Microbial Biotechnology (2008) **1**(5), 416–424 doi:10.1111/j.1751-7915.2008.00043.x

Hypersensitive response and acyl-homoserine lactone production of the fire blight antagonists *Erwinia tasmaniensis* and *Erwinia billingiae*

Vladimir Jakovljevic, Susanne Jock, Zhiqiang Du and Klaus Geider*
Max-Planck-Institut für Zellbiologie, Ladenburg and Julius Kühn Institut für Pflanzenschutz in Obst- und Weinbau, Schwabenheimer Str. 101, 69221 Dossenheim, Germany.

Summary

Fire blight caused by the Gram-negative bacterium *Erwinia amylovora* can be controlled by antagonistic microorganisms. We characterized epiphytic bacteria isolated from healthy apple and pear trees in Australia, named *Erwinia tasmaniensis*, and the epiphytic bacterium *Erwinia billingiae* from England for physiological properties, interaction with plants and interference with growth of *E. amylovora*. They reduced symptom formation by the fire blight pathogen on immature pears and the colonization of apple flowers. In contrast to *E. billingiae*, *E. tasmaniensis* strains induced a hypersensitive response in tobacco leaves and synthesized levan in the presence of sucrose. With consensus primers deduced from *lsc* as well as *hrpL*, *hrc*C and *hrc*R of the *hrp* region of *E. amylovora* and of related bacteria, these genes were successfully amplified from *E. tasmaniensis* DNA and alignment of the encoded proteins to other *Erwinia* species supported a role for environmental fitness of the epiphyti*c* bacterium. Unlike *E. tasmaniensis*, the epiphytic bacterium *E. billingiae* produced an acyl-homoserine lactone for bacterial cell-to-cell communication. Their competition with the growth of *E. amylovora* may be involved in controlling fire blight.

Introduction

Fire blight is a devastating disease of apple and pear fruit trees, and is caused by the Gram-negative bacterium *Erwinia amylovora*. The pathogen affects apples and pears as well as other rosaceous plants, including some ornamentals. Fire blight is responsible for severe losses in apple and pear production in North America, Europe, the Mediterranean region and New Zealand. Strategies to control fire blight include chemical control, breeding of resistant plants and biological control. None of the proposed strategies reviewed in details elsewhere is completely satisfactory (Vanneste, 2000).

Colonization of flowers by *E. amylovora* is considered as a primary step in the development of most fire blight infections of apple and pear trees (Stockwell *et al*., 1999). Blossoms remain susceptible to bacterial colonization for few days after opening and the presence of antagonistic bacteria in this period can suppress colonization by *E. amylovora* (Stockwell *et al*., 2002; Temple *et al*., 2004; Stockwell and Stack, 2007).

The non-pathogenic epiphytic bacterium *Pseudomonas fluorescens* strain A506 has been used as a fire blight biocontrol agent, and it is commercially available (BlightBan A506). The epiphyte *Pantoea agglomerans* (syn. *Erwinia herbicola*) has been investigated as a potential antagonist (Vanneste *et al*., 1992; Wright *et al*., 2001; Giddens *et al*., 2003), and strain C9-1 has been applied experimentally in the USA (Johnson and Stockwell, 1998). *Bacillus megaterium* and *Bacillus pumilus* strains have been reported to inhibit growth of *E. amylovora* (Jock *et al*., 2002). Isolation of *Enterobacter agglomerans* from clinical specimens and its classification together with *E. herbicola* (Gavini *et al*., 1989) has restricted the use of *P. agglomerans* (*E. herbicola*) strains as a control agent in most countries with fire blight. There are also reports that *P. fluorescens* can bind to human nerve cells and fibronectin (Picot *et al*., 2001; de Lima Pimenta, 2003). Obviously, it is of great importance to look for additional antagonists to control fire blight preferentially with bacteria of the genus *Erwinia*. Here, we investigate properties of two novel *Erwinia* species to act as antagonists against fire blight.

Results

Growth inhibition of E. amylovora

The levan-positive epiphytic strains Et1/99 and Et2/99 from Australia were classified into the novel species

Received 21 April, 2008; revised 19 May, 2008; accepted 22 May, 2008. *For correspondence. E-mail Klaus.Geider@jki.bund.de; Tel. (+49) 6221 86805 53; Fax. (+49) 6221 86805 15

