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Chapter 5

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Characterization of *Bacillus subtilis* HC8, a novel plant-beneficial endophytic strain from giant hogweed

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Abstract

Thirty endophytic bacteria were isolated from various plant species growing near Saint-Petersburg, Russia. Based on a screening for various traits, including plant-beneficial properties and DNA fragment patterns, potential siblings were removed. The remaining isolates were taxonomically identified using 16S rDNA sequences and potential human and plant pathogens were removed. The remaining strains were tested for their ability to promote radish root growth and to protect tomato plants against tomato foot and root rot (TFRR). One strain, *Bacillus subtilis* HC8, isolated from the giant hogweed *Heracleum sosnowskyi* Manden, significantly promoted plant growth and protected tomato against TFRR. Metabolites possibly responsible for these plant-beneficial properties were identified as the hormone gibberellin and (lipo)peptide antibiotics, respectively. The antibiotic properties of strain HC8 are similar to those of the commercially available plant-beneficial strain *B. amyloliquefaciens* FZB42. However, thin layer chromatography profiles of the two strains differ. It is speculated that endophytes such as *B. subtilis* HC8 contribute to the fast growth of giant hogweed.

Introduction

Bacteria which associate with plants include rhizobacteria, epiphytic bacteria and endophytic bacteria. Endophytic bacteria are defined as bacteria that can be detected within the tissues of apparently healthy plants (Schulz and Boyle, 2006). Although the majority of research on plant-associated bacteria has been focused on rhizobacteria, interest in the diversity and role of endophytic bacteria is increasing. The main reason for the interest in endophytes is the realization that, if these bacteria can be re-introduced in the endophytic stage, a more stable relationship can be established between plant-beneficial endophytic bacteria and plants than for rhizospheric or epiphytic bacteria and plants. Therefore, endophytes with the plant-beneficial traits are potentially excellent plant growth promoters and/or biological control agents for sustainable crop production (Di Fiore and Del Gallo, 1995; Strobel, 2006).

The best studied host plants of bacterial endophytes are species of agricultural importance, such as rice (Baldani et al., 2000; Okunishi et al., 2005), maize (McInroy and Kloepper, 1995; Rijavec et al., 2007), cotton (Misaghi and Donndelinger 1990; McInroy and Kloepper, 1995), potato (Sturz et al., 1998; Krechel et al., 2002), and sugar cane (Rennie et al., 1982; James and Olivares, 1997). The most common taxa of isolated heterotrophic endophytes include *Bacillus* (Bai et al., 2003), *Enterobacter* (Torres et al., 2008), *Pseudomonas* (Reiter et al., 2003; Rai et al., 2007), *Serratia* (Gyaneshwar et al., 2001; Berg et al., 2005), and *Streptomyces* (Sessitsch et al., 2002; Coombs and Franco, 2003).

It is assumed that bacterial endophytes use the same mechanisms of biological control and plant growth promotion as their rhizospheric counterparts (Berg and Hallmann, 2006). Widely recognized mechanisms of biocontrol mediated by plant growth-promoting microbes are antibiosis (Thomashow and Weller, 1995; Chin-A-Woeng et al., 1998; Haas and Défago, 2005; Lugtenberg and Kamilova, 2009), induced systemic resistance (Van Peer et al., 1991; Kloepper et al., 2004; Van Loon, 2007), competition for niches and nutrients (Kamilova et al., 2005; Validov, 2007) and predation and parasitism (Ordentlich et al., 1998; Harman et al., 2004).

Beneficial bacterial endophytes which use the above-mentioned mechanisms of biocontrol include (i) *Bacillus* sp. CY22 which produces the antibiotic iturin A and suppresses root rot of balloon flower caused by *Rhizoctonia solani* (Cho et al., 2003), (ii) *B. pumilus* SE 34 which induces systemic resistance against *Fusarium* wilt of tomato (Benhamou et al., 1998), and (iii) *P. fluorescens,* carrying the chitinase-encoding gene *chi*A, which is able to control the phytopathogenic fungus *Rhizoctonia solani* on bean seedlings (Downing and Thomson, 2000). In addition to protecting against pathogens, a number of endophytic bacteria is supposed to promote plant growth directly by the production and/or modulation of plant hormones (Bastian et al., 1998; Spaepen et al., 2009), by fixing atmospheric nitrogen (Baldani et al., 2000; Oliveira et al., 2002) and by solubilization of bound phosphates (Verma et al., 2001; Kuklinsky-Sobral et al. 2004). Using these mechanisms, some endophytic bacteria can significantly contribute to the growth of plants on low-fertility soils (Sevilla et al., 2001).

