Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease

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ABSTRACT

Representatives of *Bacillus amyloliquefaciens* were shown to possess biocontrol activity against fire blight, a serious disease of orchard trees caused by *Erwinia amylovora*. Genome analysis of *B. amyloliquefaciens* FZB42 identified gene clusters responsible for synthesis of several polyketide compounds with antibacterial action. We show here that the antibacterial polyketides difficidin and to a minor extent bacillaene act efficiently against *E. amylovora*. Surprisingly, a mutant strain blocked in the production of difficidin (CH8 Δdfn) inhibited growth of *E. amylovora* and suppressed fire blight disease nearly in the same range as the wild type. In addition, a *sfp* mutant (CH3 Δsfp) unable to synthesize non-ribosomally lipopeptides and polyketides did still suppress growth of *E. amylovora*, suggesting that besides action of polyketides another antagonistic principle exist. A double mutant (RS06 $\Delta sfp \Delta bac$) devoid in polyketide and bacilysin synthesis was unable to suppress growth of *E. amylovora* indicating that the additional inhibitory effect is due to production of bacilysin, a dipeptide whose synthesis does not depend on *Sfp*. We propose to use *B. amyloliquefaciens* strains with enhanced synthesis of difficidin and/or bacilysin for development of biocontrol agents efficient against fire blight disease.

1. Introduction

Fire blight caused by *Erwinia amylovora* is the most serious bacterial disease in apple and pear. During the last four decades it has spread throughout Europe. In 2007 heavy outbreaks of fire blight led to severe losses for apple growers in Germany, Austria and Switzerland. After being deposited on blossom surfaces by insects, *E. amylovora* multiplies on stigmas before it enters the plant tissue through nectarthodes of the blossoms or by wounds of leaves and twigs under warm and moist weather conditions. Since sanitation methods could not stop the spread of the disease, fire blight management by using appropriate biocontrol agents are a pressing need. Effective control can be achieved through application of streptomycin sulphate a method widely used in North America. However, the use of streptomycin has been banned by the European authorities, due to the risk of development of antibiotic resistance in non-target bacteria. Search of an environment friendly biological alternative is a permanent task of present research. Use of *Aureobasidium pullulans* ("BlossomProtect") in organic fruit growing has been recommended (Kunz and Haug, 2006; Kunz et al., 2008).

Unfortunately, BlossomProtect is not compatible in its application with fungicides used for apple scab control. Recently, two products based on *Bacillus subtilis* have been registered for fire blight control in Europe: Serenade®, based on strain QST 713 and Biopro®, based on strain BD 170 (Broggini et al., 2005). Spore-forming *Bacillus* based bioformulations offer advantages for biocontrol because of their long-term viability (Haas and Defago, 2005). A moderate efficiency of the two *Bacillus* based bioformulations has been established in several field trials in the USA and Germany (Aldwinckle et al., 2002; Laux et al., 2003; Kunz and Haug, 2006) suggesting a promising potential of *Bacillus* sp. in control of blossom infections by *E. amylovora*. However, results obtained with both bioproducts were found to be erratic, varying between 71% and 0% disease suppression. Therefore, we initiated this study to select effective *Bacillus* strains to develop an environmentally friendly, cost effective product with a high efficiency in fire blight control. Due to the limited capacity for field testing and the high costs of field trials, a fast but reliable two step screening procedure consisting of laboratory tests *in vitro* and an *in vivo* test system with detached apple blossoms (Kunz and Haug, 2006; Pusey, 1997) was applied.

Two compounds with strong antagonistic effect against *E. amylovora*, the polyketide difficidin and the dipeptide bacilysin were identified in this study. Both compounds were produced

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by *Bacillus amyloliquefaciens* FZB42, a representative of the group of plant-associated *B. amyloliquefaciens* strains, which proved to be especially efficient against fire blight in our screening procedure.

2. Materials and methods

2.1. Bacterial strains used

The indicator strains *Erwinia amylovora* Ea385 and *Bacillus megaterium* were provided by LfP Stuttgart and the Institute of Biology, Humboldt University, respectively.