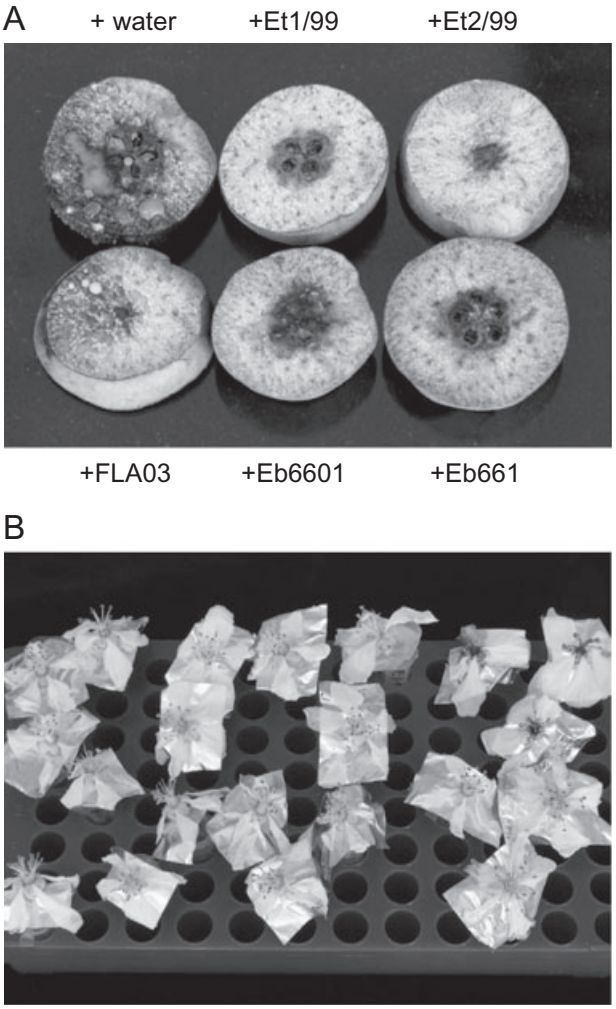

Fig 1. Antagonistic effects of *E. billingiae*, *E. tasmaniensis* on pear slices and apple flowers inoculated with *E. amylovora*.
A. Symptoms after treatment of pear slices with Eb660, Eb661, FLA03, Et1/99 and Et2/99. One slice from a set of four similar slices with the same treatment is shown.
B. Fire blight symptoms on apple flowers sprayed with Eb660, Eb661, Et1/99 and Et2/99, and then inoculated with 500 cells of *E. amylovora* Ea1/79Sm. Control was treatment with water before inoculation with *E. amylovora* (three flowers top right). Evaluation for growth of Ea1/79Sm is given in Table 1. The experiments were repeated at least twice producing similar results.

Erwinia tasmaniensis and did not affect apple seedlings nor immature pear slices to produce symptoms resembling fire blight (Geider *et al*., 2006). The *E. tasmaniensis* strains and the recently classified epiphytic bacterium *Erwinia billingiae* (Mergaert *et al*., 1999) were tested for their antagonistic effects against *E. amylovora* in assays with immature pear. Pear slices, which were soaked in a suspension of *E. tasmaniensis* or *E. billingiae* cells and then inoculated with *E. amylovora* (500 cfu ml^{-1}), showed absence or a significant reduction of ooze production and necrosis compared with controls with water (Fig. 1A). The *E. tasmaniensis* strains showed often enhanced effects in 5% sucrose solution while *E. billingiae* Eb660 and Eb661 were as efficient in symptom reduction when pear slices were soaked with bacteria in water. *Erwinia billingiae* still showed strong antagonistic effects when high levels (up to 50 000 cfu ml^{-1}) of *E. amylovora* were applied. The German isolate FLA03, classified as *E. tasmaniensis*, weakly interfered with growth of *E. amylovora*.

In similar growth competition assays with apple flowers, the antagonistic strain was diluted in water to 1×10^8 cfu ml^{-1} and sprayed on detached apple flowers about 1 or 2 days after opening with cells of several *E. billingiae* and *E. tasmaniensis* strains. After drying at room temperature, 5000 *E. amylovora* in 10 μl of water were applied to the pistil and the flowers then incubated for 5 days (Fig. 1B). The number of recovered *E. amylovora* cells (Sm^r) was quantified by plating serial dilutions on agar plates (Table 1). In contrast to control flowers sprayed with water, the number of *E. amylovora* cells diminished in flowers treated with suspensions of *E. billingiae* Eb660 and Eb661. The *E. tasmaniensis* strains Et1/99 and Et2/99 also largely reduced growth of *E. amylovora* in apple flowers. It should be emphasized that flowers are not equally protected after application of antagonistic bacteria. In two of three flowers, Et2/99 reduced growth of *E. amylovora* to a low level, whereas another flower showed an atypical intermediate colonization by the pathogen (Table 1).

The *E. tasmaniensis* strains Et1/99 and Et2/99 as well as the *E. billingiae* strains Eb660 and Eb661 did not produce growth inhibition zones on a lawn of Ea1/79. Although weak effects of culture supernatants of *E. billingiae* on growth of *E. amylovora* were occasionally observed, interference with *E. amylovora* in flowers should mainly be due to a dominant population of an antagonistic bacterial species.

Table 1. Effect of *E. billingiae* and *E. tasmaniensis* on colonization of apple flowers with *E. amylovora*.

Inoculation with *E. amylovora* Ea1/79Sm	
Flowers treated with	Ea1/78Sm recovered (cfu)
E. billingiae Eb660	0
E. billingiae Eb661	2×10^2
E. tasmaniensis Et1/99	1×10^3
E. tasmaniensis Et2/99	3×10^{3a}
Water control	4×10^7

a. Mean of three flowers, single flowers: $0/1 \times 10^4/0$.
The numbers refer to the average of Ea1/79Sm cells recovered on Stl agar with streptomycin and cycloheximide from three flowers at 5 days. The antagonistic bacteria were sprayed at 1×10^8 cfu ml^{-1} in water. Inoculation was done with 10 μl droplets containing 5000 cfu of Ea1/79Sm.


```
Et1/99    1 AACCAACGCTGTGGACTCGTGCCGATGCGTTGAAAGTGCATTCAGATGATCCAACCACAACTCAACCTCTTGTTGATGTTG
Et2/99    1 ................................................................................
Et4/99    1 ................................................................................
Ea1/79    1 ..........................A........T...GAG.....C.................GG..A....CA...

Et1/99   82 CATTTCCAGTAATGAGCGAAGAGGTGTTTATTTGGGATACCATGCCACTGCGTGACTTCGACGGTGATATTGTCTCGGTAA
Et2/99   82 ................................................................................
Et4/99   82 ................................................................................
Ea1/79   82 ....C..G........T.....A..C.....................T....A...........A..G...A....T....

Et1/99  163 ACGGCTGGTGCGTTATATTTACGCTGACGGCGGATCGCAATACGAATAATCCAGATTTCCAGGATGAAAATGGGAACTACG
Et2/99  163 ................................................................................
Et4/99  163 ................................................................................
Ea1/79  163 .T..T.....TA....T........A..A..A........C..TG.......GC.A.................C..T..T.

Et1/99  244 ATATTAAGCGTGACTGGGAAGACAGGCACGGCCGTGCACGTATTTGTTATTGGTACTCACGCACCGGTAAAGACTGGATCT
Et2/99  244 ................................................................................
Et4/99  244 ................................................................................
Ea1/79  244 ......CT.................A..T..T.....G.....................................T.

Et1/99  325 TTGGCGGTCGGGTTATGGCTGAAGGTGTCTCACCGACGACTCGTGAATGGGCCGGAACCCCGATCCTTTTAAATGATGAAG
Et2/99  325 ................................................................................
Et4/99  325 ................................................................................
Ea1/79  325 ............A.....C.........G..........G.....G.........................C...CGG.

Et1/99  406 GTGATATTGACCTTTATTACACCTGTGTCACTCCTGGTGCAACGATTGCCAAAGTGCGCGGTAAAATTGTGACGTCTGATG
Et2/99  406 ................................................................................
Et4/99  406 ................................................................................
Ea1/79  406 .C...........G.....T.............G........C.....................C..C..T..C...C

Et1/99  487 AGGGTGTGAGCCTGGAAGGTTTCCAGCATGTTAAATCACTTTTCTCTGCTGATGGTAAAATTTACCAGACGGAAGAGCA
Et2/99  487 ...............................................................................
Et4/99  487 ...............................................................................
Ea1/79  487 .AA....A.............T.....G....C.................C..G.CT.....................
```

