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ORIGINAL ARTICLE

Anti-Quorum Sensing Potential of Potato Rhizospheric Bacteria

Adeleh Sobhanipour[1], Keivan Behboudi[*1], Esmaeil Mahmoudi[2,3], Mohsen Farzaneh[4]

[1]Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

[2]Department of Plant Protection, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran

[3]Transgenesis Center of Excellence, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan

[4]Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran

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KEYWORDS

Antibiotic resistance;
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ABSTRACT: The occurrence of antibiotic-resistant pathogenic bacteria is becoming a serious problem. The rise of multiresistance strains has forced the pharmaceutical industry to come up with new generation of more effective and potent antibiotics, therefore creating development of antivirulence compounds. Due to extensive usage of cell-to-cell bacterial communication (QS) systems to monitor the production of virulence factors, disruption of QS system results in creation of a promising strategy for the control of bacterial infection. Numerous natural quorum quenching (QQ) agents have been identified. In addition, many microorganisms are capable of producing smaller molecular QS inhibitors and/or macromolecular QQ enzymes. In present survey, anti QS activity of 1280 rhizosphere bacteria was assessed using the *Pectobacterium carotovorum* as AHL-donor and *Chromobacterium violaceum* CV026 as biosensor system. The results showed that 61 strains had highly AHL-degrading activity. Both Lux I and Lux R activity were affected by some isolates, suggesting that the rhizobacteria target both QS signal and receptor. These soil microorganisms with their anti-QS activity have the potential to be novel therapeutic agents for reducing virulence and pathogenicity of antibiotic resistant bacteria.

INTRODUCTION

Antibiotic are known as antimicrobial agents for controlling diseases caused by pathogenic bacteria. Conventional antibiotics have either bacteriostatic or bactericide effect by targeting key process of bacterial growth, including cell wall synthesis, DNA replication, RNA translation, as well as protein synthesis [1]. However, due to the life-or-death selective pressure imposed by antibiotics on the selected pathogen, resistant strains of antibiotic agents are emerging continuously. The excessive and unaccounted use of antibiotics expedites the emergence of antibiotic resistance bacterial strains. Unfortunately and in

* Corresponding author: behbodi@ut.ac.ir (K. Behboudi).

comparison to the increasing levels of antibiotic resistance, the rake of novel antibiotic development has extremely slowed in the recent decades. This situation leads to the urgent attention and development of new antimicrobial agents aiming at bacterial virulence rather than vital processes of pathogens [2, 3].

In reality, antivirulence therapy may decrease antibiotic resistance [1]. Quorum sensing is the controller of some gene expression in reaction to alteration in cell-population density. Bacteria produce and release chemical signal molecules known as autoinducers (AIs) or QS signals that their concentration being parallel of cell density. The identification of specific threshold stimulatory levels of QS signals can result in an induction of some response [4, 5]. Once reaching a specific level of concentration, the signaling molecules can bind to protein receptors and activate them. These activated receptors are inside bacterial cells and can change gene expression to appear behaviors that are useful under the specific condition. "As this phenomenon is depending on cell-density, it has been termed quorum sensing" [6].

N-acylated-L-homoserin lactone (AHLs) is the most popular class of AIs used by gram negative bacteria. They are produced by Lux-type synthase enzymes and attach to LuxR-type receptors [7, 8]. Each bacterial species detect one or more type of AHL molecules result in perception the cell-density. [9, 10]. The LuxR-AHL complexes bind DNA and activate their specific genes [11]. In the second mechanism, AHLs are identified by two-component histidine kinase-type proteins termed as LuxN [12-14]. The process monitored by QS is very various and have many significant influence upon healthcare, agriculture and the environment [6]. Because QS is not necessary for the growth of bacteria, quenching QS (quorum quenching, QQ), weaken the selective pressure enforced on such pathogens and appearance of resistance to QQ compounds. As a result there has been special attention in establishing methods to penetrate this signaling process in pathogenic bacteria [2, 15]. QQ agents contain QS inhibitors (natural and/or synthesis) and QQ enzymes. Such enzymes classified into lactonazes, acylases and oxidoreductase, that using them in bacterial diseases treatment is very useful [1].

