Quorum sensing blockade in *Pseudomonas aeruginosa*: biodegrading of N-Acyl homoserine lactone by *Bacillus cereus* UT26 and its consequent effects on their interaction

Maryam Zamani*, Keivan Behboudi, Masoud Ahmadzadeh

Department of Plant Protection. Faculty of Agricultural Science and Engineering, University of Tehran, Iran.

*Corresponding Author: email address: mzamany@ut.ac.ir (M.Zamani)

ABSTRACT

Bacteria use a chemical language to communicate with each other which enables them to synchronize gene expression as a result of cell density. This special language which is called quorum sensing (QS) is based on producing and sensing the small signals and eventually leads them to perceive when a minimum population unit is reached. On the contrary, quorum sensing blockade (quorum quenching) has been recently grabbed scientists attention as a promising tool in controlling bacterial pathogens which utilize N-acyl homoserine lactone (AHL) to regulate their virulence factor production. Biodegradation of AHL molecules is an efficient way in QS interrupting which normally happens in the natural environment. In this survey, the effect of *Bacillus cereus* UT26 strain as an efficient quorum quencher on degrading both signal molecules (C4-HSL and 3oxoC12-HSL) of *Pseudomonas aeruginosa* was investigated. The strain strongly degraded both AHLs and hereby inhibited pyocyanin production in the lab condition. Moreover, co-culture experiments revealed that quorum quenching activity has an important role in interaction between different bacterial species; since the wild type (wt) strain of *B. cereus* UT26 reached 1000 fold higher growth yield comparing *aiiA* mutant strain of which has lost quorum quenching activity by a site directed mutation in AiiA lactonase gene.

Keyword: Quorum sensing; Quorum quenching; Acyl homoserine lactone

INTRODUCTION

Quorum sensing (QS) is one of the most important regulatory systems which is found in the vast majority of bacteria. QS is usually responsible for cell-cell communication phenomenon and synchronizes gene expression as a result of cell density. This system is based on diffusion and response to low molecular weight signals. Aside molecular signals, QS has another active component which is a signal repressor (LuxR protein) [1]. When a signal molecule binds to its cognate receptor, the result complex actives the expression of QS-regulated genes. So, the principal behind QS system is shared between Gram-negative and Gram positive bacteria but the signal molecule and molecular mechanism is different. Gram negative bacteria mainly use N-acyl-homoserine lactone (AHL) signals which contain the same homoserine lactone moiety but differ in the length and structure of the acyl chain. The AHL mediated QS system regulate various biological functions such as biofilm formation, antimicrobial compound production, swarming, conjugation, virulence, bioluminescence etc [2]. In Proteobacteria AHL-mediated QS has a crucial role on controlling antibiotic production. For example the production of violacein, a purple antibiotic, in *Chromobacterium violaceum* and phenazine in *P. aureofaciens* is controlled by QS [3, 4] . In *Pseudomonas aeruginosa* QS involves in production of different antimicrobial compounds including, pyocyanin, hydrogen cyanide, rhamnolipid and HQNO [5, 6].

P. aeruginosa is a Gram-negative bacterium (γ-proteobacteria), very tolerant to a wide range of temperatures (4-42˚C) and a very much common bacterium found in nosocomial and life-threatening infections of immune compromised patients [7]. It can cause various chronic and acute [8]: nosocomial peneumonia cases, surgical

wound, bloodstream and burn wound infections, ventilator-associated peneumonia in incubated patients, etc. It is also involved in chronic infections of respiratory system including bronchiectasia and cyctic fibrosis. *P. aeruginosa* can be isolated from different habitats from mammalian tissues to soil. *P. aeruginosa* produces a wide range of virulence factors which renders it highly pathogenic for susceptible patients and as it mentioned before the synthetizing of many of the key virulence factors as well as biofilm formation is controlled by QS system [7, 8]. Although it is more common between the bacteria to have only one AHL type involving in QS system, in *P. aeruginosa*, QS is organized in hierarchical way and does its responsibility at two level using two distinct types of AHLs; the short acyl chain C4-HSL (for the *rhlI/rhlR* system) and the long acyl chain 3oxoC12-HSL (for the *lasI/lasR* system). In fact, the synthesis of C4-HSL is triggered by 3oxoC12-HSL to double to make sure that all needed environmental factors as well as bacterial cell density are in an appropriate condition [8].