Fig 2. Sequence comparison of parts from *lsc* genes of the *E. tasmaniensis* strains Et1/99, Et2/99 and Et4/99 with *lsc* of *E. amylovora* strain Ea1/79.

The levansucrase gene and PCR detection of E. tasmaniensis

The epiphytic *E. tasmaniensis* strains from Australia produce large amounts of levan. To amplify the *lsc* gene, the PCR primers LSC1 and LSC2c were designed from the *lsc* gene of *E. amylovora* (Accession Number X75079). They amplified a 565 bp fragment from *E. tasmaniensis*, which was sequenced for strains Et1/99, Et2/99 and Et4/99. Alignment with the corresponding part of *lsc* from *E. amylovora* showed significant differences for the structural fragment, but the *E. tasmaniensis* sequences were identical among each other (Fig. 2). These primers could therefore be applied for detection of *E. tasmaniensis* in bacterial populations on plant surfaces. From parts of *E. tasmaniensis lsc*, which are divergent to *E. amylovora*, PCR primers were designed, and produced a PCR band of 0.3 kb. The primers LSCa1 and LSCa2c for amplification of DNA from several strains or bacterial species only amplified DNA from *E. tasmaniensis*. No signal was obtained for DNA from *Brenneria rubrifaciens*, *B. nigrifluens*, *B. quercina*, *B. salicis*, *E. amylovora* CFBP1232^T, *E. amylovora* Ea1/79, *E. amylovora* Ea273, *E. billingiae* Eb661, *Erwinia mallotivora*, *Erwinia papayae*, *Erwinia persicina* including an isolate from apple of our laboratory, *Erwinia psidii*, *Erwinia pyrifoliae* Ejp557 (Japan), *E. pyrifoliae* Ep1/96 (Korea), *Erwinia rhapontici*, *Erwinia toletana*, *Erwinia tracheiphila*, *Pantoea stewartii* DC283 and *Pectobacterium cypripedii* (data not shown; the species name only indicates a type strain). We therefore conclude a specific amplification of the *lsc* gene from *E. tasmaniensis* strains with these primers in respect to other *Erwinia* species, and they are therefore useful to detect this species among other bacteria.

Hypersensitive response and hrp *genes of* E. tasmaniensis

The production of a hypersensitive response (HR) is a typical property of most plant-pathogenic bacteria. When

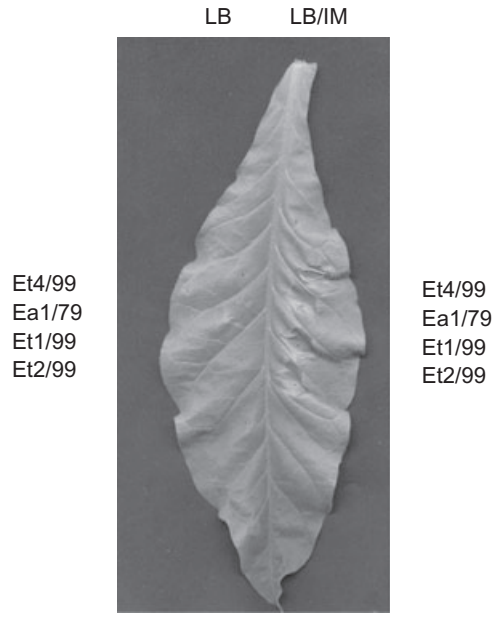

Fig 3. Induction of HR on tobacco leaves. Ea1/79, Et1/99, Et2/99 and Et4/99 were cultured in inducing medium and produced local necrotic HR lesions (right) in a leaf of cultivar 'SR1'. No hypersensitive response was found for *E. billingiae* Eb661 or with the *E. tasmaniensis* strains Et1/99, Et2/99 and Et4/99 without conditioning in IM (left).

the cells of the three *E. tasmaniensis* strains Et1/99, Et2/99 and Et4/99 were infiltrated into tobacco leaves (cv. 'SR1') at a density of 1×10^8 cfu ml^{-1}, they caused HR in leaves (cv. 'SR1') only after conditioning in an inducing medium (IM) (Fig. 3). No HR was observed for the *E. billingiae* strain Eb661. After cultivation in nutrient broth, the *E. tasmaniensis* strains induced HR in leaves of other tobacco cultivars such as 'Samsun'.

