Biocontrol Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cbst20

Quorum quenching by Bacillus cereus U92: a double-edged sword in biological control of plant diseases

Maryam Zamani, Keivan Behboudi & Masoud Ahmadzadeh

Department of Plant Protection, Faculty of Agriculture and Natural Resources, University of Tehran, Tehran, Iran

Accepted author version posted online: 02 Apr 2013. Published online: 09 May 2013.

To cite this article: Maryam Zamani, Keivan Behboudi & Masoud Ahmadzadeh (2013): Quorum quenching by Bacillus cereus U92: a double-edged sword in biological control of plant diseases, Biocontrol Science and Technology, 23:5, 555-573

To link to this article: http://dx.doi.org/10.1080/09583157.2013.787046

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.tandfonline.com/page/terms-and-conditions

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae, and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand, or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.
RESEARCH ARTICLE

Quorum quenching by *Bacillus cereus* U92: a double-edged sword in biological control of plant diseases

Maryam Zamani*, Keivan Behboudi and Masoud Ahmadzadeh

*Department of Plant Protection, Faculty of Agriculture and Natural Resources, University of Tehran, Tehran, Iran*

(Received 26 September 2012; returned 15 December 2012; accepted 14 March 2013)

In the present survey, quorum quenching activity was examined from a biocontrol point of view. Acyl-homoserine lactone (AHL) degrading bacteria were isolated from tomato rhizosphere using two standard bioreporter strains and different synthetic AHLs and then identified according to 16S rDNA sequences. Five isolates capable of inactivating both short and long 3oxo-substituted AHLs showed high similarity with the genera *Bacillus*, *Microbacterium* and *Arthrobacter*, and thereby *Bacillus cereus* U92 was determined as the most efficient quorum quencher strain. In the quantitative experiments, this strain remarkably inactivated all synthetic AHLs up to 80%. In the laboratory co-cultures, *B. cereus* U92 efficiently quenched QS-regulated phenotypes in *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, *Pseudomonas chlororaphis* and *Chromobacterium violaceum*. The strain successfully reduced the frequency of Ti-plasmid conjugal transfer in *A. tumefaciens* by about 99% in the binary cultures. Meanwhile, in a more natural environment, this strain acted as a biocontrol agent, efficient in alleviating QS-regulated crown gall incidence on tomato roots (up to 90%) as well as attenuating *Pectobacterium* soft rot on potato tubers (up to 60%). On the other hand, reducing phenazine production in *P. chlororaphis* operated as a suppressor of its QS-regulated biocontrol activity and also inhibited pyocyanin production in *P. aeruginosa*, a plant growth-promoting bacterium, by 75%. In general, *B. cereus* U92 seems very promising in the biological control of pathogenic bacteria; however, its broad AHL-degrading activity has a detrimental role on beneficial microbes which should not be neglected.

**Keywords:** quorum sensing; quorum quenching; N-acyl-homoserine lactone; *Bacillus cereus*; biological control

1. Introduction

Bacterial cells are able to change some phenotypes in order to adjust to the environmental conditions via cell-to-cell communication (or Quorum sensing, QS). QS modulates gene expression as a function of population density and relies upon the production and the response to the low molecular weight autoinducers in order to measure cell density. This phenomenon was first described in Gram-negative marine bacterium, *Vibrio fischeri* (Eberhard et al., 1981; Kaplan & Greenberg, 1985; Swift, Rowe, & Kamath, 2008). Since then this process has been considered as the most important intracellular signalling reaction functioning between bacteria. Bacterial QS system is based on two groups of signal molecules: Fatty acid

*Corresponding author. Email: mzamany@ut.ac.ir*

© 2013 Taylor & Francis
derivatives in Gram-negatives and peptide derivatives in Gram-positives. In Gram-negative bacteria, QS uses small signals, almost all belonging to the chemical family, called N-acyl-homoserine lactones (AHLs), which contain a conserved homoserine lactone ring and an acyl chain (4–18 carbons, with or without a C-3 hydroxy- or oxo-substituent). AHLs are excreted in a very low amount and diffused freely out of the cell membrane. AHLs diffuse out of the bacterial cells in very low amounts and at quorum concentration interact with LuxR family transcriptional factors. This AHL–LuxR complex can then modulate the expression of QS target genes. In most cases, AHL–QS systems are involved in regulating the expression of public goods such as antimicrobial factors and exoenzymes (Miller & Bassler, 2001; Fuqua & Greenberg, 2002). For example, in *Chromobacterium violaceum*, QS regulates the production of violacein, a purple antibiotic, and in *Pseudomonas aeruginosa*, QS acts an important role in competition and pathogenicity by modulating the production of different antimicrobial toxins such as hydrogen cyanide and pyocyanin (Williams, Winzer, Chan, & Camara, 2007). In symbiotic bacteria of legumes belonging to the *Rhizobium* spp. nodulation efficiency and symbiosome development as well as nitrogen fixation are related to QS (Rosemeyer, Michiels, Verreth, & Vanderleyden, 1998; De Kievit & Iglewski, 2000). Many plant pathogens use AHL-mediated QS to regulate their pathogenicity. As a case in point, AHL-mediated QS of *Pectobacterium carotovorum*, the causal agent of soft rot disease in many plant species, induces the production of several exoenzymes macerating plant cell wall. N-3-(oxooctanoyl)-L-homoserine lactone (3oxoC8-HSL) mediated QS is essential in *Agrobacterium tumefaciens* pathogenicity; QS in this bacterium controls the copy number of Ti plasmid and its horizontal transfer by conjugation, and thereby acts a key role in dissemination of virulence genes. Furthermore, there is a positive correlation between 3oxoC8-HSLs and the severity of symptoms on plant hosts suggesting QS contributes to *A. tumefaciens* aggressiveness. (Zhang, Murphy, Kerr, & Tate, 1993; Lojkowska, Masclaux, Boccara, Robert-Baudouy, & Hugouvieux-Cotte-Pattat, 1995; De Kievit & Iglewski, 2000; Pappas & Winans, 2003; Williams et al., 2007).