The main aims of this study are: (i) to collect different endophytic bacteria from different plants, (ii) to screen these bacteria for a number of plant-beneficial traits, such as secretion of the exo-enzymes chitinase, cellulase, β-glucanase and protease, and production of hormones and antifungal metabolites, (iii) to test the selected potentially beneficial strains for their abilities to promote growth of radish and to control tomato foot and root rot (TFRR) caused by the fungus *Fusarium oxysporum* f.sp. *radicis-lycopersici* (*Forl*), and (iv) to evaluate the putative compounds responsible for the plant growth promotion and antifungal activities of (a) selected endophytic strain(s). The results are reported in this paper.

Materials and Methods

Isolation of endophytic bacteria

Endophytic bacteria were isolated from several plant species. These include four vegetable plants [beet (*Beta vulgaris* L.), carrot (*Daucus carota* L.), potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* L.)], two grasses [maize (*Zea mays* L.) and millet (*Panicum miliaceum*)], and the weed plant *Heracleum sosnowskyi* Manden. Plants were collected from experimental fields of the All-Russia Research Institute for Agricultural Microbiology (ARRIAM) which is located near Pushkin, Saint-Petersburg, Russia.

To isolate endophytes, different surface sterilization procedures were developed (see Results) which are modifications of previously published ones (Misaghi and Donndelinger, 1990). Briefly, plant samples were disinfected and subsequently crushed with a pestle in a mortar under sterile conditions. Aliquots of 100 µl of the resulting plant juices were plated on 1/20 tryptic soy agar (TSA, Difco Laboratories, MI, USA) plates. The sterility check consisted of aliquots of water from the last rinsing which were plated on 1/20 TSA. Plates were incubated at 28°C for 3 days. Colonies derived from plant juice were further analyzed.

Microbial strains and growth conditions

All isolated bacterial strains were grown in, and maintained on, full strength TSA. Strain *Bacillus amyloliquefaciens* FZB42 (Idriss et al., 2002), and its mutants AK1 and AK2 (Koumoutsi et al., 2004) were purchased from the *Bacillus* Genetic Stock Center (BGSC, http://www.bgsc.org/). Strain FZB42 was used for comparison studies as a known antibiotic-producing *Bacillus* strain. This strain is also commercialized as a biofertilizer, biocontrol and plant-growth promoting agent (RhizoVital®, ABiTEP, Berlin, Germany). Its two mutants, AK1 (Δ*bmy*A, defective in the production of bacillomycin D) and AK2 (Δ*fen*A, defective in the production of fengycin), were used to attempt to localize these antibiotics on TLC plates.

The fungi *Aspergillus niger, Forl*, *F. solani* and the oomycete *Pythium ultimum* were routinely cultivated on potato-dextrose agar (PDA, Difco Laboratories). To obtain spores of *Forl* to be used in biocontrol experiments, the fungus was grown in Czapek-Dox liquid medium (Difco Laboratories) for 4 days at 28°C at 150 rpm.

For the extraction of antibiotics and gibberellins, strains were grown in Brain Heart Infusion broth (BHI, Difco Laboratories, MI, USA). To extract cytokinins and to check the ability of strains to sulubilize phosphates, bacteria were grown in minimal medium (MM) containing per liter of distilled water: NH_4Cl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; glucose, 10 g; yeast extract, 50 mg, and agar, 18 g. For the evaluation of ACC (1-aminocyclopropane-1-carboxylate) utilization, bacteria were grown in sucrose-malt extract-yeast extract medium (SMY) which has the following composition (weight/L): glucose, 1.2 g; KH_2PO_4, 0.4 g; K_2HPO_4, 2.0 g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.1 g; $FeSO_4$, 5.0 mg; H_3BO_3, 2 mg; $ZnSO_4$, 5.0 mg; Na_2MoO_4, 1.0 mg; $MnSO_4$, 3.0 mg; $CoSO_4$, 1.0 mg; $CuSO_4$, 1.0 mg; $NiSO_4$, 1.0 mg; yeast extract, 50 mg; pH 6.4.

