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The plant pathogen *Erwinia amylovora* produces acyl-homoserine lactone signal molecules in vitro and in planta

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Abstract

We report for the first time the production of acyl homoserine lactones (AHLs) by *Erwina amylovora*, an important quarantine bacterial pathogen that causes fire blight in plants. *E. amylovora* produces one *N*-acyl homoserine lactone [a *N*-(3-oxo-hexanoyl)-homoserine lactone or a *N*-(3-hydroxy-hexanoyl)-homoserine lactone] quorum sensing signal molecule both in vitro and in planta (pear plant). Given the involvement of AHLs in plant pathogenesis, we speculate that AHL-dependent quorum sensing could play an important role in the regulation of *E. amylovora* virulence.

Keywords: Quorum sensing; *Erwinia amylovora*; Acyl-homoserine lactone; Fire blight

1. Introduction

Erwinia amylovora is a necrogenic phytopathogenic bacterium, the causal agent of fire blight in apple, pear and other rosaceous plants [1]. The bacteria colonize the intercellular spaces of bark, causing the death of the plant cells associated with distortion of cell walls and the formation of lysogenic cavities. In the susceptible, succulent shoots, the necrosis spreads downwards from the apex with browning of the tissues. In recent years, new light has been thrown on the molecular mechanisms of the virulence [2]. Proteins injected into the plant cells through type III secretion appear to be crucial for establishment [3], while abundant extracellular polysaccarides are essential for tissue colonization [1]. The production of virulence factors of phytopathogenic *Erwinia* species is regulated by global systems which respond to stimuli like cell density (quorum sensing) or to other unknown signals resulting in the control by the Rsm global regulatory system [4].

In the last decade, it has become apparent that in bacteria a major level of regulation exists involving cell-cell communication via the production and detection of small signaling molecules called autoinducers. This allows bacteria to monitor their population density by responding to the extracellular concentration of the autoinducer they produce. This mechanism is known as quorum sensing (QS). In gram-negative bacteria, *N*-acyl homoserine lactone (AHL) autoinducers appear to be the most common signaling molecules, produced by an autoinducer synthase belonging to the LuxI protein family. The AHLs are diffusible and different bacterial species produces different AHLs

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which vary in the length and structure of the acyl chain [5]. A transcriptional regulator, belonging to the LuxR family, then forms a complex with the cognate AHL at high threshold levels affecting the transcriptional activity of target genes. QS regulated gene expression is most beneficial when a community of bacteria expresses them as for example is the case for biofilm formation, conjugation, bioluminescence, secretion of enzymes, virulence factors and pigment production ([6] and references therein). Several phytopathogenic bacteria have integrated QS regulation in the regulatory cascades that control expression of pathogenicity-related genes in plant-pathogenic bacteria. Among the best characterized examples are the AHL-dependent systems controlling conjugation in *Agrobacterium tumefaciens* and extracellular enzymes in *Erwinia carotovora* ([7] and references therein).

In this study we demonstrate that *E. amylovora* has at least one quorum sensing system present since several Italian isolates produce either a *N*-(3-oxo-hexanoyl)-homoserine lactone (3-oxo-C6-AHL) or a *N*-(3-hydroxy-hexanoyl)-homoserine lactone (3-OH-C6-AHL) in vitro. Importantly, we have also demonstrated that production of 3-oxo-C6-AHL by one of the isolates also occurs in infected tissues in planta, indicating that AHL-quorum sensing could have a role in pathogenesis. To our knowledge this is the first report demonstrating the presence of an AHL-dependent system in *E. amylovora* thus raising the question of its possible role in pathogenesis, as the AHL molecule produced was also detected in planta.