Bacterial infections cause tremendous personal and economical lost annually and needless to say that more information in bacterial biology, better approaches to fight bacterial disease. The discovery and then invention of the antibiotics in 1920s was a great progress in treating fetal bacterial disease but by developing resistance against them, they became non-sustainable and let the pathogens again to grow rapidly and cause death and illness worldwide [9]. Since conventional antibiotics has targeted the essential life process or growth in bacteria and hereby kill bacterial cells or inhibit bacterial growth, they has acted as a selective pressure and eventually leaded to arise antibiotic resistance in their bacterial targets [7]. Fortunately, the fact that many bacteria such as *P. aeruginosa* use AHL mediated quorum sensing to synchronize essential activities for infection or surviving in the host suggests a promising alternative way to control of bacterial disease which is interrupting their quorum sensing system or quorum quenching [10]. Quorum quenching activity is cell-cell communication blockade. There are different potential ways to interrupt QS system which are mainly based on signal degradation or repressor inactivation. de Nys et al., 1993 [11] showed the first example of QS-blocking compounds in *Delisea pulchra*, a red alga, produces halogenated furanones which inhibit bacterial QS by mimicking AHL molecules and consequently rapid turnover of LuxR protein [12, 13]. Moreover, the presence of QS inhibitors found in natural foods such as garlic is extremely interesting [7].

In more complex microbial communities such as soil, there are very many bacterial species with the ability of quorum quenching [14]. It has been showed that this natural quorum quencher strains which degrade AHLs very efficiently using specific enzymes such as AiiA lactoanses in some of *Bacillus* species [14-16]. The effect of quorum quenching ability is still unclear. One possibility could be increasing their fitness in competition with the bacteria which utilize AHLs to regulate antibiotic production. In this survey we investigated the quorum quenching activity of *B. cereus* UT26, a very efficient native quorum quencher strain [17],on degrading AHL signals of *P. aeruginosa* and interrupting of one of following QS functions (pyocyanin production) and finally the effect of UT26 quorum quenching activity on its resistance against *P. aeruginosa* in laboratory co-cultures.

MATERIALS AND METHODS

Chemicals, bacterial strains, media and culture condition

N- Butyryl-L-homoserine lactone (C4-HSL) and N-3-(oxododecanoyl) -L-homoserine lactone (3oxoC12-HSL) were purchased from Sigma-Aldrich Chemicals Co., (MO, USA). Stock solution (1 mM) of each compound was prepared in acetic acid acidified ethyl acetate (0.01% v/v). Bacterial strains used in this study are shown in Table 1. The media used was modified Luria-Bertani (LB) buffered with 50 mM MOPS, pH 7.0.

Quantification of synthetic AHL degrading ability of Bacillus cereus UT26

The *Bacillus cereus* UT26 was previously reported as an efficient wild type quorum quencher strain with AiiA lactonase activity [17]. Acyl-HSL degradation capability of *B. cereus*