Characterization of exo-enzymes produced by endophytic bacteria

Production of the exo-enzymes cellulase, chitinase, β-glucanase, and protease was judged as the appearance of clear zones around the growth of a bacterium on the following solid media. Cellulase activity was tested on 1/20 TSA agar plates supplemented with 1% carboxymethylcellulose (Hankin and Anagnostakis, 1977). Production of chitinase were evaluated on 1/20 TSA agar plates supplemented with 1% colloidal chitin (Wirth and Wolf, 1990). ß-glucanase activity was detected on 1/20 TSA agar plates supplemented with 0.1% lichenan (Walsh et al., 1995). Protease secretion was evaluated after growing the strains for 48 hours on 1/20 TSA supplemented with 5% skimmed milk according to Brown and Foster (1970).

Characterization of antifungal metabolites produced by bacteria

To test production of antifungal metabolites *in vitro,* a plug of mycelium, 5-mm in diameter, was taken from an actively growing culture on solid medium and stabbed in the center of a PDA agar plate which was subsequently inoculated with up to 6 individual bacterial strains at a distance of 3 cm from the fungus. All plates were incubated at 28°C for one week and subsequently scored for inhibition of fungal growth.

The method of Chittara et al. (2002) was used with some modifications to extract antibiotics produced by *Bacillus subtilis* HC8. Briefly, the strain was grown in BHI medium for 60 h. Subsequently cells were removed by centrifugation at 13 000 rpm for 10 min. The supernatant fluid was divided into two equal parts, one part (100 ml) was freeze-dried and the other was acidified to pH 2.0 with concentrated HCl. The resulting dry biomass and precipitate, respectively, were extracted twice with methanol. The methanolic extract was concentrated by vacuum evaporation, dissolved in methanol and stored at -20°C.

The methanolic extracts were analyzed by thin layer chromatography (TLC) on silica gel 60 F254 plates with a 20 x 2,5 cm concentrating zone (Merck, Darmstadt, Germany). Plates were developed in chloroform/methanol/water 65:25:4 (v/v/v) for 1.5 h at room temperature. After drying, the pattern of compounds on the developed plate was visualized using UV_{254} and stained in an iodine chamber for 5 min at room temperature followed by dipping in 1% aqueous starch. The putative antifungals were preliminarily characterized by their Retention factor (R_f) values. Pure iturin A from *B. subtilis* (Sigma-Aldrich, Steinheim, Germany) was used as a reference.

To analyze the antifungal activity of the different spots, TLC plates were run in duplicate, one was used for staining and the other one to recover the fractions by extraction. To extract the spots, silica regions were scratched off the plate and were extracted with methanol. The activity of the individual extracts was tested against *Forl* in *in vitro* assay as follows. A plug of mycelium was placed in the center of a PDA plate and pre-grown for 2 days. Subsequently six wells, 8 mm in diameter, were made in the agar plate at a distance of 1.0 cm from the growing fungus. The bottom of the wells was sealed using melted agar, and each of the wells was filled with 80µl of an individual extract. Methanol was used as a control. The plates were incubated for 2 days at 28°C and the inhibition of the *Forl* growth was judged. All experiments were carried out at least three times. To compare the activity and R_f values of the HC8 extract with those of a known antibiotic-producing strain, methanolic extracts of *B.*

amyloliquefaciens FZB42 were prepared and profiled on TLC plates as described for strain HC8.

Characterization of bacterial phytohormone production

The production of auxin (IAA, indole-3-acetic acid) was determined as described by Kamilova et al. (2005) using Salkowski reagent (Gordon and Weber, 1951). A modified method of Gutierrez-Manero et al. (2001) was used for the extraction of gibberellins from the supernatant fluid of *Bacillus subtilis* HC8. Bacteria were grown in 100 ml BHI medium for 60 h at 28°C at 150 rpm. Bacterial cells were removed by centrifugation for 15 min at 5 000 rpm and the supernatant fluid was subsequently filtered through a 0.22-µm Millipore filter. Bacteria-free supernatant was then acidified to pH 2.5 with concentrated HCl and partitioned four times with water-saturated ethyl acetate (v/v). The organic phase, containing the gibberellins, was dried by vacuum evaporation and subsequently dissolved in water-saturated ethyl acetate and stored at -20°C. A modified method of Jones and Varner (1967) was used for the evaluation of the biological activity of the crude extract. Briefly, seeds of barley cv. Triumph, 1989 harvest, were transversely cut in half and the embryo part was removed. The embryo-free halves were then surface-sterilized with 70% ethanol for 2 min followed by 4% sodium hypochlorite for 2 min and several rinses with sterile water. The disinfected half seeds were stored in sterile water at +4°C for 2 days. For gibberellin assays, 10 half seeds were transferred to a 100 ml Erlenmeyer flask with 6 ml of test solution containing: (i) 20 mM sodium succinate buffer, pH 5.3, (ii) 20 mM $CaCl_2$, and (iii) the sample to be assayed. Chloramphenicol at a final concentration 10µg/ml was added to each flask to prevent bacterial growth. After incubation for 27 h at 25°C in the dark, 1.0 ml of the solution was added to a tube containing 1.0 ml of starch reagent (Jones and Varner, 1967) and incubated for 10 min at room temperature. The reaction was stopped by adding 1.0 ml of iodine reagent (Jones and Varner, 1967). A volume of 2.0 ml of distilled water were added and, after mixing, the intensity of blue colour was measured at 620 nm. The gibberellin concentration in the crude extract was determined by using a calibration curve with pure gibberellic acid (GA_3) as a standard. The experiment was performed three times.