Since QS-regulated genes are often important in virulence, antimicrobial production, biofilm formation and colonisation of eucaryotic hosts, there is a great interest in finding ways to disrupt or manipulate QS signalling (Quorum quenching) in bacteria. Quorum quenching can be achieved in different ways. It has been shown that *Delisea pulchra*, a red alga, secretes halogenated furanones which interrupt the QS system in many bacteria by imitating AHL structure. These compounds are able to inhibit AHL-mediated synthesis of plant cell wall degrading enzyme in *P. carotovorum* (Manefield et al., 1999, 2002). Enzymatic degradation of AHLs is another well-studied QS inhibition mechanism. According to the chemical structure of AHLs, lactone ring cleavage occurs by lactonases and decarboxylases, and cleavage of AHLs to a free fatty acid moiety and a lactone ring can be caused by acylases and deaminases (De Kievit & Iglewski, 2000; Dong & Zhang, 2005). Previous investigations have led to isolation and identification of several bacterial strains with the ability of enzymatically inactivating AHLs. *Bacillus* sp. 240B1 (Dong, Xu, Li, & Zhang, 2000) and *B. thuringiensis* (Lee et al., 2002) are well-known bacterial species with the ability of degrading AHLs using AHL lactonases. Some quorum quenchers use multiple enzymes to inactivate AHL signals; for example, in *Rhodococcus erythropolis* W2 oxidoreductase (which modifies AHLs to their 3-hydroxy derivatives) and amidolytic activity are involved in quorum quenching.
ability (Uroz et al., 2005). The effect of quorum quenching ability is generally viewed as an encouraging way to control bacterial pathogens. For instance, application of AHL lactonase producing strains inhibits soft rot of plant tissues by Pectobacterium. (De Kievit & Iglewski, 2000). Recent studies have focused on stimulating AHL-degrading activity by adding special compounds to the rhizosphere. It has been demonstrated that gamma-caprolactone (GCL), a structural analogue of AHLs, significantly increases the population density of AHL-degrading bacteria (Cirou, Diallo, Kurt, Latour, & Faure, 2007). Application of GCL in the plant rhizosphere contributes to select R. erythropolis populations, which are capable of degrading both AHL signals and GCL. The success of quorum quenching activity, as a biocontrol approach, depends on the massive establishment of QS-interfering agents around the pathogen. So, biostimulating GCL promotes the emergence and quorum quenching activity of R. erythropolis in the potato rhizosphere and thereby inhibits soft rot incidence (Crépin et al., 2012).

The aims of the present study were the isolation and identification of a native bacterial strain capable of degrading AHL molecules, and the characterisation of its quorum quenching ability from biological control point of view. We examined a very potent quorum quencher strain against some AHL-mediated phenotypes in rhizospheric bacteria, including violacein production in C. violaceum, conjugal transfer of Ti-plasmid in A. tumefaciens, pyocyanin expression in P. aeruginosa and phenazine production in Pseudomonas chlororaphis. Moreover, we tested the capability of AHL-degrading activity in biological control of potato soft rot caused by P. carotovorum, and Agrobacterium crown gall on tomato roots in a tomato gnotobiotic system.

2. Materials and methods

2.1. Chemicals

The following homoserine compounds were purchased from Sigma-Aldrich Chemical Co., (MO, USA): N- Butyryl-L-homoserine lactone (C4-HSL), N-hexanoyl-L-homoserine lactone (C6-HSL), N-3-(oxohexanoyl)-L-homoserine lactone (3oxoC6-HSL), N-3-(oxooctanoyl)-L-homoserine lactone (3oxoC8-HSL) and N-3-(oxododecanoyl)-L-homoserine lactone (3oxoC12-HSL). Stock solutions (1 mM) of each compound were prepared in acidified ethyl acetate (4 ml glacial acetic acid to 40 ml ethyl acetate).

2.2. Bacterial strains, media and culture condition

Bacterial strains used in this study are shown in Table 1. A. tumefaciens strains were grown in mineral salt mixture (Petit & Tempé, 1978) supplemented with Manitol (2 g l⁻¹). Luria-Bertani (LB) was the main media for other bacterial strains. In all experiments, Bacillus subtilis 3610 (Table 1) was used as negative control, which was not able to degrade AHL molecules; however, its growth pattern and doubling time was the same as Bacillus cereus U92 strain. All quorum quenching assays were performed in LB and AT medium buffered with 50 mM MOPS, pH 7.0. and AHLs to avoid alkaline degradation of AHLs (Yates et al., 2002).
Table 1. Strains used in the present study.