UT26 strain was evaluated by incubating the strain with synthetic C4-HSL and 3oxoC12-HSL in a time course assay. The bacterial cultures were established in buffered LB (pH 7). DH5α/pECP61.5 and DH5α/pJN105-*lasR*/pSC11 were used as C4-HSL, 3oxo C12-HSL reporters, respectively according to [6, 18] with slight modifications. An overnight culture of UT26 was diluted 1:100 in fresh medium and grown to mid-log phase (OD_{600} 0.4-0.5). Mid log culture was centrifuged and the pellet resuspended in 20 ml PBS (pH 7). The result suspension (OD_{600} 0.5) was added to clean flasks containing adequate signals to reach the AHL concentration of 5 µM and then incubated at 30°C. Three ml aliquots were removed 15, 30, 60, 90 and 120 min after beginning the experiment, and residual signals were re-extracted by two extraction with 1X acidified ethyl acetate. Solvent was evaporated from extracts under nitrogen and remaining signals were quantified using Beta-gal assay kit (Invitrogen, USA) and quantification of AHL residual content in each sample was evaluated by measuring luminescence emitted from each sample on plate reader (100-500 ms read per well). Standard curves for bioassay were generated using synthetic C4-HSL or to 3oxoC12-HSL. Treatments consisted of 4 replicates and the experiment repeated 3 times.

Effect of B. cereus UT26 on pyocyanin production in P. aeruginosa in co-culture

P. aeruginosa PAO1 and *B. cereus* UT26 were grown separately overnight in LB (buffered with MOPS) and then diluted 1:100 in fresh medium and subsequently incubated at 30°C to reach an OD600 0.2-0.5. One ml of each culture was spun down at 11000 rpm for 3 min and pellet was re-suspended in fresh LB. Co-culture of *B. cereus* UT26 with *P. aeruginosa* was started with initial optical density (at 600 nm) of 0. 1 ($2\text{-}3\times 10^7$) for PAO1 and 0.1 ($2\text{-}4\times 10^7$) for *B. cereus*. The cultures were extracted using equal volume of chloroform. After vortexing for 2s, the organic layer was transformed to a micro tube and spun down for 5 min at full speed. One ml of chloroform fraction carefully transferred to the fresh tubes. Two hundred µl of HCl (0.01 N) was added to each tubes and vortexed for 2s. The aqueous fraction was removed and the OD of each sample was measured at 520 nm. Treatments consisted of 4 replicates and the experiment repeated 3 times. *B. cereus* UT26-Δ*aiiA* (A derivate mutant of *B. cereus* UT26 wt disable in AiiA lactonase production) was also tested as a control to make sure all the observations is only due to quorum quenching ability of UT26. Treatments consisted of 4 replicates and the experiment repeated 3 times.

Table 1. Bacterial strains used in this study.

Bacterial strain	Relevant characteristic	Reference
Bacillus cereus UT26	Wild type AHL degrading strain	[17]
B. cereus UT26-Δ*aiiA*	*aiiA* deficient strain. Disable in AHL degrading, derivate of UT26	[17]
Pseudomonas aeruginosa PAO1	Wild type strain	[22]
Pseudomonas aeruginosa DA6 strain	*lasR*$^-$*rhlR*$^-$ derivate of PAO1	[23]

Effect of quorum quenching ability on fitness of B. cereus UT26 in co-culture with P. aeruginosa

This experiment was done in 3 three different steps; First the antagonistic activity of *B. cereus* UT26 against *P. aeruginosa* PAO1 was evaluated. 20 ml of *B. cereus* UT26 wt strain was grown overnight and then pelleted. Supernatant was filter sterilized and the fresh culture of PAO1 at mid-log phase (OD_{600} between 0.3-0.5) was diluted in either 3 ml sterilized supernatant of in 3 ml half and half LB and *Bacillus* supernatant to reach a starting OD of 0.5 at 600 nm. After 16 h incubating at 30°C, the colony forming units (CFU) of PAO1 strain was counted.