A modified method of Vereecke (*personal communication*) was used for the extraction of cytokinins secreted by *B. subtilis* HC8. Briefly, bacteria were grown in 50 ml MM for 96 h at 28°C and 150 rpm. Subsequently the bacterial cells were removed by centrifugation for 20 min at 10 000 rpm and subsequently filtering the supernatant through a 0.22-µm Millipore filter. The cell free supernatant fluid was transferred to a Sep-Pak®Plus C18 column (Waters, USA), which had previously been activated with 5 ml 100% methanol and equilibrated with 0.1% acetic acid. Subsequently the cytokinins were eluted with 3 ml of 80% methanol-2% acetic acid, concentrated *in vacuo* and re-suspended in water before further use. The method of Biddington and Thomas (1973) was used for the evaluation of the biological activity of the eluate. Briefly, seeds of *Amaranthus caudatus* L. (purchased from Sluis Garden http://www.gardenseeds.nl/) were allowed to germinate on wet filter paper at 25°C in the dark for 72 h. The seed coats and the roots were subsequently removed and ten explants consisting of cotyledons and the upper part of the hypocotyl were placed on filter paper which had been moistened with 2 ml 0.2 M phosphate buffer (pH 6.3) containing 1 mg/ml tyrosine and the sample to be tested. After an incubation period of 18 h at 25°C in the dark the seedlings were placed in 1.0 ml distilled water. Betacyanin was extracted from the samples by 3 cycles of freezing and thawing and the optical density of the supernatant fluids was measured at 542 nm. The amount of cytokinins was determined by using a calibration curve with pure trans-zeatin as a standard. The experiment was performed three times.

Utilization of ACC and solubilization of bound phosphates by endophytic bacteria

The ability of bacteria to utilize ACC as the sole nitrogen source was monitored by screening for growth on plates according to Belimov et al. (2005).

The ability of bacteria to solubilize phosphates was evaluated on hydroxyapatite medium as described by Kim et al. (1997) with some modifications. Briefly, endophytic strains were grown in MM in which the phosphorus was present in the form of hydroxyapatite ($Ca_5HO_{13}P_3$, Sigma-Aldrich, Steinheim, Germany) at 12 g/L and the pH was adjusted to 7.0. Plates were incubated at 28°C for one week. Phosphorus-solubilizing activity was judged as the appearance of clear zones around the growth area of a bacterial sample spotted on the plate.

Molecular characterization of endophytic strains

Amplified ribosomal DNA restriction analysis (ARDRA) in combination with phenotypic characterization was applied to eliminate putative siblings as described by Validov et al. (2007). Briefly, portions of the 16S rRNA genes were obtained via PCR amplification with primers 27 fm (5'- AGA GTT TGA TCM TGG CTC AG-3') and 1522R (5'-AAG GAG GTG ATC CAG CCG CA-3') (Weisburg et al., 1991). The amplified DNA fragments were subsequently digested with the four nucleases TaqI, BsuRI, HinfI and Hin6I. The resulting fragments were subsequently separated on a 2% agarose gel and the profiles of the endophytic strains were compared.

For nucleotide sequence determination, PCR products were separated on a 1% agarose gel, recovered and purified from agarose using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany). Sequencing was performed by ServiceXS (Leiden, The Netherlands). Similarity searches in GenBank were performed using BLAST (http://www.ncbi.nlm.nih.gov/blast/; Altschul et al., 1990).