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>Reporter strain</td>
<td>Luo et al. (2003)</td>
</tr>
<tr>
<td>NTL4 pZLR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. tumefaciens C58.00</td>
<td>Ti-plasmid recipient</td>
<td>Uroz et al. (2003)</td>
</tr>
<tr>
<td>A. tumefaciens R10</td>
<td>Ti-plasmid donor</td>
<td>Uroz et al. (2003)</td>
</tr>
<tr>
<td>Bacillus subtilis 3610</td>
<td>WT strain, Disable in degrading AHLs</td>
<td>Bacillus Genetic Stock centre</td>
</tr>
<tr>
<td>Chromobacterium violaceum</td>
<td>Purple pigment-producing strain with intact quorum sensing system</td>
<td>McClean et al. (1997)</td>
</tr>
<tr>
<td>CV017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromobacterium violaceum</td>
<td>cviI derived from CV017, disable in producing AHLs</td>
<td>McClean et al. (1997)</td>
</tr>
<tr>
<td>CV026</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectobacterium carotovorum</td>
<td>Plant pathogen, AHL producer</td>
<td>Our collection</td>
</tr>
<tr>
<td>ssp. carotovorum PF13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PAO1</td>
<td>Wild type strain</td>
<td>Holloway (1955)</td>
</tr>
<tr>
<td>E. coli DH5α/pJN105-lasR/</td>
<td>Reporter strain</td>
<td>Chugani et al. (2001)</td>
</tr>
<tr>
<td>pSC11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli DH5α/pECP61.5</td>
<td>Reporter strain</td>
<td>Pearson et al. (1995)</td>
</tr>
<tr>
<td>Pseudomonas chlororaphis O6</td>
<td>Biocontrol agent, AHL producer</td>
<td>Our collection</td>
</tr>
</tbody>
</table>

2.3. Isolation of AHL-degrading bacteria from tomato rhizosphere

The roots with adhering soil were collected from tomato plants. The samples were suspended in sterile distilled water (1 g/10 ml) and centrifuged briefly to remove root and soil particles. Serially diluted supernatants were spread on LB agar plates and incubated at 28°C for 24 h. Bacterial colonies with different morphologies were isolated and purified by single colony isolation on LB plates. For screening quorum quencher strains, N-Butyryl-DL-homoserine lactone (C4-HSL), N-hexanoyl-L-homoserine lactone (C6-HSL) and N-3-(oxooctanoyl)-L-homoserine lactone (3oxoC8-HSL) were used as the target molecules in degradation assays. Bacterial strains were grown in 3 ml of LB broth containing 5 μM AHLs with shaking at 28°C for 16 h and then cells were recovered by centrifugation. 50 μl of each suspension was transferred into the wells of 96-well plate. The fresh culture of *C. violaceum* CV026 (biosensor strain for C4-HSL and C6-HSL) and *A. tumefaciens* NTL4 (biosensor strain for 3oxoC8-HSL) at mid-log phase (OD600 0.4–0.5) were diluted 1:50 in a fresh medium and 250 μl of resulted suspension was added to each well. After 16 h incubation at 28°C, the intensity of purple colour produced by CV026 reporter strain or blue colour produced by NTL4 (in the presence of X-gal) was visually inspected. The bacterial strains with the ability of inhibiting purple or blue colour appearance in reporter strains were considered as potential quorum quencher strain. In order to prevent alkaline degradation of AHLs, the medium was buffered with MOPS (pH 7).

2.4. Identification of quorum quencher isolates

Genomic DNA of each isolate was extracted from broth cell cultures using DNA easy Blood and Tissue kit (Qiagen, Germany) and their 16S rRNA gene was amplified with universal bacterial primers, 8f (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492r
(5’-TACGGTTACCTTGTTACGACTT-3’) using Phusion DNA polymerase (Finnzymes, Finland). The Polymerase Chain Reaction (PCR) condition was denaturation at 98°C for 30s, followed by 30 cycles at 98°C for 10s, 50°C for 30s and 72°C for 45s. The products were purified using QIAquick PCR Purification kit (Qiagen, Germany) and sequenced by an ABI3700 automatic sequencer (Applied Biosystems) using 8f, 787r (5’-CTTATCTACCRGGGTATCTAAT-3’) and 1492r primers. Sequences were assembled by means of DNASTar programme and compared with Ribosomal Database Project. The final candidate strain chosen for the subsequent experiments was identified by further biochemical tests (cellulose degradation, motility, nitrate reduction, tyrosine decomposition, lysozyme resistance, egg yolk reaction, anaerobic utilisation of glucose, acid production from mannitol etc.).