2. Materials and methods

2.1. Bacterial strains, culture conditions and recombinant DNA techniques

Erwinia amylovora strains used are listed in Table 1 and were grown at 27 °C in either M9CA medium [8] with the addition of 0.3% w/v casamino acids and sucrose or in LB medium [8]. *Escherichia coli* JM109 (pSB401) was grown in LB medium plus tetracycline 10 μg/ml at 37 °C. Plasmid pSB401 contains the following genetic arrangement: *luxR* gene, the promoter of *luxI* fused to a promoterless *luxCDABE* [9]. Providing exogenous AHL inducer molecules to *E. coli* (pSB401) results in the induction of bioluminescence. *E. coli* DH5α (pSCR2) was grown in LB medium plus ampicillin 100 μg/ml at 37 °C. Plasmid pSCR2 contains the following genetic arrangement: *cepR* gene, the promoter of *cepI* fused to a promoterless *lacZ* [10]. Providing exogenous AHL inducer molecules to *E. coli* (pSCR2) results in the induction of β-galactosidase production. *Chromobacterium violaceum* CVO26 was grown in LB medium and is a double mini-Tn*5* mutant derived from ATCC 31532, this mutant is non-pigmented and production of the purple pigment can be induced by providing exogenous AHL inducer molecules [11]. PCR was performed using genomic DNA of *E. amylovora* OMP-BO 1077/7 and oligonulceotides designed on regions of *E. carotovora expIR* locus [12]. Using the following two oligonucleotides, EAM1 5′ttaaatttcacatcggcaa-3′ and EAM2 5′ttatctacttttttacct-3′ (using conditions, 5 m at 98 °C initially, then 30 s 95 °C, 30 s 45 and 30 s at 72 °C for 30 cycles), resulted in the amplification of a 223-bp which was cloned in pMOSBlue (Amersham Pharmacia Biotech, Amersham, UK) yielding pGEAM. DNA sequencing of this fragment revealed that it contained part of the *luxIR* homologs of *E. amylovora* (Accession No. AJ841286).

2.2. Inoculation of plant material

Two-year old pear cv. Abate Fetel (most common used in Italy and it is sensitive to *E. amylovora*), grafted onto quince BA29, were grown in individual pots in the open. Three weeks before the experiment, in mid April, the plants were placed in a climatic chamber at 24 °C so that the shoots reached a length of 23–25 cm. For inoculation, 5 l of bacterial suspension in distilled water with a turbidity of $0.100 A_{660}$ were placed on a cross cut on each shoot at 20 cm from the base. Control shoots were

Table 1
Erwinia amylovora strains

E. amylovora strains	Host plant, year of isolation, origin	Reference
E. amylovora OMP-BO 1077/7	Pear, 1994, Emilia Romagna region, Italy	[16]
E. amylovora IPV-BO 5039	Hawthorn, 2003, Friuli Venezia Giulia region, Italy	This study
E. amylovora IPV-BO 5357	Cotoneaster, 2003, Piemonte region, Italy	This study
E. amylovora IPV-BO 5359	Pear, 2003, Piemonte region, Italy	This study
E. amylovora IPV-BO 5452	Hawthorn, 2003, Veneto region, Italy	This study
E. amylovora IPV-BO 5466	Pear, 2003, Veneto region, Italy	This study
E. amylovora IPV-BO 5468	Cotoneaster, 2003, Veneto region, Italy	This study
E. amylovora IPV-BO 5010	Pear, 2003, Lombardia region, Italy	This study
E. amylovora IPV-BO 5011	Pear, 2003, Lombardia region, Italy	This study
E. amylovora IPV-BO 4853	Apple, 2003, Bolzano province, Italy	This study

treated with distilled water. After 4 days in the climatic chamber, the shoots were cut at the base, stripped of their leaves, washed in tap water, dried rapidly on paper towels and immediately frozen in liquid nitrogen. Twelve shoots were not frozen, and were used to determine bacterial growth in planta.

2.3. In planta bacterial growth

Shoot segments, 20 cm, were washed with tap water, dried on paper towels, cleaned with denatured alcohol under sterile flow hood, rinsed with distilled sterile water and dried again with sterile blotting paper. One-cm shoot segments were cut with a sterile scalpel at a distance of 1.5, 5.5, 8.5, 11.5, 14.5, and 17.5 cm from the edge of the visible necrotic area of each shoot. For each distance two 1 cm segments have been crushed in a pestle containing 1.5 ml sterile distilled water. The suspension obtained and tenfold dilution were used for plate counting on sucrose nutrient agar [13].