In the second step in order to investigate the effect of quorum quenching ability on fitness of *B. cereus* UT26 strain, its competitiveness in the presence of *P. aeruginosa* PAO1 (as QS regulated antibiotic producer) was evaluated in mixed liquid cultures. *P. aeruginosa* PAO1 wt strain, QS

deficient *P. aeruginosa* DA6, (Table 1) wt *B. cereus* UT26 and *B. cereus* UT26-Δ*aiiA* were grown overnight separately and then spun down for 3 min at 11,000 rpm. Pellets were resuspended in 1 ml fresh buffered LB, following with diluting 1:100 in fresh medium. The strains were grown till the cultures reached an OD_{600} between 0.3-0.5. The liquid co-cultures of wt UT26+ PAO1, wt UT26+ DA6, UT26-Δ*aiiA*+PAO1 and UT26-Δ*aiiA*+DA6 were started with initial optical density (at 600 nm) of 0. 1 (2-3× 10^7) for *Pseudomonas* strains and 0.1 (2-4 ×10^7) for *Bacillus* strains. In order to quantification of *B. cereus* strains population in each co-culture, the colony forming units (CFU) *of B. cereus* strains were counted using the selective medium (LB + polymyxin (10 µg/ml) after 16 h incubating at 30°C. Treatments consisted of 4 replicates and the experiment repeated 3 times.

Finally to double check that all the observations in *B. cereus* UT26 competitiveness were only due to AHL degrading activity, the liquid co-cultures were started like the previous step, while this time 5µM exogenous C4-HSL and 3oxoC12-HSL were added to each co-culture.

Data analysis

All data were subjected to the analysis of variance (ANOVA) and statistical mean comparisons within experiments were conducted using Tukey's method.

RESULTS

Evaluation of the ability of Bacillus cereus UT26 on degrading AHL signals

The results revealed that *B. cereus* UT26 efficiently both C4 and 3oxoC12HSL, while its AiiA mutant strain (UT26-Δ*aiiA*) was not successful in degrading both AHLs. In lab condition this strain degraded about 80% of both synthetic signals (Fig 1).

Quorum quenching ability of B. cereus UT26 interrupts in pyocyanin production

Pyocyanin production as one of QS controlled phenotypes in *P. aeruginosa* was inhibited completely in presence of UT26 strain. It showed that this effect was only due to AHL degrading ability of UT26, since the *aiiA* mutant strain couldn't inhibit pyocyanin production as much as wild type strain (Fig. 2).