116 bacterial strains of different origin were included in the first screening procedure. 77 *Bacillus* strains ("BS" and "MB") were kindly supplied by Dr. Bloemberg (Institute of Biology, Leiden University, The Netherlands). Other strains used in this study are from the laboratory stock of the chair of Phytopathology, University of Konstanz, Germany; ABiTEP GmbH, Berlin, Germany ("FZB"); and the Institute of Biology Humboldt University, Berlin, Germany. The *Bacillus* strain QST 713 isolated from the commercial product Serenade WPO (AgraQuest) was used for comparison.

2.2. Mutant construction

Construction of FZB42 mutant strains CH3, CH8, CH14 devoid in production of polyketides has been previously described (Chen et al., 2006). Mutants RS01 (Δbac) and RS06 ($\Delta bac \Delta sfp$) devoid in bacilysin production were obtained in the following manner.

A PCR-fragment, involving *bacA* was amplified by the primer pair bclFW 5'...ACAGAAGAGGAAAACGGACGGC, and bclRV 5'...ACGTCGAATCCGCCGAAGTTG. The PCR-fragments were ligated into the vector pGEM-T to obtain the plasmid pbac12. The *bacA* gene was replaced by the cm^R resistance cassette yielding plasmid pRS1. The linearized pRS1 was integrated into the genome of FZB42 by double cross-over recombination generating the knockout mutant RS1. Chromosomal DNA of mutant RS1 was integrated into the genome of the CH3 mutant by double cross-over recombination yielding mutant RS06.

2.3. Growth conditions

Bacillus strains were grown in LB (Chen et al., 2006). Cultures for the production of polyketides were grown for 40 h at 30 °C and 150 rpm in Landy medium (Landy et al., 1948). For bacilysin production, the GA-medium was used. One litre GA-medium consisted of K_2HPO_4 , 7 g, KH_2PO_4 , 2 g, $MgSO_4 \cdot 7H_2O$, 0.1 g, $(NH_4)_2SO_4$, 1 g, yeast extract, 0.05 g, $Fe(SO_4)_3 \cdot 6H_2O$, 0.15 mg, $MnSO_4 \cdot H_2O$, 5 mg, $CuSO_4 \cdot 5H_2O$, 0.16 mg and sucrose, 13 g. Colonies of FZB42 and the mutant strains were inoculated in 10 ml GA-medium. The cultures were grown at 29 °C and 165 rpm for 24 h. The indicator strains *B. megaterium* and *E. amylovora* were grown in Luria broth (LB), if not otherwise stated.

2.4. Dual culture

Bacillus strains were grown overnight in NBS medium (Sifin, Berlin-Weissensee, Germany) consisting of pancreatic peptone (casein, meat) 2.25%, glucose 0.1%, KH_2PO_4 0.2%, K_2HPO_4 0.2%, NaCl 0.3% at 27 °C. Ea385 was grown for 48 h on 2% NBS agar. Before inoculating the mixed-species culture, Ea385 was diluted to a concentration of 1×10^7 cells/ml and the *Bacillus* strain was adjusted to a concentration of 5×10^7 cells/ml. After 24 h growth, concentration of Ea385 in the dual culture was determined by serial dilutions plated onto McConkey agar.

2.5. Purification of polyketides by XAD16

For preparation of samples used for HPLC and bioautography, 7 ml culture volume of each *Bacillus* strain were centrifuged for 10–40 min at 11,000 rpm and purified on an amberlite XAD16 column (6 g) by washing with 400 ml distilled water and eluting by 14 ml 100% methanol. Eluates were dried in a rotation evaporator and resolved in 550 μ l methanol containing 10% distilled water.

2.6. Bioautography and agar diffusion test

When cultures of the indicator strains *B. megaterium* and *E. amylovora* reached an $OD_{600\text{ nm}}$ of 1.0 culture was diluted 1:40 in appropriate medium solidified with 1% agar. 20 μ l of the *Bacillus* and 60 μ l of *E. amylovora* supernatant were used in agar diffusion test.