In order to increase the capacity for HR induction, the *hrpL* gene of *E. amylovora* was introduced into an *E. tasmaniensis* strain. When Et1/99(pGThrpL-Ea1) or Et4/99(pGThrpL-Ea1) were infiltrated into tobacco leaves (cv. 'SR1'), a reliable HR was observed without conditioning of the bacteria in IM. It is assumed that the IM medium increases expression of *hrp* genes.

Genes in the *hrp* cluster of *E. tasmaniensis* were analysed with primers from the two conserved genes *hrc*C/*hrc*R by comparison with sequences of *E. amylovora* and *E. pyrifoliae.* PCR bands of 0.7 and 0.5 kb were obtained with DNA of Et1/99, Et2/99 and Et4/99 for *hrc*C and *hrc*R fragments respectively. These products were cloned into pGEM-T, sequenced and aligned to the corresponding genes from other pathogenic bacteria. The alignment of 187 amino acids of HrcC showed 90% similarity to *E. amylovora* and 91% to the related Asian pear pathogen. Alignments of 146 amino acids of HrcR showed 91% similarity to *E. amylovora* and 95% to the proteins of the Asian pear pathogen (Fig. 4). Amino acid similarities greater that 70% were also found for HrcC proteins of other plant-pathogenic bacteria, such as *Dickeya* (*Erwinia*) *chrysanthemi*, *Pectobacterium carotovorum* and *Pseudomonas syringae.* Conserved domains homologous to the proteins of the flagella biosynthesis and type III secretion pathways were found, which is a common feature of Hrp/Hrc proteins.

The *hrpL* gene of Et1/99 was amplified by PCR applying primers HRPL1 and HRPL2c derived from the *hrpL* sequence of *E. amylovora*. Gene comparison on the protein level revealed high similarity to the HrpL proteins of *E. amylovora* and *E. pyrifoliae* (Fig. 4). HrpL of *P. stewartii* (Accession Number AF282857) was only 75% similar to HrpL of *E. tasmaniensis*. Nevertheless, the presence of *hrp* genes in the genome of *E. tasmaniensis* strain Et1/99 supports its ability to induce HR in tobacco leaves and may add to its epiphytic fitness.

Acyl-homoserine lactone production by E. billingiae

The *E. tasmaniensis* and *E. billingiae* strains were investigated for production of acyl-homoserine lactones (AHLs) involved in cell-to-cell communication of many bacteria. The screening was performed by using different sensor strains *Chromobacterium violaceum* CV026 and *E. coli* MT102 (pJBA132) as described in *Experimental procedures*. Synthesis of an AHL was detected for the plant-pathogenic bacterium *E. rhapontici*, for three *E. billingiae* strains assayed (Fig. 5), Eb660, Eb661 and Eb1261, for *P. stewartii* (DC283) and for *D. chrysanthemi*, but not for *E. amylovora*, the Asian pear pathogen *E. pyrifoliae*, nor for *E. tasmaniensis* and *E. mallotivora*. In particular, a complementation of *C. violaceum* CV026 was not observed for strains Ea1/79, Ep1/96, nor for Et1/99, Et2/99 and Et4/99 (Table 2). The genus *Erwinia* is apparently heterogeneous for AHL production.

We introduced an internal AHL reporter into cells of *E. amylovora* Ea1/79 and *P. stewartii* DC283, which is plasmid pJB132, where the *gfp* gene is fused to a *luxI* promoter. No fluorescence was observed for Ea1/79(pJBA132). Cells of DC283 (pJBA132) showed intensive green fluorescence due to AHL activation of *gfp* gene in pJBA132. When Ea1/79(pJBA132) was cross-streaked with DC283 or other AHL-producing bacteria, they produced fluorescence proofing the ability of *E. amylovora* to respond to exogenous AHL. Others such as *E. tasmaniensis* did not restore fluorescence. In addition, the *esaR* gene of *P. stewartii* was cloned with and without *esaI* into plasmid pSU23 and expressed under control of the *lacZ* promoter. When strains Ea1/79(pesaR) and Ea1/79(pesaIR) were cross-streaked with CV026, colour formation was restored with Ea1/79 (pesaIR), but not with Ea1/79 (pesaR). These experiments show the ability of

HrcC

```
Et2/99     1 MTFPLRYASVADRTIRYRDQTVVIPGVATMLNELMNGKRPAPASASGSDGTAGAPDTGSMMQNTQSLLSRLSSRNKTPGRAGDRDSGIDDLSGR
Ea1/79     1 ..............H.....................A.......I.S.P.G...N.......T..........SN...G..NE.E V...
Ep4/96     1 ..............H.....................A.........SAP.G...T.......T..........SANK..G...D...V...

Et2/99    95 ISADVRNNALLIRDDDKRRDEYSQLIGKIDVPQNLVEIDAVILDIDRTALNRLEANWQATLGGVTGGSSLMSGSGTLFVSDFKRFFADIQALE
Ea1/79    95 .................H.......A.....................................................................
Ep4/96    95 ........................A.....................................................................
```

HrcR

```
Et4/99     1       MALYGIALAATLFVMAPVFNQMQQQFSQSPADLSSMDSLKNSVTHGVAPQQKFMTHNTDPDILIHLQENSVRMWPKEMSDSVSKDNLLLV
Ea1/79     1       ..............S.....I....T.V........N..T...N....LH.....................C........N.......
Ep4/96     1       ............................V........N..T..SN....L.....................................

Et4/99    91       IPAFVLSELQAGFKIGFLIYIPFIVIDLIVSNVLLALGMQMVAPMTLSLPLKMLLF
Ea1/79    91       ..............................H.....................
Ep4/96    91       ..........................................................
```

