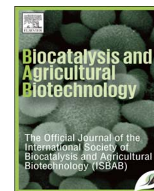
Biocatalysis and Agricultural Biotechnology 9 (2017) 48–57

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab



Evaluation of quorum quenching *Bacillus* spp. for their biocontrol traits against *Pectobacterium carotovorum* subsp. *carotovorum* causing soft rot

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ARTICLE INFO

Keywords:
AHL degrading *Bacillus* spp.
Biocontrol
Pectobacterium carotovorum subsp. *carotovorum*
Quorum quenching
Soft rot

ABSTRACT

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*) is an important broad host range, soft rot causing phytopathogen with multiple modes of infection. AHL mediated quorum sensing mechanism plays a pivotal role in pathogenesis of *Pcc*, by regulating its major virulence enzymes that degrade plant cell wall. Here, the disruption of quorum sensing as a strategy for biocontrol of *Pcc* is proposed, especially in the light of rising antimicrobial resistance. *Bacillus* based biocontrol agents have been found useful in management of bacterial and fungal plant diseases. Additionally, they are known to produce AHL lactonase that inactivates AHL signalling in quorum sensing. Since prior to use in the field, a bacterial strain should be tested for its efficacy and amenability as biocontrol agent, the focus of the present studies was to evaluate the traits that bestow the biocontrol attributes to the isolates so as to be an effective biocontrol agent. Keeping in view this, 97 isolates screened for AHL degradation from root and soil yielded 20 AHL degrading *Bacillus* spp. out of which three isolates As30, Gs42 and Gs52 were selected since they demonstrated appreciable attenuation of *Pcc* soft rot. Moreover, the attenuation was observed in both curative and preventive biocontrol studies. The root colonisation ability and *in planta* studies of prevention of mung bean spoilage further demonstrated that the isolates have high potential to reduce the disease. The studies conducted in this work suggests that soil quorum quenching soil *Bacillus* isolates have the attributes to be taken for further application studies.

1. Introduction

Pectobacteria are responsible for a range of plant diseases including blackleg and soft rot in a wide range of crop and ornamental plants that include *P. atrosepticum* (*Pa*), *P. carotovorum* subsp. *carotovorum* (*Pcc*), and the recently characterised *P. wasabiae* and *P. carotovorum* subsp. *brasiliense*. Among these, *Pcc* has widest host range of all the soft rot bacteria. Potato being one of the economically important crops affected by *Pcc* in temperate regions, the epidemiology of potato soft rot is well studied (Davidsson et al., 2013). Soft rot of potato tubers caused by *Pcc* can result in extensive post-harvest losses, especially during storage (Czajkowski et al., 2011). While in the field, the healthy plants can get infested through the microbial inoculum of *Pcc* found near seed tuber in soil (De Boer et al., 1978; H´elias et al., 2000). *Pcc* colonizes on potato roots and makes its way into the stem and progeny tubers via the vascular system. Once in the stems, the pathogen do not necessarily cause stem rot (blackleg) but can survive in latent form (Czajkowski et al., 2010). Rotten mother seed tubers release the pathogen that is transmitted through soil water to contaminate neighbouring progeny tubers. Long-distance transmission can also occur via flying insect vectors or via aerosols produced by rain (Pérombelon, 1974).

Pcc is termed as 'brute force' pathogen since it causes necrotrophic damage through physical attack on plant cell wall primarily through production of enzymes that causes the death of the host tissue. This coordinated and prolific production of virulence factors at high population density is regulated by a cell-cell communication mechanism known as quorum sensing (QS) preventing premature activation of plant defences and elevates the possibility of successful infection (Liu et al., 2008; Zhang and Dong, 2004). The signalling molecules of QS are *N*-acyl homoserine lactones (AHLs) responsible for controlling the virulence factors production which include plant cell wall degrading enzymes (PCWDE) pectate lyases, pectinase and cellulase. QS also regulates harpin and other factors secreted by Type III secretion system, small number of virulence regulators, and the antibiotic carbapenem for the full virulence in *Pcc* (Chatterjee et al., 1995; Cui et al., 2005; Jones et al., 1993; Mattinen et al., 2004; McGowan et al., 2005; Pemberton et al., 2005). QS, being a prerequisite for pathogenesis of *Pcc* disrupting its regulation could prevent the production of virulence factors which makes it an appropriate target for development

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http://dx.doi.org/10.1016/j.bcab.2016.11.004
Received 28 September 2016; Received in revised form 18 November 2016; Accepted 19 November 2016
Available online 23 November 2016
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of new phytoprotective agents (Barnard and Salmond, 2007).