Thus, the anti QS activity of rhizosphere bacteria was investigated using *Pectobacterium carotovorum* AHL-donor and *Chromobacterium violaceum* CV026 biosensor system.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

Bacterial strains were isolated from the rhizosphere of potato plants collected from different potato growing areas in Iran. *P. carotovorum* (Kindly provided from Mr. Ghasemi, Iranian Research Institute of Plant Protection) was used as soft rot pathogen and source of naturally produced NAHL (C6-HSL) molecules. *C. violaceum* CV026 [16] (provided by Yves Dessaux, CNRS, Gif-sur-Yvette, France) was used as the indicator strain for AHLs detection. Luria-Bertani (LB) and Nutrient Agar (NA) media were used [17]. The bacteria were grown at 28°C. AHL standard was purchased from Sigma (Sigma-Aldrich, Inc., St. Louis, Mo., USA).

Soil samples were collected from potato field cultivated for commercial purposes in Iran. One gram of each sample was used for isolating of culturable bacteria. Soil samples were resuspended in 10 ml of sterile 0.8% NaCl by vigorous shaking for 3 min, and the resulted suspension was serially diluted. Appropriate dilutions (10^{-4} and 10^{-5}) were spread on NA medium for isolation of total culturable bacteria. Plates were incubated at 28ºC for 72h. Thereafter, For AHL-degradation screening, the bacterial colonies were randomly picked from medium, grown to pure cultures, and kept as frozen stocks in glycerol medium at -80°C.

Screening of bacterial isolates for N-AHSL degradation activity

Screening for AHL degrading activities of rhizospheric bacterial isolates was performed as described by Morohoshi et al. [18]. Because NAHLs are sensitive to alkaline pH [19], all degradation assays were done in LB medium that was buffered at pH 6.5 using of 100 mM KH_2PO_4/K_2HPO_4. The N-hexanoyl homoserine lactone (C6-HSL) was used as first target molecule in this degradation assay. Bacterial strains were incubated into 5 ml LB medium containing 5mg/l C6-HSL on rotary shaker for 20 h at 28°C. Cells were removed by centrifuge at 12000 rpm for 10 min. the culture supernatant (50 µl) was transferred into the wells of a 96-well plate. The full-grown culture of the CV026 biosensor was diluted 1:100 in fresh LB medium, and 500 µl of the diluted culture was inoculated into each well. A control experiment involved non-inoculated degradation medium. After incubation for 24 h at 30°C on rotary shaker, the remaining C6-HSL was detected through violacein production by the *C. violaceum* CV026 reporter strain.

AHL production by Pectobacterium carotovorum

P. carotovorum was streaked as homogeneous line on LB medium and biosensor strain, *C. violaceum* CV026, was spotted at a distance of 6 to 7 mm from the Pc line. After incubation at 28°C for 24 h, appearance of violet pigment in CV026 colony revealed the production of violacein by CV026 as well as production of N-AHL by *P. carotovorum*.

Amplification and sequencing of the anti QS rhizobacteria aiiA lactonase gene

The following primer set was used for amplification of *aiiA* gene (AHL-degrading enzyme, AiiA Lactonase) from genomic DNA of NAHL degrading bacteria, using taq DNA polymerase: forward 5′ -ATG GGA TCCATG ACA GTA AAG AAG CTT TAT-3′ , and reverse 5′ -GTC GAATTC CTC AAC AAG ATA CTC CTA ATG-3′ [20]. The PCR reaction was performed as follows: one cycle of 94°C for 5 min; 30 cycles of 94°C for 30 s, 50°C for 30s, and 72°C for 1 min; and a final 7-min extension at 72°C [21]. The amplified PCR products were visualized on 1 % agarose gel by ethidium bromide (EtBr) staining. The nucleotide sequence of all amplicon was verified by sequencing of both strands by Big Dye Terminator and ABI Prism 3700 Genetic Analyzer (Macrogene, World Meridian Venture Center, Korea). Comparison of nucleotide sequences were performed using the BLASTA search in GenBank (http://www.ncbi.nlm.nih.gov/).