HrpL

```
Et1/99     1 MTEVHLLKTEKPSIGDALPLNIDWEGIFREHGRRVHHFIRKRVSHREDAEDLEQMTWLEVLRNRDKFAGASRPETWVFGIALNLVRNHFRL
Ea1/79     1 ...I..QT..ST.VN.G.....................................................................
Ep4/96     1 ...I..QS..PT.VNEG.....................................................................

Et1/99    92 QSGRPRCDELEDDIVVVQGDDPSHITEHQRILTSTLNSIAALPEDTRRLMSMLVEKDGSYQAIAAHLNIPIGTVRSRLSRARVSLKQSVFS
Ea1/79    92 ..............ILT..........Y..L.N.......N.........LN.............................T.......
Ep4/96    92 ..........D...ILT..........Y..L.N.......V....A....N..........D.....................T.......
```

Fig 4. Alignment of partial amino acid sequences HrcC, HrcR and HrpL from *E. tasmaniensis*, *E. amylovora* and *E. pyrifoliae* strains. HrcC (187 aa): *E. tasmaniensis* Et2/99, *E. amylovora* Ea1/79 and *E. pyrifoliae* Ep4/96; HrcR (146 aa): same but Et4/99; HrpL (182 aa): same but Et1/99.

E. amylovora to synthesize AHL, when the *luxI* (*esaI*) gene is provided *in trans*.

Discussion

Bacteria have developed several strategies to face competitive conditions of their environment. They may release toxic compounds described for *B. pumilus*, some *B. megaterium* strains (Jock *et al*., 2002) and also for other Gram-positive bacteria (Emmert and Handelsman, 1999). For sensing cell density, they often secrete small compounds, such as AHLs (von Bodman *et al*., 2003) or auto-

Table 2. AHL synthesis of various *Erwinia* strains.

Strain	AHL signal[a]
D. chrysanthemi Ech3937	+
D. chrysanthemi Ech540	+
E. amylovora Ea1/79	–
E. billingiae Eb660	+
E. billingiae Eb661	+
E. billingiae Eb1261	+
E. mallotivora CFBP2503	–
E. persicina CFBP3622	–
E. pyrifoliae Ep1/96	–
E. rhapontici CFBP3618	+
E. tasmaniensis Et1/99	–
E. tasmaniensis Et2/99	–
E. tasmaniensis Et4/99	–
P. stewartii DC283	+

a. Induced colour change of CV26 and fluorescence of *E. coli* MT102(pJBA132).

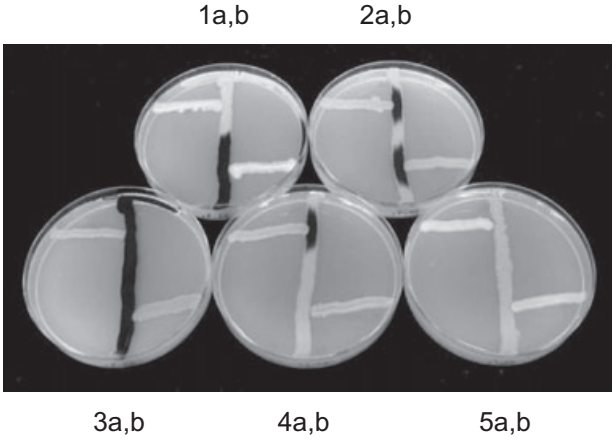

Fig 5. AHL assays with *C. violaceum* CV026 as a sensor (vertical streak). a, upper streak; b, lower streak; 1a, *E. persicina*; 1b, *E. rhapontici*; 2a, *E. billingiae* Eb1261; 2b, *E. billingiae* Eb661; 3a, *P. stewartii* DC283; 3b, *D. chrysanthemi* 3937; 4a, *E. billingiae* Eb660; 4b, *E. mallotivora*; 5a, *E. amylovora* Ea1/79; 5b, *E. pyrifoliae* Ep1/96.

inducer 2 (AI-2), a furanosyl diborate from Gram-negative bacteria or oligopeptides from Gram-positive bacteria (Federle and Bassler, 2003). These are assumed to affect gene regulation during cell growth. The AHLs can be partially selective for a species while AI-2 compounds might be more general. *Erwinia billingiae* synthesizes AI-2 (Mohammadi and Geider, 2007), but these bacteria also produce AHL. The AI-2 was detected for many *Erwinia* species, but not for *Pseudomonas* strains. The role of AI-2 in bacterial gene regulation is still open, but its accumulation in the environment as in apple flowers could interfere with cell growth of other bacteria such as *E. amylovora*.

Erwinia amylovora was described as an AHL producer (Venturi *et al*., 2004; Molina *et al*., 2005). We could not confirm release of AHL from *E. amylovora* with the sensor *C. violaceum*, nor by applying other AHL-sensor strains such as *Escherichia coli* with the *gfp*-reporter plasmid pJBA132. Also *E. coli* with plasmids pSB403 and pSB406 carrying *lux* genes from *P. aeruginosa* with the corresponding promoter fusions to the *lux* operon of *Vibrio fischeri* and *V. harveyi* BB170 did not respond to culture supernatants of *E. amylovora* (Mohammadi and Geider, 2007). On the other hand, heterologous expression of a *luxI* gene in *E. amylovora* resulted in AHL synthesis.

The virulence of plant-associated bacteria often depends on their ability to produce an HR on non-host plants (Alfano and Collmer, 1997). The HR induction is only one of the many factors required for virulence. The conversion of *P. fluorescens* into an HR producer by overexpression of the *rspL* gene (Preston *et al*., 2001) has been an example for latent *hrp* genes. HrpL activates *hrp* genes of *E. amylovora* (Wei and Beer, 1995), and *E. tasmaniensis* also increases the formation of HR lesions at high HrpL levels. Its ability of HR induction and even levan synthesis may add to the epiphytic fitness of *E. tasmaniensis*. On the other hand, the lack of both properties in *E. billingiae* is obviously not obstructive to interference with growth of *E. amylovora*. Interference of autoinducers with gene expression of *E. amylovora* may contribute to antagonism of *E. billingiae*.