Bioautography of polyketides was performed essentially as previously described (Chen et al., 2006). Samples were purified with XAD16 resin. 10 μ l of the samples purified by XAD16 as described above were loaded onto thin-layer chromatography (TLC) aluminum sheets for *B. megaterium* bioautography; whilst 30 μ l was necessary for the *E. amylovora* test system. TLC was performed with chloroform-methanol-water at 65:25:4, vol/vol. For bacilysin detection 30 μ l supernatant of the culture grown in GA medium were spotted onto TLC sheets. Mobile phase consisted of 1-butanol/acetic acid/water 4:1:1 (v/v/v).

2.7. Assay of polyketides and bacilysin

High pressure liquid chromatography was performed with the HPLC 1200 device from Agilent, Waldbronn, Germany, essentially as previously described (Schneider et al., 2007). In brief, 10 μ l sample was injected onto a HPLC column (ZORBAX Eclipse XDB-C18, 4.6 $\times$ 150 mm, 5 μ m, Agilent). The temperature was kept at 30 °C during the experiment. The run was performed with a flow rate of 1.5 ml/min and a gradient of solvents A (0.1%, v/v HCOOH) and B (CH_3CN), which reached 100% B after 10 min. Concentration of 100% CH_3CN -HCOOH was held for further two min. To equilibrate the column, it was treated with 5% CH_3CN -HCOOH for 3 min. A diode-array detector in range from 190 nm to 550 nm was used for detecting peaks. All the polyketides were detected at 280 nm; bacilysin was detected at 230 nm. For quantitative assays, the area of polyketide peaks were calculated with the "auto integrate" software of Agilent.

2.8. Efficiency of test strains against *E. amylovora* on detached blossoms

An *in vivo* test-system with detached apple blossoms was established according to Pusey (1997). Apple trees ('Gala') were stored at 2 °C in the dark from January to August. Every week a group of trees was transferred to the greenhouse to force them to bloom. The blossoms were cut and maintained with cut peduncle submerged in 10% sucrose in plastic racks (23 °C, 100% rH). Blossoms were sprayed with a suspension of Ea385 (10^6 cfu/ml) in water until runoff. Test preparations of *Bacillus* strains were sprayed to the blossoms 1 h after inoculation. The number of blossoms with bacterial ooze at the peduncle was counted 6 days after the inoculation (Fig. 1). The experiments were repeated three to four times unless otherwise stated.

3. Results and discussion

3.1. Screening of *Bacillus* strains

Initially, 116 strains were tested in competitive mixed culture experiments (see Section 2) for their ability to suppress growth of *E.*

Fig. 1. Left: Detached apple blossoms in racks prior to inoculation and treatment. Right: Blossom after 6 days of incubation at 20 °C and 100% relative humidity. Top: healthy blossom. Below: Blossom with black peduncle and bacterial ooze due to infection by *E. amylovora*.

amylovora (Supplementary material: Table 1). The efficiency of the strains depended on the incubation temperature. In general, strains showed higher efficiency at 27 °C than at 20 °C. During apple bloom in mid-Europe, temperatures of 27 °C are rarely reached. Therefore, the suppression of *E. amylovora* at 20 °C is a prerequisite for high activity in the field. Strain QST713, the active constituent of the biocontrol agent "Serenade®" was used for comparison. Around 20 strains including QST713 and FZB42 suppressed *E. amylovora* growth in mixed culture completely (Table 1). Those strains were selected for further experiments with detached blossoms.

On detached blossoms the recommended concentration of the formulated product Serenade WPO was less effective than the undiluted culture filtrate of QST713. Interestingly, six strains (FZB 45, FZB42, FZB13, FDK33, FDK21 and KF60) all belonging to *B. amyloliquefaciens*, displayed a higher efficiency than QST713 (Tab. 1).

We have shown previously that QST713, formerly misclassified as *B. subtilis*, is also a representative of the group of plant-associated *B. amyloliquefaciens* strains, which are occurring inside the plant rhizosphere (Chen et al., 2007).