Qualitative modulation of QS activity

The effect of the AHL-degrading rhizobacteria on inhibition of AHL synthesis (via LuxI) and modulation of AHL activity (via LuxR) was performed as described by Chenia [15] with some modification. This ability was determined using an agar diffusion double streak assay and the *C. violaceum* CV026 biosensor system. The AHL biosensor *C. violaceum* CV026 was streaked in a line on plates of LB agar. The AHL donor *P. carotovorum* was applied in line 16-17 mm from the *C. violaceum* CV026 line. Test bacteria were spotted in between the biosensor and the AHL donor. To test for potential LuxI inhibition, the AHL-producer was placed in close proximity to the test rhizobacteria and the AHL biosensor placed distally. To test for Lux R inhibition, the location of the AHL-producer and biosensor strain was reversed. Plates were incubated at 28°C for 2 d. Migration of AHL from the donor *P. carotovorum* was confirmed by the production of violacein and purple pigmentation in the biosensor strain. In either case, potential anti QS activity results in a lower purple violacein aiolacein production.

RESULTS AND DISCUSSION

Isolation and Screening of degrading NAHLs bacteria

Among sixty-seven rhizospheric soils samples, 1280 individual colonies with different morphologies were screened for NAHL degradation. Usage of the *C. violaceum* CV026 biosensor (pigment induction-based), make it possible to screen NAHL degraders as described earlier [16, 22]. Sixty-one isolates completely degraded 5 mg/l of C6-HSL after 20 h indicated by the absent of violacein production in the CV026 indicator (Figures 1 and 2). Slightly production of purple pigment by CV026 biosensor, demonstrated that these strains could not completely degrade 5 mg/l of N-AHLs [21]. QS systems regulate the virulence of pathogenic bacteria. Operation and efficiency of this system can be diminished by inhibition of signal synthesis or passivate of AHL-producing enzymes and enzymatic AIs degradation [23].The main aim of this work was finding bacterial strains of potato rhizosphere that degrades C6-HSL molecules. Out of 1280 examined isolates, 61 strains had the complete degradation capability of C6-HSL. One explanation could be suggested to confirm for disability of the other isolates which did not decompose AHL molecules. They might demonstrate very slow decomposition that could not be identified by our experimental conditions. All isolates completely degraded C6-HSL signal molecules, is a necessary component for QS regulatory system and regulates the production of virulence factors. Recently several species of plants and bacteria and other organisms have been shown interference with bacterial QS based virulence. These organisms have recommend as novel tools for the control of plant, animal or human pathogens [24, 25].

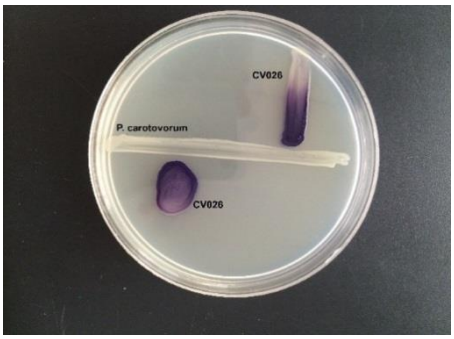

Figure 1. N-AHL production by *Pectobacterium carotovorum* (Pc) and appearance of violet pigment in CV026 colony.

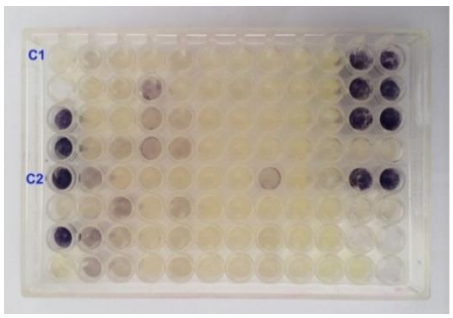

Figure 2. Detection of N-AHL-degrading isolates. The N-AHL-degrading isolates were detected as they ability to inhibition the synthesis of violacein by *Chromobacterium violaceum* CV026 in the presence of C6-HSL at 5 mgL-1. C1 (negative control): degradation assay performed without biosensor bacterium. C2 (positive control): degradation assay without rhizobacteria). The picture was taken after 24 h incubation.