The rhizosphere of plants, along with AHL producing pathogens like *Pcc*, also hosts AHL degrading bacteria (Chan et al., 2011; D'Angelo-Picard et al., 2005; Jafra et al., 2006). The degradation of AHLs inactivates the signal and leads to the disruption of QS which is termed as quorum quenching (QQ) (Uroz et al., 2009). The first QQ bacteria were identified from soil and belong to the Gram-positive *Bacillus* (Dong et al., 2000). Potato rhizosphere also showed the abundance of AHL degrading bacteria and they mainly belong to *Agrobacterium*, *Bacillus*, *Pseudomonas*, *Delftia*, *Ochrobactrum* and *Rhodococcus* genera (Cirou et al., 2007; Jafra et al., 2006). Use of AHL degrading bacteria for attenuation of soft rot on potato against *P. atrosepticum* (Smadja et al., 2004) or *P. carotovorum* has also been demonstrated (Molina et al., 2003; Uroz et al., 2003, 2009). A distinct characteristic of this strategy is that it attenuates the production of virulence factors and does not eradicate the pathogen unlike other antimicrobial methods (Faure and Dessaux, 2007).

Commonly studied biocontrol strategy against *Pcetobacterium* spp. is antagonism that involves usage of fluorescent pseudomonads and *Bacillus* strains for the control of blackleg and soft rot. The antagonistic properties exhibited by these strains were due to antibiotic synthesis, iron competition and induced systemic resistance in host (Cronin et al., 1997; De Weger et al., 1986, 1995; Sharga et al., 1998; Xu et al., 1986). These strains having antagonistic activity against pathogen were evaluated exhaustively for their biocontrol properties to establish them as biocontrol agents. Along with the *in vitro* antagonistic activity of the isolates these studies focused on their ability to colonize on roots and the persistence of *Pseudomonas* strains on treated plants. Additionally, the influence of other abiotic factors like soil texture and pH on the expression of antagonistic activities was observed to establish the antagonist as biocontrol agent (Diallo et al., 2010). Similar studies testing the bacterial QQ mechanism as effective biocontrol approach are sparse in literature. AHL degraders and their molecular mechanism of AHL degradation are being identified with great interest (Lee et al., 2002; Mei et al., 2010; Pan et al., 2008; Park et al., 2003, 2005, 2006; Wang et al., 2010; Yin et al., 2010) but evaluation of the biocontrol attributes of the AHL degrading bacteria for controlling the soft rot caused by *Pcc* has received less attention.

Bacillus spp. have many desirable properties of a biocontrol agent like ease of culturing, amenability to formulation and additionally in this case quorum quenching. Merely having the desired trait does not make a bacterium a good biocontrol agent. Meticulously designed screening procedures are necessary to select potential strains that can function as biocontrol agents. The focus of the current study is to evaluate the QQ bacteria for biocontrol properties such as adherence on seeds, colonisation ability and persistence of QQ isolates on root (mung bean model), broad host range which is susceptible to *Pcc* (Potato, Carrot and Cucumber) on which the QQ isolates can survive and exhibit the biocontrol potential. Additionally, studies showing absence of deleterious effect of the isolates on the host plant and the ability of the QQ isolates to control the disease pre-infection (preventive) and post-infection (curative) are included.

2. Materials and method

2.1. Bacterial strains and culture conditions

The QS pathogen used is a strain of *Pcc* from eggplant (*Pcc*BR1) that was found to cause soft rot in wide range of fruits and vegetables (Maisuria and Nerurkar, 2013). It produces the QS signal molecule 3-oxo-hexanoyl homoserine lactone (OHHL) that regulates its virulence factors viz. polygalturonase and pectate lyase (Maisuria et al., 2010; Maisuria and Nerurkar, 2012). The AHL biosensor strain *Chromobacterium violaceum* CV026 (McClean et al., 1997) was grown overnight under shaking conditions at 30 °C and maintained in Luria Bertani (LB) broth at 30 °C with Kanamycin (30 µg/ml). *Pcc*BR1 (Laboratory stock) and *Bacillus* isolates were grown and maintained in LB at 30 °C under shaking conditions.