The ability of *E. tasmaniensis* to form levan is advantageous to reduce high sucrose in the environment such as in nectar. The polyfructan can be a protectant against plant cell defence reactions and the released glucose is a convenient carbon source. *Erwinia amylovora* also produces levan and survives high sucrose concentrations (Geier and Geider, 1993). On the other hand, *E. pyrifoliae* from Korea and similar isolates from Japan are not able to synthesize levan (Kim *et al*., 2001). An *lsc* gene could not be detected with the *E. amylovora lsc* primers and analysis of the DNA sequence between the *pst-glmS* regions of *E. pyrifoliae* and *E. billingiae* (Kube *et al*., unpublished) reveals the absence of the *lsc* gene. Furthermore, in contrast to *E. billingiae*, *E. tasmaniensis* strains do not produce a detectable amount of capsular exopolysaccharide (K. Geider, unpublished), although a gene cluster related to the *ams* region of *E. amylovora* exists in the genome of Et1/99 (Kube *et al*., 2008). This deficiency and a lack of *E. billingiae* for HR induction may explain their inability to cause disease symptoms in plants.

In summary, *E. tasmaniensis* and *E. billingiae* have the common property to synthesize AI-2, which might interfere with *E. amylovora*, similar to AHL of *E. billingiae.* Both lack at least expression of one important virulence factor such as synthesis of capsular EPS or HR induction, but seem to be well-equipped to colonize plant surfaces and to prevent growth of *E. amylovora* in a competitive environment.

Experimental procedures

Bacterial strains used in the experiments and diagnostics of E. amylovora *and levan producers*

The bacterial strains are listed in Table 3. *Erwinia amylovora* and all other plant-pathogenic and plant-associated bacteria and *C. violaceum* were grown at 28°C, routinely in nutrient broth Standard I (StI, Merck, Darmstadt, Germany). *Escherichia coli* strains were grown at 37°C overnight. The isolation and taxonomic classification of levan-producing bacteria from Australia were described previously (Geider *et al*., 2006). *Erwinia amylovora* produces mucoid white colonies on MM2C agar, yellow colonies on MM2Cu agar and typical dome-shaped colonies with levan on Luria–Bertani (LB) agar with 5% sucrose (LB-sucrose) (Bereswill *et al*., 1998). LB-sucrose agar was also used for identification of *E. tasmaniensis* strains.

HR on tobacco leaves

Overnight cultures of bacteria in StI medium were centrifuged and cells re-suspended in sterile water to an OD_{600} of 0.1 (10^8 cfu ml^{-1}). Tobacco leaves (cv. 'SR1') were punctured with a thin needle and 0.1–0.2 ml of bacterial suspension was infiltrated. If indicated, bacteria from overnight cultures were grown for 6 h in IM [2 mM $(NH_4)_2SO_4$; 1 mM KH_2PO_4; 1 mM $MgSO_47H_2O$; 100 mM MES; 0.1% casamino acids; 1 % sucrose; pH 5.5 adjusted with NaOH; $MgSO_4$ and sucrose autoclaved separately) (Coplin *et al*., 2002). The necrotic symptoms of HR were evaluated 2 days after infiltration.

Assays with immature pear slices

Immature pears (cv. 'Bartlett') were stored at 4°C for a short time after harvest. Pear slices were cut with a sterile knife and placed in a Petri dish. Overnight cultures of bacteria to be tested for the antagonistic effects were diluted 20 times in water, or in 5% sucrose. Immature pear slices were soaked for 10 min in the bacterial suspensions and then air-dried under a laminar flow hood. Then, dilutions of *E. amylovora* were applied to each slice in 10 µl aliquots containing 500

Table 3. Bacteria used in this study.

	Description	Reference
Strain		
CFBP2503	*E. mallotivora*, Japan	Hauben *et al.* (1998)
CFBP 3618	*E. rhapontici*, UK	Hauben *et al.* (1998)
CFBP 3622	*Erwinia persicina*, Japan	Hao *et al.* (1990)
CV026	*C.* violaceum	McClean *et al.* (1997)
DC283	*P. stewartii*, USA	Coplin *et al.* (2002)
Ea1/79	*E. amylovora*, Germany	Falkenstein *et al.* (1988)
Ea1/79Sm	spontaneous Sm^r mutant of Ea1/79	
Eb1261	NCBP1261, *E. billingiae*, UK	Mergaert *et al.* (1999)
Eb660	NCBP660, *E. billingiae*, UK	Mergaert *et al.* (1999)
Eb661	NCBP661, *E. billingiae*, UK	Mergaert *et al.* (1999)
Ech 3937	*D. chrysanthemi*	Kazemi-Pour *et al.* (2004)
Ejp557	*E. pyrifoliae*, Japan	Kim *et al.* (2001)
Ep1/96	*E. pyrifoliae*, South Korea	Kim *et al.* (1999)
Et1/99	*E. tasmaniensis*, Tasmania, Australia	Geider *et al.* (2006)
Et2/99	*E. tasmaniensis*, Victoria, Australia	Geider *et al.* (2006)
Et4/99	*E. tasmaniensis*, Queensland, Australia	Geider *et al.* (2006)
FLA03	*E. tasmaniensis*, Heidelberg, Germany	This work
Plasmids		
pJBA132	Tc^R, *in E. coli* MT102, AHL sensor, *gfp* controlled via *luxR/I* from V. fischeri	Andersen *et al.* (2001)
pGThrpL-Ea1	Ap^R, *hrpL* gene from Ea1/79, P_{lac}	Jock *et al.* (2003)

cells. Slices were incubated at 28°C in small plastic boxes or Petri dishes sealed with Parafilm, until evaluation after 5 days.