Amplification and sequencing of the anti QS rhizobacteria aiiA lactonase gene

Enzymatic disruption of AHLs has been the most acknowledged mechanism for QS disruption. The aiiA gene has the enzymatic decomposition ability of AHL signals in many bacteria. This gene has been found in many Bacteria, specially Bacillus sp. Hydrolysis of the lactone ring of AHL molecules is the mode of action of AiiA enzymes or AiiA-lactonases [20]; Thus altering the respective configurational structure of the AHL molecoules and prohibits binding to the LuxR transcriptional regulator protein [26,28]. In present survey, amplification of the aiiA gene from anti QS rhizobacteria using specific PCR primers showed that AHL-degrading property was encoded in these strains by a gene whose DNA sequence was similar to some previously identified as aiiA sequences. A potential *aiiA* homologue was PCR-amplified from our strains using aiiA-7 F and aiiA-7R primers. These primers were amplified about 800 bp fragment in all strains (Figure 3). By using the online FASTA search engine (http://www.ncbi.nlm.nih.gov), DNA sequences of amplified *aiiA* gene were compared to those found in the data Genebanks The BLAST search results showed high similarity (92%) of *aiiA* gene sequences of tested strains with the reference strains *aiiA* gene, 240B1 [20] and A24 [27].

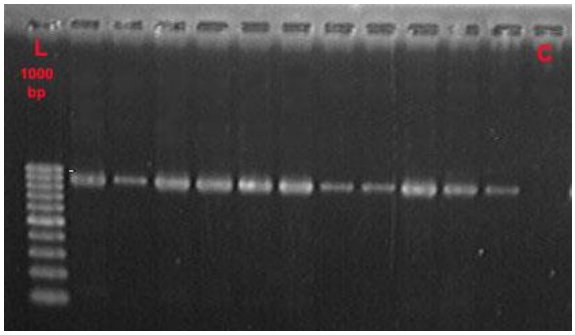

Figure 3. PCR amplification of the aiiA gene (about 800 bp) from acyl homoserine lactone (AHL)-degrading rhizobacteria strains using specific primers aiiA-7F/aiiA-7R. Lines: L, 100 bp DNA ladder; C, PCR reaction without DNA template as negative control.

Qualitative modulation of QS activity

Most Gram negative bacteria use AHLs type of auotoinducer in their QS system. AHLs are produced by LuxI synthase enzymes and bind to cytoplasmic LuxR receptors to regulate specific behaviours [10]. In the QQ methods, the LuxI (as signal generator), and the LuxR (as signal receptor) are potential targets. The QS procedur can be destroyed by various mechanisms: (i) lesening the efficiency of AHL receptor or AHL synthase proteins, (ii) preventing the production of QS signal molecules, (iii) breakup of the AHL, and (iv) simulating the signal molecules basically by using synthetic analogues of AIs. Antibodies have been suggested as a new method for anti-QS therapy [29, 30]. Many natural compounds such as plant extracts and microorganism enzymes inhibit QS by competing with AHLs due to precipitate the degradation of the receptor proteins and/or their structural similarity to the AHL signals [1, 2].