Plant growth promotion

Endophytic bacteria were tested for their ability to promote the growth of radish plants. To do this, seeds of radish cv. Duro (Russkiy Ogorod – NC, Moscow, Russia) were allowed to germinate for 24 hours on moist filter paper at room temperature. The germinated seeds were then soaked in a suspension of bacterial cells in 0.85% NaCl adjusted to 10^6 cfu/ml for 15 minutes. As a negative control, seedlings were treated with 0.85% NaCl without added bacteria. The treated seedlings were subsequently planted in non-sterile potting soil (Terravita, Russia) mixed with field podsol soil in the ratio 4:1 and grown under agroindustrial conditions in the summer greenhouse of ARRIAM. Each variant consisted of four replicates with five seedlings each. After 31 days of growth, the fresh weight of the roots was determined.

Biocontrol of tomato foot and root rot

Biocontrol of TFRR was carried out in stonewool substrate as described by Validov et al. (2007). Briefly, 120 stonewool plugs were soaked in 1.0 L of commercial Plant Nutrient Solution (PNS, Wageningen UR Greenhouse Horticulture, Bleiswijk, the Netherlands) supplemented with *Forl* spores (10^7 spores/L) and bacterial cells (10^6 cfu/ml). In the negative control PNS was supplemented with spores only. Seeds of tomato cv. Carmello (Syngenta, B.V., Enkhuizen, the Netherlands) were placed in the stonewool plugs (one seed per plug) and grown for 14 days under greenhouse conditions at 80% humidity and 16 h of daylight. The plants were then removed from the stonewool and examined for symptoms of foot and root rot. Only roots without any brown spots or lesions were referred to as healthy. Dead plants, wilting plants or plants with symptoms of foot and root rot were considered as diseased. All experiments were performed twice.

Statistics

Homogeneity of variance and analysis of variance (ANOVA) at P = 0.05 were conducted with the program DIANA (Saint-Petersburg, Russia) and SPSS software (Chicago, IL, USA) for the plant-growth promotion and biocontrol assays, respectively.

Results and Discussion

Isolation and preliminary characterization of endophytic bacteria

Procedures of chemical sterilization of plant parts from different plants were developed (Table 1) to kill non-endophytic microorganisms. Validation of the surface sterilization procedure was done by culturing aliquots of water from the last rinsing onto nutrient media. Bacterial growth was never detected on such control plates, indicating the efficiency of the developed sterilization protocols.

A total of 30 morphologically different strains was chosen from a larger collection of isolates obtained after plating plant juices on 1/20 TSA. The strategy described by Validov et al. (2007) was used for the elimination of siblings and potential pathogens.

Table 1. Origin of endophytes and protocols for surface sterilization of plant samples[a]

Host plant	Part of isolation[b]	Sterilization procedure[c]
Beta vulgaris L. (beet) *Daucus carota* L. (carrot) *Lycopersicon esculentum* L. (tomato) *Solanum tuberosum* L. (potato)	Beetroot Taproot Fruit Tuber	A: 1. tap water for 30 sec 2. 70% ethanol for 5 min 3. 15% H_2O_2 for 10 min 4. sterile water 2 min ×5
Heracleum sosnowsky Manden[d] (hogweed)	Stem	B: 1. 70% ethanol for 10 min 2. 15% H_2O_2 for 15 min 3. sterile water 2 min ×5
Panicum miliaceum (millet)[d] *Zea mays* L. (maize)[d]	Stem Stem	C: 1. tap water for 30 sec 2. 70% ethanol for 7 min 3. 15% H_2O_2 for 10 min 4. sterile water 2 min ×5

[a] Plant samples were collected from experimental fields of St-Petersburg suburbs.

[b] The disinfected plant samples were crushed with a pestle in a mortar under sterile conditions.

[c] Validation of the surface sterilization procedure was done by culturing aliquots of water from the last rinsing onto nutrient medium. Bacterial growth was never detected on control plates.

[d] Plants were analyzed at the stage of flowering.

To eliminate siblings, the 30 strains were compared for their motility, their ARDRA patterns and production of the exo-enzymes chitinase, cellulase, β-glucanase and protease. Strains originating from the same sample which were indistinguishable with respect to these mentioned traits were considered as likely siblings. Eighteen isolates were removed from the collection as possible siblings. This left us with 12 strains for further analysis.