3.2. Quantitative HPLC analysis of polyketide production

FZB42 and other plant-associated *B. amyloliquefaciens* strains are known for their production of antibacterial polyketides. Polyketide megasynthases responsible for synthesis of bacillaene, macrolactin and difficidin are encoded by three giant gene clusters that are located at different sites of the FZB42 genome (Chen et al., 2007). Those polyketides, especially difficidin, have been shown to act against Gram-positive and Gram-negative bacteria, including the plant pathogen *Erwinia carotovora* (Chen et al., 2006) and might be good candidates for being the "antagonistic principle" acting against the causative agent of fire blight. A previous screening for presence of those polyketides in bacilli revealed that occurrence of all those three antibiotics is limited to the group of plant-associated *B. amyloliquefaciens*. Notably, difficidin, the most efficient antibacterial compound in FZB42, is restricted in its occurrence to that selected group of bacteria (Schneider et al., 2007).

For identification and a quantitative analysis of polyketides by high pressure liquid chromatography (HPLC), we took advantage that distinct peaks obtained after the HPLC run can be distinguished. Using mutant strains deficient in production of difficidin (CH8), macrolactin, and bacillaene (CH14) or completely devoid in non-ribosomal synthesis of lipopeptides and polyketides (CH3) and supported by the availability of the specific UV-absorption maxima of those compounds we were able to assign the profile generated by HPLC to distinct polyketides (see Fig. 2). The quantities corresponding to the isoforms were calculated as described in Section 2.

Our analysis revealed that nearly all the strains that have proven as especially efficient in the blossom test and in suppressing *E. amylovora*, did produce the spectrum of the three polyketides, which are unique for the *B. amyloliquefaciens* group. Whilst the main polyketide produced by *B. amyloliquefaciens* FZB42 and QST713 is difficidin, other strains with high polyketide production exhib-

Table 1
Efficiency (%) of the test strains in suppressing *Erwinia amylovora* in dual culture^a and on detached blossoms.

Strain	Dual culture		Blossom test		Species	Source
	20 °C	27 °C	100%	20%		
FZB 45	100	100	98	n.d.	<i>B. amyloliquefaciens</i>	ABiTEP GmbH
FZB 42	100	100	98	42 ^b	<i>B. amyloliquefaciens</i>	ABiTEP GmbH
FZB 13	100	100	96	95 ^b	<i>B. amyloliquefaciens</i>	ABiTEP GmbH
FDK 33	100	100	96	95 ^b	<i>B. amyloliquefaciens</i>	University of Konstanz
FDK 21	98	100	96	83 ^b	<i>B. amyloliquefaciens</i>	University of Konstanz
KF 60	99	99	87	83 ^b	<i>B. amyloliquefaciens</i>	University of Konstanz
QST713	100	100	76	n.d. ^c	<i>B. amyloliquefaciens</i>	Serenade WPO (AgraQuest)
FZB 108	100	100	66	n.d.	<i>B. subtilis</i>	ABiTEP GmbH
MB 2	100	100	n.d.	58	<i>Bacillus mojavensis</i>	Leiden University
FZB 24	100	100	65	47 ^b	<i>B. amyloliquefaciens</i>	ABiTEP GmbH
FZB 109	99	100	57	n.d.	<i>B. amyloliquefaciens</i>	ABiTEP GmbH
FDK 24	100	100	39	n.d.	<i>B. velezensis</i>	University of Konstanz
BS 19	100	100	n.d.	22	<i>Bacillus</i> spp.	Leiden University
MB 8	100	100	n.d.	21	<i>Bacillus</i> spp.	Leiden University
MB 33	100	99	n.d.	13	<i>Bacillus</i> spp.	Leiden University
MB 31	100	100	n.d.	11	<i>Bacillus</i> spp.	Leiden University
MB 23	100	100	n.d.	10	<i>Bacillus</i> spp.	Leiden University
MB 27	100	100	n.d.	6	<i>Bacillus</i> spp.	Leiden University
BS 18	100	100	n.d.	3	<i>Bacillus</i> spp.	Leiden University
MB 25	100	100	n.d.	1	<i>Bacillus</i> spp.	Leiden University

^a Dual cultures consisting of *Erwinia amylovora* and the test strain were incubated at temperatures of either 20 °C or 27 °C. The efficiency on blossoms was tested by spraying undiluted (100%) and water diluted (20%) culture broths.