To appointment whether quenching bacteria target AHL synthesis or AHL response (via LuxI and LuxR, respectively), a double streak bioassay was designed using the *C. violaceum* CV026 andicator system. In this

research 17 isolates (unknown genus and species) exhibited various of Lux I modulation (strains:32P, 4B, 7N, 18C, 3B, 21F, 20F, 52H, 2G,10H, 31P, 29H, 34S, 22G, 221H, 5N, 24S) (Figure 4) and, eight isolates displayed very different modulation of Lux R (strains: 4B, 3B, 20F, 52H, 91J, 22T, 312J, 5N), although LuxI modulation was at a higher degree. In all cases, lower appearance of violet pigment from the biosensor strain was observed, due to decreased violacein production. Moreover, five strains (4B, 3B, 20F, 52H, 5N) were lowly illustrating modulation of both LuxI and LuxR, especially in the LuxR (Figure 5).

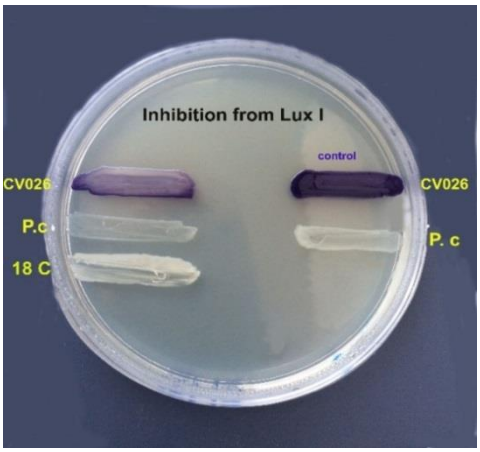

Figure 4. Lux I modulation by anti QS rhizobacteria (without LuxR inhibition).CV026: *C.violaceum* CV026 biosensor strain, P.c: *P.carotovorum* AHL-donor strain, 18C: anti QS test rhizibacteria strain.

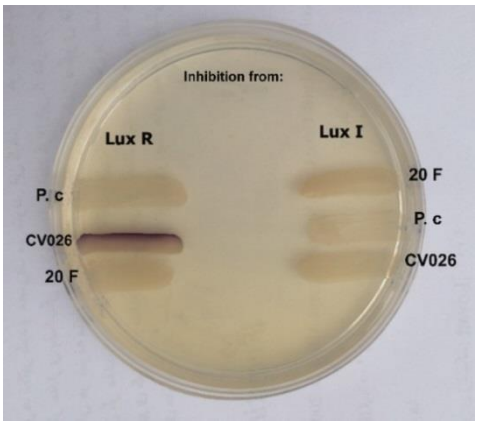

Figure 5. Both LuxI and LuxR modulation by 20F strain. CV026: *C.violaceum* CV026 biosensor strain, P.c: *P.carotovorum* AHL-donor strain, 20F: AHL-quenching test rhizobacteria strain.

Violacein production in *C. violaceum* CV026 is under the control of QS system. Violacein producer genes are expressed in response to the presence of AHL molecules secreted by the AHL-producer *P. carotovorum*. Some anti-QS compounds such as vanillin, cinnamaldehyde and halogenated furanone, decreased the production of the β-galactosidase and blue pigment appearance from the *Agrobacterium tumefaciens* A136 bioreporter in comparison to the control treatment, for both LuxI and LuxR evaluation. Similarly, *Kigelia africana* extract, showed different levels of anti-QS effect at the sub-inhibitory concentration and decreased both LuxI and LuxR activity [15].

The anti QS experiments showed that our rhizobacteria have multiple anti QS mechanisms with additive effects than other bacterial quencheror or compounds to

interfere with the reception of AHL and modulation of the AHLs synthesis. This effect has been observed with pharmaceutical plants [31], such as with *K. africana* fruit extract [15] and *Camellia sinesis* extracts [32], but no with quorum quencher bacteria (according to our knowledge). These antagonistic bacteria probably have some QS-modulating compounds, allowing them to affect and inhibit QS at multiple levels. Whether or not, detection and utilize anti-QS compounds is a novel and hopeful method for controlling bacterial diseases and decreasing their lesion.

CONCLUSIONS

The highlight of this research is that we found 17 strains of rhizospheric bacteria with 3 different mechanisms for QS disruption. Future work should to include identifying these bacteria and then identifying the exact compounds mediating the anti-QS potential and their mode of action.

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