2.2. Isolation of Bacteria from roots and soil samples

Roots of carrot (*Daucus carota)*, beetroot (*Beta vulgaris L*), maize (*Zea mays*), fenugreek (*Trigonella foenum-graecum*), potato (*Solanum tuberosum*), pigeon pea (*Cajanus cajan*), radish (*Raphanus sativus*), pearl millet (*Pennisetum glaucum*) and eggplant (*Solanum melongena*) were taken for isolating bacterial strains (Fall et al., 2004). Approximately 2.5 in. of plant root was washed with sterile Ringer's salt solution (NaCl 1.25 g, KCl 0.05 g, $CaCl_2 \cdot 2H_2O$ 0.06 g and $NaHCO_3$ 0.25 g in 500 ml distilled water) and sonicated in a sonic bath for 5 cycles of 60 s to remove adhering soil particles. Roots were heat treated at 80 °C for 20 min in a water bath to kill vegetative cells and selectively enrich for spore forming bacteria including *Bacillus* spp. Root was placed on casein mannitol (CM) semi-solid medium and colonies surrounding it were isolated after overnight incubation at 30 °C. CM medium contained 10 g/L casein digest (Sigma-Aldrich), 10 g/L D-mannitol, 10 g/L agar and 1.5 g $CaCl_2 \cdot 2H_2O$, 50 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 100 mg $CoCl_2 \cdot 6H_2O$, 100 mg $Na_2MoO_4 \cdot 2H_2O$, 50 mg $CuSO_4 \cdot 5H_2O$, 50 mg $ZnSO_4 \cdot 7H_2O$, 20 mg H_3BO_3, 50 mg $FeSO_4 \cdot 7H_2O$ and 50 mg ascorbic acid per litre.

For the isolation from soil, samples were collected from gardens in different areas of The Maharaja Sayajirao University of Baroda (Gujarat, India) campus and placed in distilled water. Soil particles were allowed to settle, the supernatant was heat treated as described above and bacteria were isolated on Luria agar (LA). All the isolates obtained from root or soil samples were maintained on LA throughout.

2.3. Bioassay for AHL degradation

N-Hexanoyl DL-homoserine lactone (HHL) degrading activity of the isolates was detected using the biosensor *C. violaceum* CV026, which produces the purple pigment violacein in response to externally added HHL. Bacterial isolates to be tested were grown overnight and centrifuged at 7000 rpm for 5 mins. Pelleted cells were resuspended in phosphate buffered saline (PBS pH 7.4). 80 µl of 25 µM HHL (Sigma Aldrich) prepared in PBS was added to 20 µl of pelleted cell suspension and the mixture was incubated at 30 °C for 2 h (Huang et al., 2012). 25 µM HHL without the cell suspension of the isolates was used as a control. For estimation of the remaining HHL the reaction mixture was added to the biosensor *C. violaceum* CV026 in 96 well microtiter plates. After overnight incubation at 30 °C under static conditions, the purple colour produced by the biosensor was expressed as Violacein Units (VU) calculated as absorbance at 585 nm / absorbance at 660 nm. The Violacein Unit is inversely proportional to the HHL degrading activity of the test isolate.

2.4. PCR amplification of 16S rRNA gene of isolates

Full length 16 S rRNA genes were amplified using the 16 S ribosomal DNA universal bacterial primer set 27 F (5'-AGAGTTTGA TCCTGGCTCAG3') and 1541 R (5'AAGGAGGTGATCCAGCCGCA3'). 50 µl PCR reactions were performed in an Eppendorf thermocycler and contained 200 µM deoxynucleoside triphosphate mix, 0.2 µM of each primer, 1.5 mM $MgCl_2$, PCR buffer, 1.5 U Taq DNA polymerase and 1 µl genomic DNA template. PCR conditions used were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. A final extension at 72 °C for 10 min was then performed.

2.5. Amplified ribosomal DNA restriction analysis (ARDRA)

The 16S rRNA gene amplicons (as described above) were used for ARDRA. Each 15 µl digestion reaction consisted of 1X Tango Buffer,

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