitored at different phases of growth at 37°C by measuring the light intensity with a Chiron luminometer and optical density at 600 nm (OD_{600}) with a SpectraMax plus Spectrophotometer. Relative Light Unit (RLU) was calculated with the bioluminescence intensity divided by the optical density in the same time of culture. The experiments were repeated at least three times.