^b The experiment was done only once.

^c n.d., not determined.

Fig. 2. HPLC analysis and identification of single metabolites in culture supernatants of FZB42 and the mutant strains CH3 (Δsfp), CH8 (Δdfn) and CH14 ($\Delta bae \Delta mln$) grown in Landy medium. mln, macrolactin; dfn, diffidin; bae, bacillaene.

Table 2
Polyketide profiles and efficiency against *E. amylovora* determined in dual culture (1) and in detached blossom (2)^a.

Strain	HPLC retention times (peak area)			% inhibition	
	Bacillaene	Macrolactin	Diffidin	(1)	(2)
<i>B. amyloquelquefaciens</i> FZB 24	6.31 (1249) , 6.47 (388), 6.63 (707), 6.78 (535), 6.84 (203)	6.02 (570), 7.27 (157)	8.37 (1367) , 9.01 (105), 10.81 (188)	100 ± 0	62 ± 3 (2)
<i>B. amyloquelquefaciens</i> FDK 21	6.47 (65), 6.55 (251), 6.62 (99), 6.705 (50), 6.78 (153)	6.01 (377), 7.27 (115)	6.55 (251), 6.78 (153), 8.43 (81)	100 ± 0	55 ± 5 (2)
<i>B. amyloquelquefaciens</i> FZB 42	6.47 (693), 6.78 (1038)	7.02 (230), 7.1 (175), 7.26 (359)	7.79 (146), 8.38 (1783) , 8.82 (118), 9.03 (128)	100 ± 0	50 ± 13 (4)
<i>B. subtilis subtilis</i> INR 937	6.58 (9639) , 6.65 (1440), 6.80 (2808) , 7.67 (119)	7.02 (234)	–	100 ± 0	39 ± 1 (2)
<i>B. amyloquelquefaciens</i> Gao B85	6.31 (1302) , 6.38 (237), 6.46 (276), 6.51 (453)	5.7 (1685) , 5.82 (175), 5.93 (61), 6.017 (481), 7.1 (178)	8.39 (522), 8.797 (60), 9.027 (54)	100 ± 0	28 ± 22 (3)
<i>B. amyloquelquefaciens</i> QST713	6.47 (460)	6.01 (498), 7.26 (530)	8.38 (1807), 8.82 (120), 9.04 (116), 10.84 (290), 10.93 (200)	100 ± 0	23 ± 4 (2)
<i>B. mojavensis</i> MB 2	6.8 (1192) , 6.63 (661), 6.56 (344), 6.47 (189)	–	–	100 ± 0	22 ± 14 (3)
<i>B. amyloquelquefaciens</i> GB 03	6.31 (509), 6.397 (44)	5.72 (366), 6.07 (252)	8.42 (128), 8.87 (10), 9.037 (6)	100 ± 0	15 ± 7 (2)
<i>B. amyloquelquefaciens</i> GaoB3	6.77 (140)	6.01 (258)	8.4 (249), 8.8 (46), 9.02 (18)	100 ± 0	7 ± 2 (2)
<i>B. amyloquelquefaciens</i> FZB 111	6.31 (448)	5.72 (213), 6.0 (228), 6.2 (58)	8.42 (94)	100 ± 0	3 ± 11 (2)
<i>B. amyloquelquefaciens</i> FZB 113	n.d. ^b	6.19 (110), 7.1 (77)	8.42 (18)	100 ± 0	0 ± 4 (2)

^a Prominent peaks of the polyketides are indicated in bold letters. For the blossom test *Bacillus* strains were grown in an optimised medium. Water diluted culture broth (1%) was directly sprayed on the detached apple blossoms. Numbers in brackets indicate number of repetitions.

^b n.d., not detected.

Table 3Effect of *B. amyloliquefaciens* FZB42 and its mutant strains CH8 and CH14 on detached blossoms infected with *Erwinia amylovora*^a.