2.5. The quantification of synthetic AHL-degrading ability of B. cereus U92

The B. cereus U92 was selected as the most efficient strain in this collection for the following experiments. AHL degradation capability of B. cereus U92 strain was evaluated by incubating the strain with synthetic C4-HSL, C6-HSL, 3oxoC8-HSL and 3oxoC12-HSL in a time course assay. The bacterial cultures were established in buffered LB (pH 7). E. coli DH5α/pSC11 and E. coli DH5α/pJN105-lasR/pSC11 were used as C4-HSL, 3oxoC12-HSL reporters, respectively, and A. tumefaciens NTL4 pZLR4 was used to quantify C6-HSL and 3oxoC8-HSL residual according to the published method (Luo, Li, & Morrison, 2003). The assay was performed according to (Pearson, Passador, Iglewski, & Greenberg, 1995) with slight modifications. A fresh culture of B. cereus U92 (OD600 0.4-0.5), resuspended in 20 ml PBS (pH 7), was added to clean flasks containing adequate signals to reach the AHL concentration of 5 μM and then incubated at 30°C. Three millilitre aliquots were sequentially removed 15, 30, 60, 90 and 120 min, and residual signals were extracted with 1X acidified ethyl acetate. Five microlitres of each acetate extracts were transferred to 2 ml tubes and dried under chemical hood. Five hundred microlitre of prepared biosensor strains (Pearson et al., 1995) was added to dried down ethyl acetate extracts or standards, and incubated at 37°C shaker. After 3 h, the cells were broken by adding 50 μl chloroform to each tube and 10s overtaxing. Ten microlitres from the top of each sample were transferred to 96-well white optiplates (Thermo Fisher Scientific Inc. USA). AHL residuals were quantified using Beta-gal assay kit (Invitrogen, USA) by adding 70 μl galactone substrate diluted 1:100 in galactone reaction buffer. Plates were wrapped in aluminium foil and incubated at room temperature for 1 h. The enhancer solution was then added to the reaction mixture and the luminescence was recorded (100–500 ms read per well). Standard curves for bioassay were generated using pure synthetic signals (Sigma-Aldrich Chemicals Co).

In all the subsequent experiments, the inhibitory effect of U92 against all target strains was examined by growing the test strains in 1:1 ratio of filter-sterilised U92 culture and fresh LB.

2.6. Inhibition of violacein production in C. violaceum

The experiment was performed according to Blosser and Gray (2000). Co-cultures were started with an initial OD600 0.01 (2–3 × 10⁶ colony forming units per ml; CFU
ml⁻¹) for CV017 and Bacillus strains. After 16 h growing at 30°C, 200 μl of each culture was placed in 1.5 ml tubes and cells were lysed by adding 200 μl of 10% sodium dodecyl sulphate and incubating at room temperature for 5 min. Violacein was extracted by adding 900 μl of water-saturated butanol and the absorbance of the upper phase, containing violacein, was measured at 685 nm. The data were normalised using the optical density of the original co-cultures at 660 nm.

2.7. Effect of AHL-degrading activity of B. cereus U92 on pyocyanin production by P. aeruginosa PAO1

The co-culture of each Bacillus strain and P. aeruginosa PAO1 (Steidle et al., 2001) was started with initial concentration of 2-3 × 10⁶ CFU ml⁻¹ (OD600 0.01) of each strain. The overnight co-cultures were extracted using equal volumes of chloroform and the organic layer was transferred to a micro tube and spun down for 5 min at full speed. 200 μl of HCl (0.01 N) was added to the tubes after a brief vortex, the aqueous fraction was removed and the OD of each sample was determined at 520 nm. The data was normalised using the optical density of the original co-cultures at 660 nm.

2.8. Effect of AHL-degrading activity of B. cereus U92 on phenazine production by P. chlororaphis O6 in co-culture

P. chlororaphis O6 and Bacillus strain cells were co-inoculated in 5 ml of fresh LB broth. To quantify phenazine, culture filtrates were extracted into acidified ethyl acetate. After evaporation of ethyl acetate under chemical hood, the resulting solids were re-suspended in 1 ml of 0.1M NaOH, and the absorbance of each sample was measured at 367 nm according to Chancey, Wood, and Pierson (1999) and Kang et al. (2004).

2.9. Effect of AHL-degrading activity of B. cereus U92 on the conjugal transfer of A. tumefaciens Ti-plasmid

To measure the effect of U92 on conjugation frequency of Agrobacterium, the spot mating method was performed according to Uroz et al. (2003). Donor (A. tumefaciens R10) and recipient strains (A. tumefaciens C58C1RS) were grown to a late exponential phase in AT liquid medium. Prior to conjugation 20 μl of the recipient strain (10⁸ CFU ml⁻¹ in 0.8% NaCl) was spread over the surfaces of the selection plates containing rifampin, streptomycin and either kanamycin or 1 mM nopaline (Sigma-Aldrich Chemicals Co.). Five μl of donor cell medium (10⁸ CFU ml⁻¹ in 0.8% NaCl) or 10 μl of 1:1 mixture of donor strain and Bacillus strain were spotted onto the surface of recipient lawn. After incubating at 28°C for 72 h, conjugation frequencies (per donor) were determined by counting the transconjugants emerging within the donor inoculum spot using a microscope.