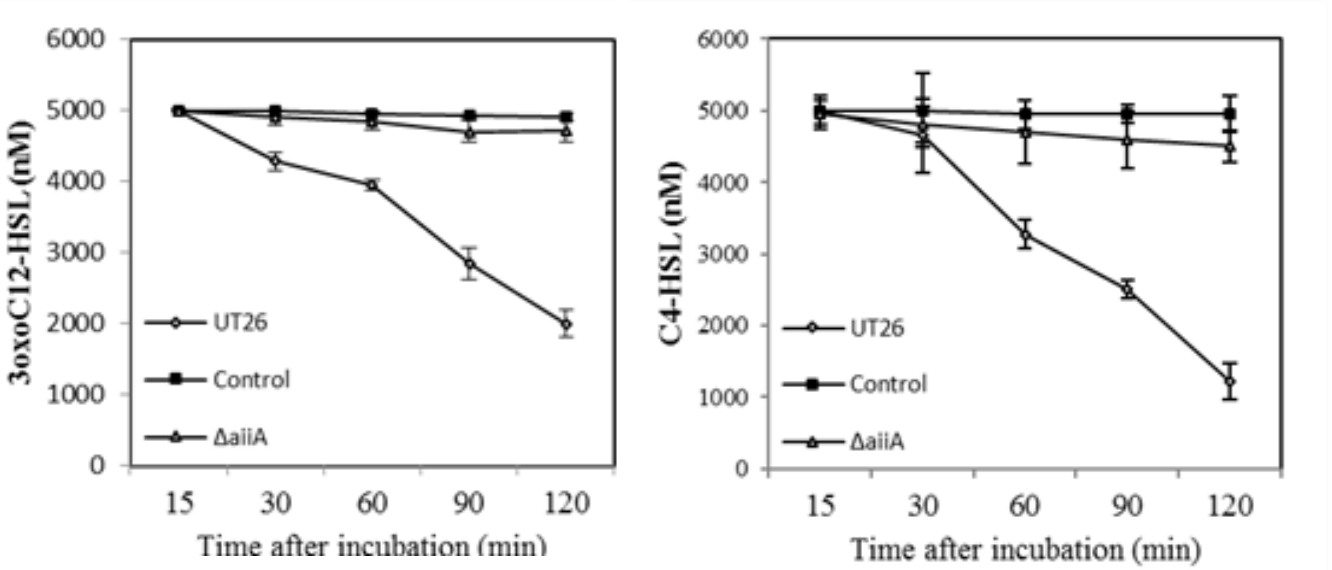

Fig 1. The effect of *Bacillus cereus* UT26 in acyl-homoserine lactones degradation after 120 min incubation. The control was 5000 nM of AHLs in PBS buffer and *B. cereus* UT26-Δ*aiiA* (disable in AHL degrading) used as negative control. Values represent the mean of 3 trials with 4 treatment replications per trial. Vertical bars represent the standard errors.

Effect of quorum quenching ability on fitness of B. cereus UT26 in co-culture with P. aeruginosa

In antagonistic assay we observed that the *B. cereus* UT26 did not have any negative effect on PAO1, since PAO1 grew properly in presence of UT26 filtered supernatant. The results showed that the population of *B. cereus* UT26 wild type strain in co-culture with PAO1 was 1000 fold higher than its AiiA mutant. Surprisingly counting *Bacillus* CFU in co-cultures showed that there was not any significant difference between this treatment (wt UT26 + PAO1) and (UT26-Δ*aiiA* + DA6). The results demonstrated that wt UT26 strain was more resistant to QS regulated antibiotics of *P. aeruginosa* since it grew better in co-culture than its *aiiA* mutant derivate. Since culture supernatant of wild type *B. cereus* UT26 didn't have any negative effect on the growth of the *Pseudomonas* strain, it showed that this QS inhibition was not due to growth inhibition of any of target strains. Moreover, the fitness of UT26 in in co-culture with PAO1 was significantly declined (about 100 fold) by adding exogenous AHL to binary culture (Fig 3). Meanwhile, it was still had a significant difference with the binary culture its AiiA derivate with PAO1, describing that it degraded the exogenous AHLs partially. As it assumed there was not any significant difference between the CFU of *B. cereus* UT26 wt or Δ*aiiA* strain in co-culture with the *lasR*$^{-}$*/rhlR*$^{-}$ mutant strain of *P. aeruginosa*, since the quorum sensing functions was silenced by a site directed mutations in R receptors.

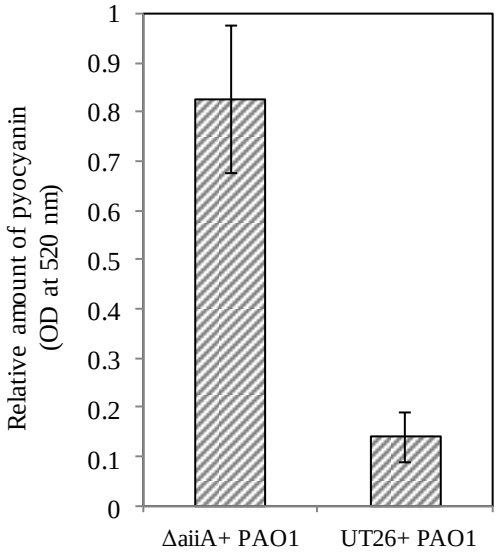

Fig 2. Inhibition of synthesizing pyocyanin in *Pseudomonas aeruginosa* PAO1 by *Bacillus cereus* UT26. Bars labeled with different letters indicate statistically significant differences (Tukey comparisons with 5% family error rate).