Strain	Geno-type	Synthesis of polyketides			Efficiency [%] on detached blossoms
		Bacillaene	Macrolactin	Difficidin	
FZB 42	Wild-type	Yes	Yes	Yes	84 ± 7 (3)
CH8	$\Delta dfn::ermAM$	Yes	Yes	No	79 ± 5 (3)
CH14	$\Delta bae::cat \Delta mln::neo$	No	No	Yes	89 ± 12 (3)

^a The number in brackets indicate number of repetitions.

ited a nearly equal ratio between difficidin and bacillaene (FZB24) or a production of either bacillaene and macrolactin was more pronounced (GaoB85, Table 2). A remarkably high production of bacillaene was registered in *B. subtilis* INR937, a strain also proven as being efficient in suppression of *E. amylovora*. However, the best results in suppressing the fire blight bacterium *in vitro* (mixed culture) and *in vivo* (detached blossom) were obtained with the *B. amyloliquefaciens* strains FZB24, FZB42 and FDK21 (Table 2).

3.3. The polyketide difficidin produced by FZB42 suppresses *E. amylovora*

To ascertain the role of difficidin and other polyketides in suppressing fire blight by *B. amyloliquefaciens*, we compared the effect exerted on *E. amylovora* infected blossoms by FZB42 with that of polyketide deficient mutants generated by targeted mutagenesis of competent FZB42 cells via homologous recombination (Chen et al., 2006). As expected, the suppressive effect exerted by CH14, the mutant strain producing only difficidin but no macrolactin and bacillaene, was found equal or slightly higher, than in FZB42 wild-strain underlining importance of difficidin. However, strain CH8, deficient in difficidin production was only slightly reduced in its ability to suppress fire blight symptoms on detached blossoms (Table 3).

The following mutant strains were used to investigate *in vitro* suppressing effects of difficidin and bacillaene on *E. amylovora* by bioautography (see Section 2): CH8 (Δdfn), deficient in difficidin but unaffected in bacillaene and macrolactin production, CH14 ($\Delta bae \Delta mln$), deficient in production of macrolactin and bacillaene

but proficient in difficidin formation and CH3 (Δsfp), unable to produce any polyketide or lipopeptide due to a deletion introduced into the gene encoding 4'-phosphopantetheinyl transferase, Sfp, an enzyme catalysing a necessary processing step in non-ribosomal synthesis of lipopeptides and polyketides. All strains were grown in Landy medium, a medium specifically designed to facilitate production of lipopeptides and polyketides. Bioautography showed that among the compounds present in the supernatant of FZB42, difficidin strongly inhibited growth of the sensitive *B. megaterium* indicator strain, whilst bacillaene and the lipopeptides surfactin had a minor effect. Growth inhibition caused by difficidin, the only polyketide produced by mutant CH14, was in the same range as in the wild-type. Slight growth repression due to presence of bacillaene and surfactin in mutant CH8 was also registered. However, strain CH3, was completely unable to produce any compound that suppress growth of the *B. megaterium* indicator strain (Fig. 3A). Similar effects were observed when *E. amylovora* was used as indicator strain in bioautography. Only growth inhibition caused by difficidin and to a smaller extent by bacillaene was detectable (Fig. 3B). However, agar diffusion tests performed with the cell-free supernatants of FZB42 and the three mutant strains mentioned above yielded a different scenario. As expected, growth of *B. megaterium* was clearly inhibited by supernatants of FZB42 and the difficidin producer CH14, whilst the inhibiting effect exerted by CH8 (devoid in difficidin) was clearly reduced and CH3 did not affect growth of *B. megaterium* at all (Fig. 3C). When *E. amylovora* was used as indicator, growth inhibiting effect exerted by CH8 still remained low underlining the main antibacterial action of difficidin. But to our surprise, the inhibiting effect exerted by CH3 on *E. amylovora* was

Fig. 3. Bioautography and agar diffusion test of FZB42 and mutant strains CH3, CH8 and CH14 grown in Landy medium. 3A: Bioautography with indicator strain *B. megaterium*; 3B: Bioautography with indicator strain *E. amylovora*; 3C: Agar diffusion test with indicator strain *B. megaterium*; 3D: Agar diffusion test with indicator strain *E. amylovora*. For details see text.

comparable with that of FZB42 and CH14, as well, although no Sfp-dependent synthesis of polyketides and lipopeptides did occur in that strain (Fig. 3D).