2.4. Purification and characterization of AHLs

A culture of *E. amylovora* IPV-BO 1077/7 was grown to early stationary phase in 10 l M9CA medium [8] in a Tecbio 10 (Tecninox, Parma, Italy) fermenter at 27 °C. The supernantant from the 10 l culture was concentrated to 200 ml by rotary evaporation at 40 °C in 2 days, acidified with 0.1% acetic acid and extracted twice with the same volume of ethyl acetate. The extract in the organic solvent was dried overnight at room temperature under a chemical flow hood and resuspended in a final volume of 1 ml methanol/water (20:80 vol/vol). This extract was fractionated by HPLC with a semipreparative C_{18} reverse-phase column (1×25 cm) using a methanol:water gradient from 20:80 to 80:20 eluent composition in 35 min at a flow rate of 2.5 ml/min. Fractions of 5 ml each were collected, dried and resuspended in 10 μl of ethyl acetate. They were applied to C_{18} reversed-phase TLC plate with fluorescent detector F_{254} (20×20 cm, Merck, Darmstadt, Germany) and separated using methanol/water (60:40 vol/vol) as eluent. After chromatography, the solvent was evaporated and the dried plates were overlaid with *E. coli* JM109 (pSB401) mixed with LB top agar, as described by [14]. Positive fractions containing the autoinducers were detected by the appearance of a dark spot, through exposure and development of an autoradiographic film. For *E. amylovora* strains IPV-BO 5039, IPV-BO 5357, IPV-BO 5359, IPV-BO 5452, IPV-BO 5466, IPV-BO 5468, IPV-BO 5010, IPV-BO 5010 and IPV-BO 4853, the supernatant from 200 ml of LB cultures of the other *E. amylovora* strains was acidified with 0.1% acetic acid and extracted twice with the same volume of ethyl acetate. The extract from the supernatant of each *E. amylovora* strain culture was finally resuspended in 50 μl of ethyl acetate and directly analyzed on TLC as described above. AHLs purification from plant material after the infection with *E. amylovora* IPV-BO 1077/7 was performed as follows; twenty cm of shoot immediately downstream the necrotic area were cut in 1-cm pieces from 75 shoots, resulting in 100 g of infected biological material. The sample was frozen in liquid nitrogen, milled and resuspended in 500 ml distilled water at 40 °C. After shaking for 2 h at 30 °C the suspension was filtered through sterile gauze and centrifuged at 6000 rpm in a Sorvall centrifuge using a GSA rotor. The resulting 450 ml solution was acidified with 0.1% acetic acid and extracted twice with the same volume of ethyl acetate. The organic phase was recovered after centrifugation at 5000 rpm (Sorvall with a GSA rotor) for 5 min and dried. The extract was resuspended and prepared for HPLC fractionation, TLC analysis and overlaid with *E. coli* (pSB401) as described above. The same procedure was performed on 75 uninfected healthy shoots.

3. Results and discussion

3.1. Detection of AHL autoinducers from E. amylovora isolates

Using bacterial biosensor strains described in Section 2, all the *E. amylovora* isolates listed in Table 1 were used to test by growth in solid media in plate streak assay [15]. The bacterial biosensor *C. violaceum* CVO26 induces the production of violacein when AHL signal molecules are present, *E. coli* (pSCR2) activates the biosynthesis of β-galactosidase, and *E. coli* (pSB401) induces light emission. All strains gave a negative result in solid media in plate streak assays meaning that they either did not produce AHLs, produced very low amounts or the biosensors displayed low sensitivity towards the putative molecule(s) that they produce. Consequently, in order to determine if *E. amylovora* produced AHLs, purification of AHLs was performed from a 10 l of *E. amylovora* IPV-BO 1077/7 spent supernatant by extraction, HPLC and TLC as described in Section 2. The detection of AHLs in TLC plates was visualized by making use of *E. coli* JM109 (pSB401) which induced light emission in the presence of AHLs. Fig. 1 depicts the result of this analysis showing that one spot was detected in one of the fractions after HPLC meaning that this *E. amylovora* strain produces at least one AHL molecule. Characterization of this AHL was performed by examination of the R_f of the spot on the TLC with respect to standard reference samples and it was deduced that this molecule is most likely a *N*-(3-oxo-hexanoyl)-homoserine lactone (3-oxo-C6-AHL) (Fig. 1). As apparently the spot on the TLC did not produce a tailing effect, it is possible that the AHL produced by *E. amylovora* is a 3-hydroxysubstituted C6-AHL as