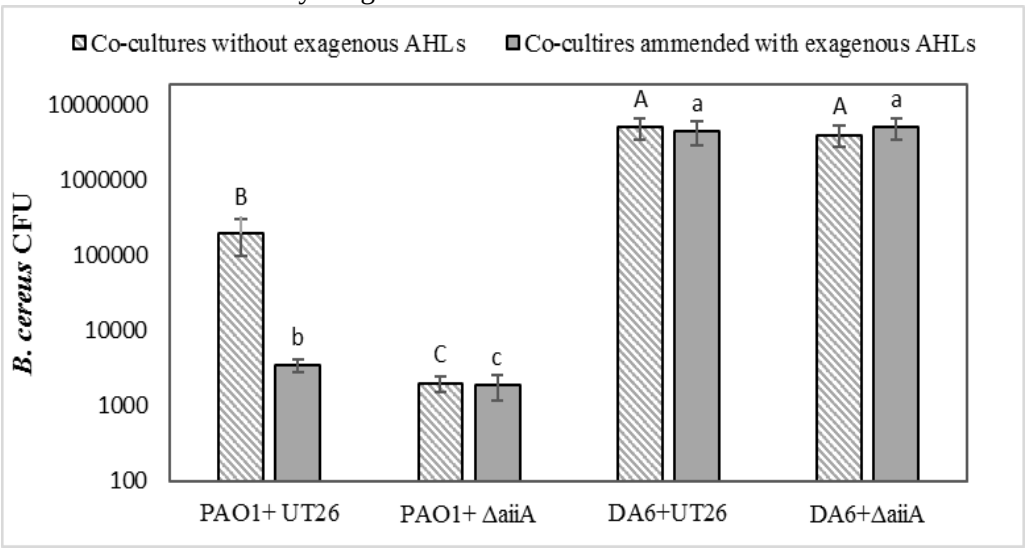

Fig. 3. The population of *B. cereus* UT26 strain (wild type or Δ*aiiA*) in co-culture with wt or QS deficient strain *P. aeruginosa* in absence or presence of 5µM of each synthetic C4-HSL and 3oxoC12-HSL. PAO1: *P. aeruginosa* PAO1 with intact QS system; DA6: *P. aeruginosa lasR*$^{-}$*/rhlR*$^{-}$ as QS deficient strain; UT26: wild type *B. cereus* UT26 strain enable in degrading AHLs; *ΔaiiA*: *B. cereus* UT26-Δ*aiiA* disable in AHL degrading. Bars labeled with different letters indicate statistically significant differences (Tukey comparisons with 5% family error rate).