3.4. The dipeptide bacilysin acts as an additional suppressor of the growth of *E. amylovora*

To identify the molecular reason for the observed suppression of *E. amylovora* by CH3, we searched in the FZB42 genome for gene clusters involved in Sfp-independent synthesis of secondary metabolites. Only one gene cluster involved in biosynthesis of the dipeptide bacilysin and its antibiotic constituent anticapsin was identified (Chen et al., 2007). The gene cluster, detected in FZB42 at 3,593,877–3,599,784, displayed the same gene organisation as the one known for *B. subtilis* 168, in which the genes *bacABCDE* and *ywfG* have been shown necessary for bacilysin synthesis (Steinborn et al., 2005). Similarity between both gene clusters was around 84–93% identity on amino acid level. The bacilysin molecule contains an L-alanine residue at the N-terminus and a non-proteinogenic amino acid, L-anticapsin, at the C-terminus (Walker and Abraham, 1970). Antibiotic action is achieved by the anticapsin moiety, which becomes released after uptake in susceptible cells and blocks glucosamine synthetase, an essential enzyme of cell wall biosynthesis (Kenig et al., 1976). Products of the *bacDE* genes function in non-ribosomal but Sfp independent amino acid ligation and immunity, respectively (Steinborn et al., 2005).

A double mutant, RS06 (Δ sfp, Δ bac), simultaneously blocked in non-ribosomal synthesis of lipopeptides and polyketides and in

production of bacilysin (Fig. 4A) was used to investigate repressing effect of bacilysin on growth of *E. amylovora*. Strains FZB42, CH8, CH14, CH3, and RS06 were grown in GA medium specifically designed for bacilysin production (see Section 2). Bioautography (Fig. 4C) revealed that bacilysin produced by all that strains under those conditions, except RS06, inhibits growth of *E. amylovora*. Agar diffusion tests performed with the supernatants of FZB42, CH8 and CH14 yielded strong inhibition zones onto the *E. amylovora* indicator lawn. The zone of inhibition caused by supernatant of CH3 was also in a comparable size. On the contrary, the double mutant RS06 did not significantly repress growth of *E. amylovora*, suggesting that bacilysin acts as an important antagonist of *E. amylovora* (Fig. 4B).

4. Conclusion

Plant-associated *B. amyloliquefaciens*, e.g. FZB42, are efficient in suppressing plant pathogenic bacteria, including *E. amylovora*, the causative agent of fire blight disease in orchard trees. Trials performed with inoculated detached blossoms and field trials (Fried, 2007) have corroborated the results of the *in vitro* experiments reported here. *Bacillus* based bioformulations have important advantages in fire blight control compared to other biocontrol agents:

1. *Bacillus* endospores with long-term viability guarantee constant product quality over a long period of time.
2. Together with the active substances, living spores can be applied to blossoms of apple or pear trees. Spores might be able to germinate and multiply there and could be carried to other blossoms by insects (Broggini et al., 2005). Given that FZB42 can produce diffidin and bacilysin during colonising the blossoms, continuous supply of antagonistic compounds, directly at the site where *E. amylovora* normally occurs, would be possible.
3. In contrast to other biocontrol agents used in fire blight control, the active principles suppressing the devastating disease of apple and pear trees have been identified as being diffidin, one of the three polyketides produced by FZB42, and the antimicrobial dipeptide bacilysin.

The identification of these two compounds as efficient suppressors of *E. amylovora* will facilitate future directed development of optimised *Bacillus* based biocontrol agents as alternatives to streptomycin sulphate and other toxic chemo-pesticides which become increasingly restricted in their application.

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Fig. 4. HPLC analysis (A), agar diffusion test (B) and bioautography (C) with FZB42 and the mutant strains CH3 (Δ sfp), CH8 (Δ dfn), CH14 (Δ bae Δ mln) and RS06 (Δ sfp Δ bac) grown in GA medium. For details see text. Bcl, bacilysin.

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