Assays with apple flowers

Flowers were taken at day 2 after opening from young apple trees raised in a climatic chamber for blooming. The bacteria were grown as for the pear assays. *Erwinia amylovora* strain Ea1/79Sm was diluted in water to 5×10^5 cfu ml^{-1} and 10 µl pipetted into the pistil area of flowers. These were placed in Eppendorf tubes with water and incubated for 5 days in a climatic cabinet at high humidity with 16 h illumination at 24°C. Flowers were extracted without petioles in 1.5 ml of water in a large Eppendorf tube for 15 min. Ea1/79Sm was selected by plating on StI agar with Sm (500 µg ml^{-1}).

DNA manipulations

The PCR primers (Table 4) were designed by the program Primer designer v.4.0 (Scientific and Educational Software, 1995) and synthesized commercially. The PCR reactions were carried out in a volume of 50 µl with lysed bacteria as DNA template (15 min at 65°C in 0.1% Tween 20), with 10 pmol of each primer and 1 U of Taq DNA polymerase in a buffer system described by Bereswill and colleagues (1992). Amplification was performed with 35 cycles (denaturation 94°C for 30 s, annealing 52°C for 30 s, extension at 72°C of 1 min) after initial denaturation of 5 min at 94°C and final extension of 5 min at 72°C. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

DNA sequencing was done commercially (Seqlab, Göttingen) and sequence analysis performed with programs Align Plus V. 4.00 and Clone Manager V. 5.20 (Scientific and Educational Software) and with programs nBLAST and pBLAST in searches in the Internet (EBI, NIH).

Table 4. PCR primers used in this study.

PCR primer	Sequence (5′-3′)	Length
HRCC1	TGATGGCGTGGTGCTGGTGA	20
HRCC2c	CTTCCAGCGCCTGGATATCG	20
HRCR	GATCACACGCAATGCCATC	19
HRCR2c	GGTCCAGCCATTGATTAGC	19
LSC1	AACCAACGCTGTGGACTC	18
LSC2c	TGCTCTTCCGTCTGGTAA	18
LSCa1	GACGGCGGATCGCAATACGAA	21
LSCa2c	CACACCCTCATCAGACGTCAC	21
HRPL1	GGCACAAGCCTTGCTAA	17
HRPL2c	CGGCAAGACAGGACACT	17

N-AHL detection assays

The AHL was detected with the *C. violaceum* mutant CV026 by restoration of the production of the purple pigment violacein in the presence of AHL (McClean *et al.*, 1997), and with the *gfp*-based fluorescent reporter strain *E. coli* MT102(pJBA132) (Andersen *et al.*, 2001). T-streaks on plates were used for *C. violaceum* and the *gfp* reporter strain. Violacein production was estimated visually and the fluorescence caused by *gfp* was observed under UV light.

Acknowledgement

We thank Annette Wensing for comments on the manuscript.

References

Alfano, J.R., and Collmer, A. (1997) The type III (Hrp) secretion pathway of plant-pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J Bacteriol* **179:** 5655–5661.

Andersen, J.B., Heydorn, A., Hentzer, M., Eberl, L., Geisenberger, O., Christensen, B.B., *et al*. (2001) *gfp*-based N-acyl homoserine-lactone sensor systems for detection of bacterial communication. *Appl Environ Microbiol* **67:** 575–585.

von Bodman, S.B., Bauer, W.D., and Coplin, D.L. (2003) Quorum sensing in plant-pathogenic bacteria. *Annu Rev Phytopathol* **41:** 455–482.

Bereswill, S., Pahl, A., Bellemann, P., Zeller, W., and Geider, K. (1992) Sensitive and species-specific detection of *Erwinia amylovora* by PCR-analysis. *Appl Environ Microbiol* **58:** 3522–3526.

Bereswill, S., Jock, S., and Geider, K. (1998) Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin. *Plant Dis* **82:** 158–164.

Coplin, D.L., Majerczak, D.R., Zhang, Y., Kim, W.-K., Jock, S., and Geider, K. (2002) Identification of *Pantoea stewartii* subsp. stewarti by PCR and strain differentiation by PFGE. *Plant Dis* **86:** 304–311.

Emmert, E.A.B., and Handelsman, J. (1999) Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiol Lett* **171:** 1–9.

Falkenstein, H., Bellemann, P., Walter, S., Zeller, W., and Geider, K. (1988) Identification of *Erwinia amylovora*, the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. *Appl Enviro Microbiol* **54:** 2798–2802.

Federle, M.J., and Bassler, B.L. (2003) Interspecies communication in bacteria. *J Clin Invest* **112:** 1291–1299.

Gavini, F., Mergaert, J., Beji, A., Mielcarek, C., Izard, D., and Kersters, K. (1989) Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing and Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. *Int J Syst Bacteriol* **39:** 337–345.

Geider, K., Auling, G., Du, Z., Jakovljevic, V., Jock, S., and Völksch, B. (2006) *Erwinia tasmaniensis* sp. nov., a non-phytopathogenic bacterium from apple and pear trees. *Int J Syst Evol Microbiol* **56:** 2937–2943.

Geier, G., and Geider, K. (1993) Characterization and influence on virulence of the levansucrase gene from the fireblight pathogen *Erwinia amylovora*. *Physiol Mol Plant Pathol* **42:** 387–404.

Giddens, S.R., Houliston, G.J., and Mahnty, K.H. (2003) The influence of antibiotic production and pre-emptive colinization on the population dynamics of *Pantoea agglomerans* (*Erwinia herbicola*) Eh1087 and *Erwinia amylovora in planta*. *Environ Microbiol* **5:** 1016–1021.

Hao, M.V., Brenner, D.J., Steigerwalt, A.G., Kosako, Y., and Komagata, K. (1990) *Erwinia persicinus*, a new species isolated from plants. *Int J Syst Bacteriol* **40:** 379–383.