Characterization of potential plant- beneficial traits

The 12 remaining strains were screened for their antagonistic activity towards four phytopathogens, their ability to produce auxin, their growth on ACC as the sole N-source and their ability to solubilize bound phosphates (Table 2).

Three strains, namely BT18, HC8 and MZ3 show strong antifungal activity against all four tested pathogens. These strains also have cellulase, glucanase and protease activity. Strain ML15 is antagonistic only towards *P. ultimum* and does not secrete cellulases and glucanases. None of the strains showed chitinase activity.

Table 2. Overview of potential plant-beneficial traits of the selected endophytic strains[a]

Strain	Host plant	Antifungal activity[b]	Exo-enzymes[c]	Auxin[d/e]	ACC[f]/PO_4[g]
BT18	*Beta vulgaris* L. (beet)	A,*Forl*,Fs,Pu	C, βG, P	-/-	-/-
CAR2	*Daucus carota* L.(carrot)	-	C,βG	+++/-	-/-
HC2	*Heracleum* sp. (hogweed)	-	-	+/+	-/+
HC8	*Heracleum* sp. (hogweed)	A,*Forl*,Fs,Pu	C, βG, P	-/-	-/-
ML15	*Panicum miliaceum* (millet)	Pu	P	+/-	-/-
ML16	*Panicum miliaceum* (millet)	-	C,βG	++/-	-/-
TM1	*L. esculentum* L. (tomato)	-	-	+++/+	-/-
TM2	*L. esculentum* L. (tomato)	-	-	-/-	-/-
PT19	*Solanum tuberosum* L. (potato)	-	P	-/-	-/-
PT20	*Solanum tuberosum* L. (potato)	-	-	-/-	-/-
MZ3	*Zea mays* L.(maize)	A,*Forl*,Fs,Pu	C, βG, P	-/-	-/-
MZ4	*Zea mays* L.(maize)	-	-	-/-	-/+

[a] After elimination of siblings.

[b] A, *Aspergillus niger*; *Forl*, *Fusarium oxysporum f.sp. radicis-lycopersici*; Fs, *Fusarium solani*; Pu, *Pythium ultimum.*

[c] C, cellulase; βG, β-glucanase; P, protease.

[d] Auxin level after growth in medium supplemented with tryptophan: +++ >60 µg/ml, ++ >30 µg/ml, + >10 µg/ml, - < 10 µg/ml.

[e] Auxin level after growth in medium without tryptophan: + >10 µg/ml, - < 10 µg/ml.

[f] ACC, 1-aminocyclopropane-1-carboxylate.

[g] Solubilization of bound phosphates.

Two strains, HC2 and TM1, produce detectable amounts of auxins in the presence and absence of tryptophan in the medium. The level of auxin secreted by strain HC2 is less than 30 µg/ml. In the case of TM1, the auxin level in the media without and with tryptophan is less than 30 µg/ml and higher than 60 µg/ml, respectively. Three strains, namely CAR2, ML15, and ML16, produce different auxin levels and only in the medium supplemented with tryptophan.

None of the twelve strains was able to utilize ACC as the sole nitrogen source. However, all of them, except ML15 and TM2, showed a poor to good growth on N-free medium. Two strains, namely HC2 and MZ4, were able to solubilize hydroxyapatite in an *in vitro* plate assay.

Molecular identification of endophytic strains

BLAST searches in the GenBank database using 16S rDNA sequences revealed that the strains belong to different bacterial species (Table 3). To see whether these strains are safe to be applied in the field as bioocontrol and/or plant-growth promoting strains, we evaluated to which risk group (Anonymous, 1998) they belong. Of the twelve

Table 3. Molecular identification of endophytic strains and risk group classification[a]

Strain	Bacterial species and accession number[b]	Phylum	Risk group[c]
BT18	*Bacillus subtilis* HQ667318	Firmicutes	1
CAR2	*Enterobacter agglomerans* HQ667319	γ-Proteobacteria	2
HC2	*Rahnella aquatilis* HQ667320	γ-Proteobacteria	1
HC8	*Bacillus subtilis* HM441224	Firmicutes	1
ML15	*Bacillus cereus* HQ667321	Firmicutes	2
ML16	*Enterobacter agglomerans* HQ667322	γ-Proteobacteria	2
MZ3	*Bacillus subtilis* HQ667323	Firmicutes	1
MZ4	*Acinetobacter baumannii* HQ667324	γ-Proteobacteria	2
PT19	*Serratia sp.* HQ667325	γ-Proteobacteria	1
PT20	*Enterobacter amnigenus* HQ667326	γ-Proteobacteria	2
TM1	*Enterobacter agglomerans* HQ667327	γ-Proteobacteria	2
TM2	*Kocuria* sp. HQ667328	Actinobacteria	2

[a] Based on comparison of their 16S rDNA sequences with those in the GenBank database sharing at least 99% homology.