2.10. Biocontrol assay for potato tuber rot caused by P. carotovorum ssp. carotovorum

Biocontrol effect of Bacillus U92 strain was investigated in potato (Solanum tuberosum) tuber assay following the protocol described before (Molina et al., 2003). Potato tubers were sterilised with sodium hypochlorite 10%. Two wells (5 × 5
mm) were cut in the tubes and inoculated with bacterial suspensions (pathogen alone, or with each Bacillus strain, at $10^5 - 10^6$ CFU ml$^{-1}$). After four days of incubation at $25^\circ$C under humid atmosphere, the results were recorded by quantifying by measuring the maceration zone.

2.11. Biological control of tomato crown gall caused by A. tumefaciens

The biocontrol activity of B. cereus U92 on attenuating crown gall symptoms was examined in a sterile gnotobiotic system, described previously by Simons et al. (1996). Tomato seeds (Lycopersicon esculentum Mill cv. Carmello) were surface sterilised and placed on plant nutrient solution (PNS) (Hoagland & Arnon, 1950) solidified with 1.5% agar. Incubation was at 4°C overnight in the dark, followed by 28°C for 2 days. After 10 days growing of each germinated seed in gnotobiotic tubes containing sterile quartz, the seedlings were carefully harvested and after pruning one-third of their roots, dipped in quorum quencher, and B. subtilis 3610 suspension as negative control ($10^6$ CFU ml$^{-1}$) for 15 min, and then blot-dried on sterile papers. Seedling roots subsequently inoculated with dipping in A. tumefaciens C58 suspension ($10^6$ CFU ml$^{-1}$) for 15 min. Then inoculated plants were transferred to gnotobiotic tubes and grown at growth rooms at 26 ± 2°C for 21 days. All plants were carefully removed from the sand and roots were excised, weighed and the ratio of total weight of galls on root weight was evaluated in all treatments. Treatments consisted of 12 replicate (one plant in each tube) and the experiment repeated four times. To assure that all observations were only due to AHL-degrading activity, inoculated tomato plants, with a mixture of C58 and U92, were cultured in the sand columns moisturised with PNS solution supplemented with 5 μM 3oxoC8-HSL.

3. Results

3.1. Screening for potential quorum quenchers

Seventeen different bacterial strains, isolated from tomato rhizosphere samples, were screened for their ability to inhibit QS system in C. violaceum and identified by amplifying 16S rRNA gene-encoding region (Table 2). Among them, the five AHL inactivating isolates were Bacillus spp. (U92 and U125), Arthrobacter spp. (US14 and UP5) and Microbacterium spp. (U124). The isolate U92 showed the highest efficiency in suppressing of QS-regulated biosensing features in C. violaceum CV026 and A. tumefaciens NTL4. Sequence analysis of U92 showed 99% similarity with B. cereus CC-149, and other B. cereus group including B. cereus, B. thuringiensis and B. anthracis. Further biochemical and molecular tests revealed this isolate to be B. cereus.

To characterise AHL-degrading activity in B. cereus U92, a time course assay of AHL degradation, using four different types of AHLs (short chain, long chain and 3oxo-substituted AHLs) was conducted. The residual AHL contents were quantified and the results revealed that B. cereus U92 was able to degrade the various structures of AHLs efficiently. B. cereus U92 cells remarkably inactivated 3oxo compounds with longer acyl-chain, and reduced the amount of 3oxoC8-HSL and 3oxoC12-HSL up to 80% and 90%, respectively. Non-AHL-degrading control, Bacillus subtilis 3610, did
not show any significant effect on the amount of residual AHLs compared with the blank (Figure 1).

3.2. B. cereus U29 efficiently quenches QS-regulated phenotypes

Pyocyanin and violacein production by P. aeruginosa PAO1 and C. violaceum CV017, respectively, were significantly reduced in co-culture with the isolate U92, which was due to interrupting their QS system via degrading AHLs, without any growth inhibitory effects. In liquid co-cultures, the relative amount of violacein and pyocyanin production decreased up to 97% and 75% in co-cultured with U29, respectively (Figures 2 and 3).

Moreover, B. cereus U29 was capable of inhibiting phenazine production by P. chlororaphis O6 about 75% in co-culture (Figure 4). In the presence of Bacillus subtilis 3610 strain as a non-AHL-degrading control, the amount of QS-controlled antibiotics was almost the same as the pure culture of antibiotic producer strains.

To investigate the ability of U92 in interfering with the conjugal transfer of Ti plasmid, crosses involving A. tumefaciens R10 isolate as pTi donor strain and A. tumefaciens C58.00RS as pTi recipient strain were performed. The results showed that B. cereus U92, efficiently prevented Ti-plasmid conjugal transfer up to 99%. Conjugation frequencies per donor decreased from 1.58 × 10^{-3} (in the absence of U92) to 1.62 × 10^{-5} (in the presence of U92). However, no significant difference in conjugation frequencies was observed when donor strain co-inoculated with B. subtilis 3610. Moreover, the quorum sensing phenotypes of all four target strains in