DISCUSSION

Although bacteria are known as unicellular, their ability in regulating gene expression as a consequence of their population, using QS system, renders them to act as multicellular organisms. Gram negative bacteria mainly produce AHLs as their QS signals to modulate various process such as antimicrobial compounds production [5]. *P. aeruginosa* is a very important opportunistic pathogen, and like most of other opportunists, QS modulation has a great influence on its virulence and survival by taking part in regulating antibiotic production and biofilm formation. QS blockage has been recently noticed as an new strategy of controlling *P. aeruginosa* infections either in preventing biofilm formation or inhibiting different virulence factor synthesizing. Most of researches in this have been based on finding quorum sensing inhibitors in plants or foods. In this research we investigated the AHL biodegrading activity of one bacterial strain (*B. cereus* UT26) against *P. aeruginosa* PAO1. The quorum quencher we used in this survey was previously reported as a very efficient native strain isolated form tomato rhizosphere enable in degrading 3oxoC8-HSL and blocking QS-regulated Ti-plasmid conjugation in *Agrobacterium tumefaciens* as well as 100% prevention of violacein production in *C. violaceum* via C6-HSL degrading (unpublished data). The results of this study showed that this strain also enables in degrading a very short acyl chain AHL (C4-HSL) and long 3oxo substituted AHL (3oxoC12-HSL). Like other reported quorum quenchers related to *Bacillus* sp., the AHL degrading activity of this strain is due to produce AiiA lactonase (*aiiA* gene product) and previous experiment has showed that this strain has only one active copy of *aiiA* gene, since site directed mutation via splicing in *aiiA* homologue removed the AHL degrading activity of the strain [17]. Since this strain inactivated AHLs of *P. aeruginosa* it seems this strain can effect on downstream QS regulated functions, such as antimicrobial production. As a case in point we tested the effect of quorum quenching ability of this microorganism on pyocyanin (1-hydroxy-5-methylphenazine) production of PAO1 in laboratory co-cultures. Results showed that this strain strongly inhibited pyocyanin production comparing UT26 *aiiA* mutant strain. Pyocyanin, a blue pigment, is one of the important antibiotics secreted by *P. aeruginosa* which has antimicrobial activity against a variety of microorganisms. Furthermore, it has been demonstrated that under limited conditions the growth of *P. aeruginosa* and the subsequent production of pyocyanin, alter the microbial community structure by inhibiting the growth of microorganisms sensitive to pyocyanin [19]. Our co-culture experiment showed that AHL degrading UT26 strain had a better fitness in co-culture with PAO1 while knocking out the *aiiA* gene in mutant strain declined the *B. cereus* population 1000 fold in co-culture. Several indications support the suggestion that the quorum quenching ability of *B. cereus* UT26 was a direct responsible for its better fitness in co-culture; First and foremost, the *aiiA* mutant derivate of UT26 (*B. cereus* UT26- *ΔaiiA*) which had the same growth pattern and doubling time with wt UT26 strain was not successful in co-culture as wild type strain. Second, filter sterilized supernatant of UT26 wt did not inhibit the growth of target strain (PAO1). Third, adding exogenous AHL signal to the co-culture of wt UT26 and PAO1 decreased colony forming unit cells of UT26 significantly, since apparently UT26 was not able to degrade all amount of AHL (consisted of biological concentration AHL produced by PAO1 and 5µM exogenous synthetic signals) and could not inhibit QS regulated functions completely both still did a better job than *ΔaiiA*. Finally in all experiments the culture was buffered with MOPS (pH 7.0) and no changes was observed in pH medium, excluding the possibility of a spontaneous alkaline hydrolysis of AHLs. It is better to mention that *P. aeruginosa* DA6 is a *lasR*$^{-}$*/rhlR*$^{-}$ strain which means that it has the ability of producing both AHL types, but because of site directed mutations in both AHL receptor proteins, it has lost the ability of sensing AHLs. So the QS system in this strain is deactivated and any increment in exogenous AHL does not have any positive effect on its QS system functions. It is why that this strain acted the same in presence and absence of exogenous AHLs. These results support the hypothesis stating that since QS has a very important role in interaction between

bacterial species by modulating antimicrobial production to compete better with sensitive species, not only can quorum quenching increase the fitness of sensitive strains but also can tip the balance in favor of the third parties which are sensitive to QS regulated antibiotics but disable in QS interrupting. Zamani et al., 2012 using three component co-culture showed that AHL degrading activity of a quorum quencher strain promoted the fitness of a *Bacillus subtilis*, a bystander strain which was neither resistant to QS-controlled antibiotics nor able to degrade AHLs, in co-culture with *C. violaceum* in both lab condition and gnotobiotic tomato rhizosphere [20].

CONCLUSION

As implied before, extensive use of antibiotics has resulted in the emergence of "superbugs" [21] which have a great amount of resistance to conventional antibiotics and render the scientists to look for new approaches in controlling bacterial disease either in medical science or plant pathology. Signal interference, which abolishes bacterial infections by interrupting cell-cell communication between bacteria, is a promising alternative strategy of disease control. Meanwhile, we should never neglect the potential of biological quorum sensing inhibitors which naturally produced by other microorganisms. It seems beneficial and necessary to start doing more fundamental experiments in order to purification of these biocompounds and working on other aspect of their usage such as their compatibility with human body as well as their side effects to see whether they are worthwhile to synthesis and use or not.

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