[b] All sequences have been submitted to GenBank. Sequences were obtained by sequencing the 5'end using primer 27fm for HC8 and the 3'end using primer R1522 for all other strains. Sequences are between 600 and 800 bp long.

[c] Risk group 1 includes bacteria which are safe to be applied in the field; risk group 2 includes potential human and plant pathogens.

remaining strains as many as seven strains belong to risk group 2 (Table 3), indicating a high percentage of potential human and/or plant pathogens among these endophytes. Therefore, they were excluded from further experiments. High levels of potential pathogens have been found earlier for rhizosphere bacteria (Berg et al., 2005; Egamberdiyeva et al., 2008).

The remaining five endophytic strains were BT18, HC8 and MZ3, identified as *Bacillus subtilis*, HC2 (*Rahnella aquatilis*), and PT19 (*Serratia* sp.). All of them have been found earlier as endophytes (Bai et al., 2003; Berg et al., 2005; Torres et al., 2008). Of these, strains BT18, HC8 and MZ3, which possess strong antifungal activity *in vitro* against *A. niger*, *Forl*, *F. solani* and *P. ultimum* as well as strain HC2, which produces auxin, can be considered as potential beneficial strains.

Plant growth promotion by B. subtilis HC8 and possible mechanism of action

Four endophytic strains, namely BT18, HC2, HC8 and MZ3 were tested for their ability to promote the growth of radish plants in non-sterile potting soil (Fig. 1). Radish was chosen as the model plant because its roots secrete a high level of tryptophan (Kamilova et al., 2006) which can be used by many beneficial bacteria as the precursor of auxin. The only tested strain which was able to increase the root weight of radish plants was *Bacillus subtilis* HC8 (Fig. 1). The root weight was chosen since this is the commercially interesting plant part. Strain HC8 significantly enhanced fresh root biomass, with as much as 46% compared with uninoculated control plants. Inoculation

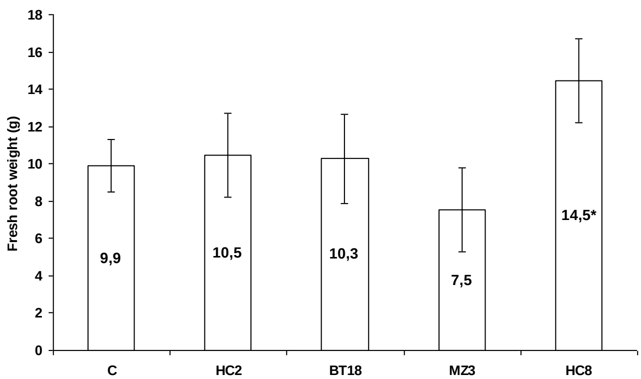

Fig. 1. Plant growth promotion mediated by endophytic bacteria. Seedlings of radish were inoculated with a suspension of bacterial cells except for the control (C) and planted in soil. Each variant consisted of four replicates with five seedlings each. Numbers inside the columns represent the mean fresh weight of the root system scored 31 days after inoculation. Bars indicate confidence interval (p = 0.05). The asterisk indicates a significantly different value.

with the auxin-producing strain *Rahnella aquatilis* HC2 and with *B. subtilis* BT18 did not show a significant increase of root growth. *B. subtilis* strain MZ3 decreased the root biomass, but not significantly.

One of the mechanisms of stimulation of plant growth by bacteria involves the production of phytohormones, such as auxins, gibberellins and cytokinins. Auxins are known to be essential for plant physiology directly affecting the root and shoot architecture (Spaepen et al., 2009). Since HC8 did not produce auxin in the tested laboratory media (Table 2) its ability to produce the plant hormones cytokinin and gibberellin was tested. Indeed, gibberellin but not cytokinin was found to be produced by HC8 (150 ng per 10^9 cells). Previously, microbial production of similar amounts of gibberellins (appr. 200 ng per 10^9 cells) has been reported for *B. lichenoformis* and *B. pumilus* (Gutierrez-Manero et al., 2001). Gibberellin is not known to enhance root growth directly (Spaepen et al., 2009). A possible explanation of the results is that gibberellin acts synergistically with another, unknown compound.