<table>
<thead>
<tr>
<th>Strain</th>
<th>Related genera according to 16S rDNA</th>
<th>AHLs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C4-HSL</td>
</tr>
<tr>
<td>U92</td>
<td>Bacillus spp.</td>
<td>+</td>
</tr>
<tr>
<td>U125</td>
<td>Bacillus spp.</td>
<td>+</td>
</tr>
<tr>
<td>U154</td>
<td>Bacillus spp.</td>
<td>+</td>
</tr>
<tr>
<td>U171</td>
<td>Bacillus spp.</td>
<td>+</td>
</tr>
<tr>
<td>U2</td>
<td>Paenibacillus spp.</td>
<td>+</td>
</tr>
<tr>
<td>U9</td>
<td>Paenibacillus spp.</td>
<td>+</td>
</tr>
<tr>
<td>U17</td>
<td>Paenibacillus spp.</td>
<td>+</td>
</tr>
<tr>
<td>U124</td>
<td>Microbacterium spp.</td>
<td>+</td>
</tr>
<tr>
<td>UZ1</td>
<td>Exiguobacterium spp.</td>
<td>+</td>
</tr>
<tr>
<td>UO22</td>
<td>Flavobacterium spp.</td>
<td>+</td>
</tr>
<tr>
<td>UO91</td>
<td>Flavobacterium spp.</td>
<td>+</td>
</tr>
<tr>
<td>US2</td>
<td>Agrobacterium spp.</td>
<td>+</td>
</tr>
<tr>
<td>US4</td>
<td>Pseudomonas sp.</td>
<td>+</td>
</tr>
<tr>
<td>US5</td>
<td>Pseudomonas spp.</td>
<td>+</td>
</tr>
<tr>
<td>US12</td>
<td>Pseudomonas sp.</td>
<td>+</td>
</tr>
<tr>
<td>US14</td>
<td>Arthrobacter spp.</td>
<td>+</td>
</tr>
<tr>
<td>UP5</td>
<td>Arthrobacter spp.</td>
<td>+</td>
</tr>
</tbody>
</table>

Note: − = no AHL-degrading activity; + = low AHL-degrading activity; ++ = high degrading activity.
3.3. Biological control assay for potato tuber rot caused by *P. carotovorum* ssp. *carotovorum*

*B. cereus* U92 was also able to attenuate the virulence in *P. carotovorum* strain PF13. The maceration zone decreased from 229 ± 28 mm² (without U92) to 80 ± 8.9 mm² (U92-treated). The non-AHL-degrading *B. subtilis* 3610 had no effect on reducing tissue rot (Figure 5).

3.4. Biological control of tomato crown gall caused by *A. tumefaciens*

The application of U92 quorum quencher strain on tomato pruned roots inoculated with *A. tumefaciens* revealed that this strain is an ideal biocontrol agent to inhibit grown gall on tomato roots. In the presence of this strain, the incidence of galls was reduced up to 90% (Figure 6). While the non-quorum quencher *Bacillus* strain did not have any significant effect on reducing disease symptoms compared to the positive control (*A. tumefaciens* alone). These indications show that the high level of protection of tomato roots against *A. tumefaciens* is only due to AHL-degrading
activity. As it was predicted, the crown gall severity (in co-inoculation with U92) was restored in presence of 5 μM exogenous 3oxoC8-HSL in sand columns.

4. Discussion
This work first aimed at isolating and identifying an efficient wild-type strain with the ability of degrading AHL from tomato rhizosphere. Among five different isolates capable of degrading AHLs, *B. cereus* U92 was selected to continue subsequent experiments as the most efficient quorum quencher strain in our collection.

This strain exhibited a remarkable ability of degrading different types of AHL structures, including short chain and long 3oxo-substituted. Several indications support the view that the quorum quenching ability of *B. cereus* U92 is directly responsible for attenuating QS functions in all target strains; First and foremost, in the co-culture experiments the growth pattern of target strains was similar in the presence and absence of filter-sterilised overnight culture of U92; Moreover, QS-regulated functions of target strain was restored by adding more signals to the co-cultures, suggesting that the quorum quencher strain was not able to degrade...
whole amount of AHLs, and the AHL-regulated functions were turned on again in presence of exogenous AHL.

Second, in quantification assays, all AHL residuals were reduced dramatically; however, this effect was not due to growth inhibition of reporter strains by quorum quencher strain. In other words, the filter-sterilised overnight culture of U92 strain did not inhibit the growth of bioreporters; the last but not the least, no changes was observed in the pH of the medium, excluding the possibility of a spontaneous alkaline hydrolysis of AHLs.

AHL-degrading ability in *Microbacterium* and *Arthrobacter* species have been reported previously (Dong et al., 2000; Morohoshi, Someya, & Ikeda, 2009; Park et al., 2005). It has also been shown that AHL-degrading ability in *Bacillus* species is due to the expression of AHL lactonase encoded by *aiiA* gene (Mäe, Montesano, Koiv, & Palva, 2001). Time-course incubation of U92 with either C3-substituted or substituted AHLs shows that this strain does not act specifically in degrading AHLs and is able to inactivate a broad range of AHLs regardless of the chain length and 3oxo-substitution. The culture supernatant of U92 did not have any effects on degrading AHLs and degradation of all 4 types of AHLs required active growing cells (data not shown). One possible explanation could be that AiiA looks to be a
cytoplasmic enzyme. Lack of signal peptide on its N-terminus (Dunn & Handelsman, 1999) as well as absence of the quorum quenching activity in the supernatant of Bacillus culture suggests that AHLs are being degraded in cytoplasm, after diffusing inside the U92 cells. The AHL-degrading activity in U92 did not result in molecules that could serve as the sole source of carbon and nitrogen, because this strain was not able to utilize these molecules as a sole source of carbon and nitrogen.