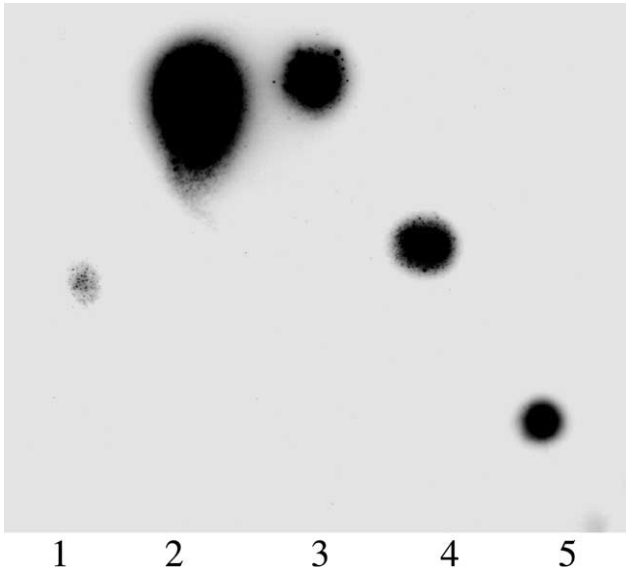

Fig. 1. TLC analysis of AHLs produced by *E. amylovora* strain IPV-BO 1077/7. *E. coli* (pSB401) AHL biosensor was used for the detection of the spots. Lane 1, 45 ng of synthetic 3-oxo-C8-AHL (provided by P. Williams, University of Nottingham, UK), Lane 2, 17 ng of synthetic 3-oxo-C6-AHL (Fluka, Buchs, CH), Lane 3, AHL purified from *E. amylovora* IPV-BO 1077/7 spent supernatant Lane 4 and 5, synthetic 19 ng of C6-AHL and 90 ng of C8-AHL, respectively. See text for details.

this molecule migrates with the same mobility as its 3-oxo analog but the spot does not tail [14]. Finally, since the spot shown in Fig. 1 corresponds to the amount present in 650 ml of spent supernatant, it is concluded that under conditions of controlled in vitro growth this *E. amylovora* strain produced very little amount of this AHL molecule. This direct biodetection must be used with caution as detection is limited to which AHL the biosensor will respond. Plasmid pSB401 is a LuxR based biosensor which is rather sensitive and detects well C6 type of AHL molecules [9] thus also providing a good indication of the amount of AHL molecules present in the TLC. This low level production of AHL by *E. amylovora* raises the question of whether this AHL-quorum sensing system requires very low levels for its functioning or possibly that this system is under tight regulation. The characterization of AHL molecules produced was also determined in other nine *E. amylovora* strains isolated from various host plants and regions of Italy (Table 1); namely the strains were IPV-BO 5039, IPV-BO 5357, IPV-BO 5359, IPV-BO 5452, IPV-BO 5466, IPV-BO5468, IPV-BO 5010, IPV-BO 5010 and IPV-BO 4853. AHLs were purified from 200 ml liquid cultures in rich medium and all strains as detected in TLC with *E. coli* (pSB401) produced the same spot as detected with *E. amylovora* IPV-BO 1077/7 (data not shown). It was concluded that production of this AHL was conserved in *E. amylovora* and to our knowledge this is the first report of AHL production in this species. In addition, from the purification and analysis of 200 ml of spent supernatant it was concluded that all *E. amylovora* strains produced similar low amounts of AHLs.

3.2. Evidence for the presence of luxIR homologs in E. amylovora

After having demonstrated the presence of AHL molecules, it was of interest to verify if the corresponding *luxIR* homologs of *E. amylovora*, which are responsible for the synthesis and response to the AHL molecule, were present in the chromosome. In order to demonstrate this, two oligonucleotides were designed based on the *expIR* sequence of closely related *E. carotovora* [12]. Consequently, using two oligonucleotides as primer pairs in a PCR reaction (see Section 2) resulted in the amplification of an expected fragment of 223 bp (Accession No. AJ841286) which displayed 90% identity with *expIR* corresponding to the last 120 bp of a *luxI* homolog and 100 bp of the end of a *luxR* homolog (data not shown). This indicated that like in *E. carotovora*, also in *E. amylovora* the *luxIR* homologs are convergently transcribed and being highly identical in primary structure. Interestingly, *E. carotovora* produces a *N*-(3-oxo-hexanoyl)-homoserine lactone (3-oxo-C6-AHL) [12], the probable AHL produced by *E. amylovora* (see above).