Hauben, L., Moore, E.R.B., Vaterin, L., Steenackers, M., Mergaert, J., Verdonck, L., and Swings, J. (1998) Phylogenetic position of phytopathogens within *Enterobacteriaceae*. *Syst Appl Microbiol* **21:** 384–397.

Jock, S., Völksch, B., Mansvelt, L., and Geider, K. (2002) Characterization of *Bacillus* strains from apple and pear trees in South Africa antagonistic to *Erwinia amylovora*. *FEMS Microbiol Lett* **211:** 247–252.

Jock, S., Kim, W.-K., Barny, M.-A., and Geider, K. (2003) Molecular characterization of natural *Erwinia pyrifoliae* strains deficient in the hypersensitive response. *Appl Env Microbiol* **69:** 679–682.

Johnson, K.B., and Stockwell, V.O. (1998) Management of fire blight: a case study in microbial ecology. *Annu Rev Phytopathol* **36:** 227–248.

Kazemi-Pour, N., Condemine, G., and Hugouvieux-Cotte-Pattat, N. (2004) The secretome of the plant pathogenic bacterium Erwinia chrysanthemi. *Proteomics* **4:** 3177–3186.

Kim, W.-S., Gardan, L., Rhim, S.L., and Geider, K. (1999) *Erwinia pyrifoliae* sp. nov., a novel pathogen that affects Asian pear trees (Pyrus pyrifolia Nakai). *Int J Syst Bacteriol* **49:** 899–906.

Kim, W.-S., Hildebrand, M., Jock, S., and Geider, K. (2001) Molecular comparison of pathogenic bacteria from pear trees in Japan and the fire blight pathogen *Erwinia amylovora*. *Microbiology* **147:** 2951–2959.

Kube, M., Migdoll, A., Müller, I., Kuhl, H., Beck, A., Reinhardt, R., and Geider, K. (2008) The genome of *Erwinia tasmaniensis* strain Et1/99, a novel non-pathogenic bacterium in the genus *Erwinia*. *Environ Microbiol* **10**, (doi: 10.1111/j.1462-2920.2008.01639.x.)

de Lima Pimenta, A., Di Martino, P., Le Bouder, E., Hulen, C., and Blight, M.A. (2003) *In vitro* identification of two adherence factors required for in vivo virulence of *Pseudomonas fluorescens*. *Microbes Infect* **5:** 1177–1187.

McClean, K.H., Winson, M., Fish, L., Adrian, T., Chhabr, S.R., Camara, M., *et al*. (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology* **143:** 3703–3711.

Mergaert, J., Hauben, L., Cnockaert, M.C., and Swings, J. (1999) Reclassification of non-pigmented *Erwinia herbicola* strains from trees as *Erwinia billingiae* sp. nov. *Int J Syst Bacteriol* **49:** 377–383.

Mohammadi, M., and Geider, K. (2007) Autoinducer-2 of the fire blight pathogen *Erwinia amylovora* and other plant-associated bacteria. *FEMS Microbiol Lett* **266:** 34–41.

Molina, L., Rezzonico, F., Defago, G., and Duffy, B. (2005) Autoinduction in *Erwinia amylovora*: evidence of an acylhomoserine lactone signal in the fire blight pathogen. *J Bacteriol* **187:** 3206–3213.

Picot, L., Abdelmoula, S.M., Merieau, A., Leroux, P., Cazin, L., Orange, N., and Feuilloley, M.G. (2001) *Pseudomonas fluorescens* as a potential pathogen: adherence to nerve cells. *Microbes Infect* **3:** 985–995.

Preston, G.M., Bertrand, N., and Rainey, P.B. (2001) Type III secretion in plant growth-promoting *Pseudomonas fluorescens* SBW25. *Mol Microbiol* **41:** 999–1014.

Stockwell, V.O., and Stack, J.P. (2007) Using *Pseudomonas* spp. for integrated biological control. *Phytopathology* **97:** 244–249.

Stockwell, V.O., McLaughlin, R.J., Henkels, M.D., Loper, J.E., Sugar, D., and Roberts, R.G. (1999) Epiphytic colonization of pear stigmas and hypanthia by bacteria during primary bloom. *Phytopathology* **89:** 1162–1168.

Stockwell, V.O., Johnson, K.B., Sugar, D., and Loper, J.E. (2002) Antibiosis contributes to biological control of fire blight by *Pantoea agglomerans* strain Eh252 in orchards. *Phytopathology* **92:** 1202–1209.

Temple, T.N., Stockwell, V.O., Loper, J.E., and Johnson, K.B. (2004) Bioavailability of iron to *Pseudomonas fluorescens* strain A506 on flowers of pear and apple. *Phytopathology* **94:** 1286–1294.

Vanneste, J.L. (2000) Erwinia amylovora. In *Fire Blight – The Disease and its Causative Agent*. Vanneste, J. (ed.). Oxon/UK, New York, USA: CABI publishing. pp. 199–358.

Vanneste, J.L., Yu, J., and Beer, S.V. (1992) Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora*. *J Bacteriol* **174:** 2785–2796.

Venturi, V., Venuti, C., Devescovi, G., Lucchese, C., Friscina, A., Degrassi, G., *et al*. (2004) The plant pathogen *Erwinia amylovora* produces acyl-homoserine lactone signal molecules in vitro and in planta. *FEMS Microbiol Lett* **241:** 179–183.

Wei, Z.-M., and Beer, S.V. (1995) *hrpL* activates *Erwinia amylovora hrp* gene transcription and is a member of the ECF subfamily of σ factors. *J Bacteriol* **177:** 6201–6210.

Wright, S.A.I., Zumoff, C.H., Schneider, L., and Beer, S.V. (2001) *Pantoea agglomerans* strain Eh318 produces two antibiotics that inhibit *Erwinia amylovora in vitro*. *Appl Environ Microbiol* **67:** 284–292.