Figure 4. Inhibition of phenazine production in Pseudomonas chlororaphis O6 by Bacillus cereus U92. O6 strain was incubated with either AHL-degrading B. cereus U92 strain or non-AHL-degrading B. subtilis 3610 strain and AHL-degrading U92 plus 5 μM exogenous C6-HSL (total concentration 5 μM). Absorbance of phenazine extracted from supernatants of overnight cultures resuspended in 0.1 N NaOH and diluted 10⁻¹ was measured. Values represent the mean of three trials with four treatment replications per trial. Vertical bars represent the standard errors. Bars labelled with different letters indicate statistically significant differences (Tukey’s comparisons with 5% family error rate).

Table 3. The effect of B. cereus U92 on conjugal transfer of Ti-plasmid.

<table>
<thead>
<tr>
<th>Treatments*</th>
<th>The conjugation frequency per donor**</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. tumefaciens R10</td>
<td>1.58 × 10⁻³ a</td>
</tr>
<tr>
<td>A. tumefaciens R10 + B. cereus U92</td>
<td>1.62 × 10⁻⁵ b</td>
</tr>
<tr>
<td>A. tumefaciens R10 + B. subtilis 3610</td>
<td>1.78 × 10⁻⁵ a</td>
</tr>
<tr>
<td>A. tumefaciens R10 + B. cereus U92 + 5μM 3oxoC8-HSL</td>
<td>1.68 × 10⁻³ a</td>
</tr>
</tbody>
</table>

*A. tumefaciens R10 (pTi donor strain) was mated with C58C1RS (pTi recipient) on solid medium. **Expressed as transconjugant cells recovered per donor. Values represent the average of five replicates for each treatment. The experiment was repeated twice, and the data were statistically analysed by Tukey’s comparisons (P <0.05).
able to grow in minimal medium supplemented with AHL molecules, as sole carbon and nitrogen source (data not shown). Metabolising AHLs has already been observed in different bacteria such as *Variovorax paradoxus* or *Arthrobacter* sp. (Leadbetter & Greenberg, 2000; Park et al., 2003). So, in this strain, AHL degradation may not be the primary function of AHL lactonase. The effect of AiiA lactonases in biology of *Bacillus* species is far from clear. It has been proposed that AHL-degrading activity of bacterial species can improve their fitness to compete with AHL utilising bacteria in natural ecosystems, especially against those which use AHLs to regulate antibiotic production (Uroz et al., 2005; Zamani, Peterson, & Parsek, 2011). Furthermore, 3oxo AHLs and their tetrameric acid derivatives possess bactericidal activity towards Gram-positives and AHL lactonase in Gram-positive bacteria is likely involved in preventing antibacterial activity of AHLs to enhance bacterial survival in natural environment (De Kievit & Iglewski, 2000; Moré et al., 1996).

The excellent quenching abilities of this strain *in vitro* were correlated with its ability to degrade a broad range of AHL structures. In binary cultures, we verified this quorum quenching ability by adding appropriate AHLs to the co-cultures as well as testing the inhibitory effect of U92 on target microbes. Quenching pathogenicity-related functions of QS system by degrading AHLs has been already reported, either using AHL-degrading micro-organisms or AHL lactonase expressing plants (D’Angelo-Picard et al., 2004; Holloway, 1955; Kang et al., 2004; Måe et al., 2001; Morohoshi et al., 2009; Sio et al., 2006). Limited data are available related to the biological control based on AHL degradation. Our biocontrol assays demonstrated that AHL-degrading ability of U92 strain is a very efficient strategy in
biological control of two important plant diseases (*Pectobacterium* soft rot and *Agrobacterium* crown gall). AiiA lactonase activity of *B. cereus* U92 suppressed *P. carotovorum* pathogenicity up to 66%. It has been proved that secretion of exoenzymes in *P. carotovorum* is an AHL-regulated function which happens only at high cell density to ensure that the bacteria do not prematurely wound the host prior to achieving a sufficient bacterial cell number (Miller & Bassler, 2001). There are several reports about other quorum quencher strains enable to attenuate *Pectobacterium* diseases on plant tissues; such as *R. erythropolis* W2 which is able to suppress both *P. carotovorum* and *Pectobacterium atrosepticum* (Uroz et al., 2003, 2005).

In biocontrol experiments, *B. cereus* U92 strain proved itself as an ideal wild-type strain in biological control of *Agrobacterium* crown gall by reducing tumour incidence up to 90%. This data was congruent with the observations by Molina et al. (2003), who tested the biocontrol activity of a genetically manipulated strain of *Pseudomonas*, enable to produce AHL lactonase, against *A. tumefaciens* on tomato roots and observed the same results.