3.3. Detection of AHL autoinducers from E. amylovora IPV-BO 1077/7 in planta

Following the observation that *E. amylovora* OMP-BO 1077/7 produces one AHL molecule (see above), it was of interest to determine if it also produces the signal molecule in planta. After 4 days at 24 °C, all the inoculated shoots showed apical necrosis with dark brown discoloration which spread downwards for approximately 1–3 cm below the inoculation point. Six segments, 1 cm long, were cut from the symptomless section of each shoot at increasing distances from the border of necrosis and were used to assess the CFU/g of plant tissue (Fig. 2(a)). As depicted, the bacteria colonized the plant very efficiently also detecting 2×10^8 CFU/g at 17.5 cm from the necrosis front. Organic extracts of the plant infected material were made and then subjected to HPLC and TLC resulting in the detection of a AHL molecule with the same R_f value of the AHL produced from the same *E. amylovora* strain when grown in vitro (Fig. 2(b)). The same purification procedure was made using the same amount of healthy uninfected plant tissue material and this did not reveal any positive spot on the TLC overlaid with sensor *E. coli* (pSB401) (data not shown). This observation of AHL presence in planta, indicates that the AHL-dependent

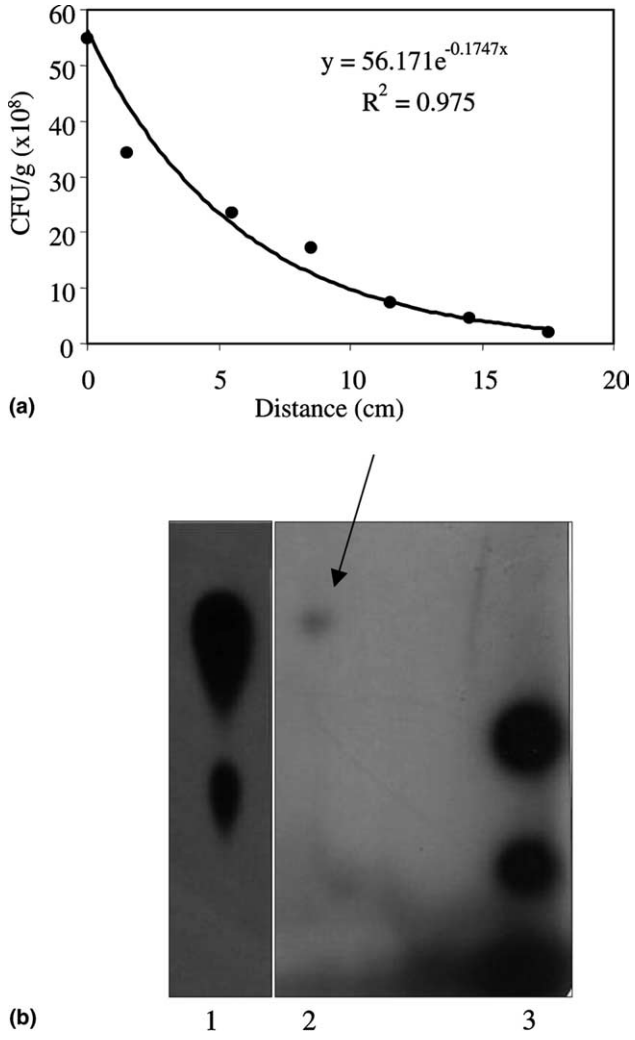

Fig. 2. Analysis of AHLs produced by *E. amylovora* strain IPV-BO 1077/7 in planta. (a) Determination of CFU/g of plant tissue at various cm distances from the front of necrosis. Each point represents a quadruplicate value triplicate experiments. (b) TLC analysis of AHLs produced by *E. amylovora* strain IPV-BO 1077/7. *E. coli* (pSB401) AHL biosensor was used for the detection of the spots. Lane 1 synthetic 45 ng of 3-oxo-C8-AHL and 17 ng of 3-oxo-C6-AHL (provided by P. Williams, University of Nottingham, UK), this lane was part of the same TLC but was exposed with auto radiographic film for less time. Lane 2 the HPLC fraction of the AHL purified (spot corresponding to AHL is indicated by an arrow) from *E. amylovora* IPV-BO 1077/7 infected plant tissue. Lane 3 synthetic 19 ng of C6-AHL and 90 ng of C8-AHL (Fluka, Buchs, CH). See text for details.

quorum sensing system is functional in planta raising the important question of its role in plant colonization and pathogenesis. Future work will determine the role of quorum sensing in the disease of this important plant pathogen possibly providing a target for the control of the disease.

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