Figure 6. Biocontrol of tomato crown gall caused by *A. tumefaciens* C58 by *Bacillus cereus* U92. Tomato roots were inoculated with either the pathogen alone or a mixture with AHL-degrading *B. cereus* U92 strain, non-AHL-degrading *B. subtilis* 3610 strain and AHL-degrading U92 complemented with 5 μM 3oxoC8-HSL. Values represent the mean of four trials with 12 replications for each treatment per trial. Vertical bars represent the standard errors. Bars labelled with different letters indicate statistically significant differences (Tukey’s comparisons with 5% family error rate).
Several different biotic and abiotic factors, including growth substrates, humidity, pH and temperature as well as other micro-organisms, can affect root colorisation of bacteria. Therefore, we used a very well-controlled gnotobiotic system consisting of a sand column moisturised with a certain amount of plant nutrient solution in order to assure that any reduction in disease incidence was only due to AHL-degrading ability. It has been proved that the gnotobiotic system successfully limits the environmental variations and culminates in more reproducible and reliable results. Although the interesting results should be definitely examined in more realistic environment such as field, it has been shown that gnotobiotic results also appear to be the case in soil almost all the time (Simons et al. 1996). Therefore, it will hold true to re-observe the interesting results of biocontrol activity of U92 against A. tumefaciens in further field experiments.

AHL-degrading activity of U92 strain seems to be an efficient mechanism in biological attenuation of virulence in human pathogens such as P. aeruginosa. The fact that AHL-degrading ability suppresses QS-regulated antibiotic production is very important but ambiguous in ecological context. As a case in point, QS-regulated antibiotics in P. aeruginosa have an exemplary positive effect on its fitness by inhibiting other bacterial species. Suppression of antibiotic production, on one hand, may have a negative effect by decreasing the fitness of this bacterium either as one of the important plant growth-promoting bacteria; and on the other hand, likely have a positive effect to protect other antagonisms, such as Bacillus subtilis, which are sensitive to QS-regulated antimicrobials. In one recent study, it has been shown that AHL-degrading activity of a bacterial species not only protects this micro-organism against QS-regulated antimicrobials but also promotes the fitness of a bystander strain which is neither resistant to QS-controlled antibiotics nor able to degrade AHLS (Zamani et al., 2011).

Our results indicate that U92 strain is generally a promising strain to study of biological control of different plant bacterial diseases; however, its quorum quenching ability does not act specifically and targets AHL-QS system in beneficial micro-organisms such as P. chlororaphis. Since phenazine synthesis is the main antagonistic mechanism of P. chlororaphis against fungal pathogen agents, it seems that AHL-degrading activity of U92 probably contributes to suppress biocontrol activity of P. chlororaphis. Moreover, AHL-mediated quorum sensing is also involved in the establishment and maintenance of symbiotic relationships between leguminous plants and N-fixing bacteria, such as Sinorhizobium meliloti (De Kievit & Iglewski, 2000; Rosemeyer, Michiels, Verreth, & Vanderleyden, 1998); so, the non-specific AHL-degrading activity of U92 may have a negative effect on QS-regulated symbiosis via degrading both AHL signals produced by rhizobia and AHL mimics produced by plants. There is a hypothesis that both AHL-mediated quorum sensing and quorum quenching activities play a role in bacterial interactions and competitiveness and thus are important in shaping microbial community structure. This hypothesis was tested in potato hydroponic system, which potato microfloras were modified to promote AHL-degrading populations via adding biostimulating molecules. In this study, AHL-producing bacteria did not increase quorum quencher populations (Cirou et al., 2007). In other research, the impact of a genetically engineered plant, Nicotiana tabacum line expressing the lactonase AttM (which degraded AHL signals) was examined on a selected bacterial population composed of pseudomonads and agrobacteria. The results of DGGE analysis targeting 16S
rRNA gene did not show any significant difference between bacterial population in AttM plant rhizosphere and its wild-type parent line, suggesting that bacterial population was not affected by AttM-lactonase activity of the plant (D’Angelo-Picard, Chapelle, Ratet, Faure, & Dessaux, 2011). Notwithstanding, the risk of interfering in cross-talk between beneficial bacteria seems to be the major criticism to AHL-degrading ability of this strain and highlights the importance of caring about designing effective mixtures of biocontrol agents.

All in all, in this study, we presented some fundamental AHL-degrading characteristics of B. cereus U92, as a potent quorum quencher strain from tomato rhizosphere and a possible candidate for biocontrol programmes. In our preliminary experiments, we tried to knock out aiiA gene of U92 to provide a lactonase mutant strain. Although we successfully created a site-directed mutation in aiiA homologue gene and confirmed the splicing by sequencing analysis, the AHL-degrading activity in this strain did not completely disappeared. There are two possible explanations for this matter; first, it suggests that there are likely more than one active copy of aiiA in this quorum quencher strain. Another possibility suggests that some other quorum quenching mechanism in addition to aiiA lactonase may be active in this strain. Further experiments may focus on more efforts to eliminate quorum quenching activity of U92 strain in order to study the ecological effects of using this strain as a biocontrol agent, precisely. In our current study, we are working on AHL lactonase expression regulation in this strain to see whether AiiA is expressed constitutively or induced in presence of AHLs.

Acknowledgements
The authors thank Dr Denis Faure, Institut des Sciences du Végétal, CNRS, France, for kindly providing us with several bacterial strains.

References


Rhodococcus erythropolis W2 by both amidolytic and novel oxidoreductase activities. *Microbiology, 151*, 3313–3322. doi:10.1099/mic.0.27961-0