Biocontrol of TFRR by B. subtilis HC8 and possible mechanism of action

The three *B. subtilis* strains, BT18, HC8 and MZ3, were selected as the best antagonists (Table 2). Therefore, their ability to control TFRR was evaluated. Seed bacterization with only HC8 significantly decreased disease symptoms, from 91 to 42% (Fig. 2a). Significant biocontrol activity of HC8 was also found in a second experiment (Fig 2b).

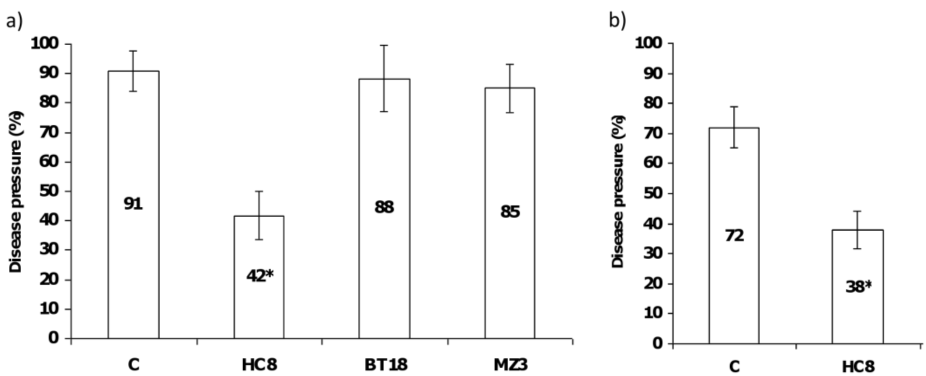

Fig. 2. Biocontrol of TFRR in stonewool substrate by endophytic bacteria. Tomato seeds were inoculated with a suspension of bacterial cells except for the control (C) and grown in stonewool plugs with added spores. Each variant consisted of 4 replicas with 30 plants each. Numbers inside the columns present the percentage of sick plants scored 2 weeks after inoculation. Bars indicate confidence interval ($p < 0.05$). Statistically different values are indicated with asterisks. (a) and (b) represent different experiments.

Although the two other antagonistic strains, MZ3 and BT18, did not show significant biocontrol of TFRR (Fig. 2a) plants bacterized with these strains did show reduced disease severity (results not shown).

For the detection of one or more compounds responsible for the antifungal activity, and therefore probably for biocontrol, the crude methanolic extracts from the dried and acid precipitated supernatant fluid of *B. subtilis* HC8 were profiled on thin layer chromatography (TLC) plates, using iturin A as a reference antibiotic (Fig. 3a). We have also profiled *B. amyloliquefaciens* FZB42 to evaluate the similarity/difference between two beneficial strains.

The iodine-starch pattern of supernatant fluids of FZB42 and HC8 are very similar. Dried supernatant fluids and acid precipitated supernatant fluids had indistinguishable patterns (results not shown). We found for both HC8 and FZB42 major spots in positions ***t***, ***u***, ***w***, ***x*** and ***y***. The R_f values of these spots shown in Fig. 3a are ***t***, 0.10; ***u***, 0.16; ***w***, 0.21; ***x***, 0.23; and ***y***, 0.26. Although the two strains produce very similar antibiotic patterns, there are also clear differences, not only in taxonomy. Spot ***v*** (R_f = 0.18) is present in HC8 but always missing in FZB42. In addition, FZB42 lacks spot ***z*** with the R_f value similar to that of iturin A (Rf_z=0.47). Spot ***s*** (Rf=0.31) of strain FZB42 is not visible in HC8 material. To test which spots are active against *Forl*, we extracted the whole HC8 and FZB42 strips (major and minor spots as well as the regions without visible spots) and checked their antibiotic activity against *Forl in vitro* (Fig. 3b). Also for biological activity dried supernatant fluids and acid precipitated supernatant fluids have indistinguishable patterns. We found for both HC8 and FZB42 that four spots, namely ***t***, ***u*** (in case of HC8 it was ***u***/***v*** since spot ***v*** sometimes migrates very close to spot ***u***, which makes it difficult to analyze them separately), ***w*** and ***x***, clearly inhibit the growth of *Forl*. Spot ***y*** does not show any antibiotic activity. Interestingly, both spot ***z*** of HC8 and spot ***s*** of FZB42 had very low antibiotic activities when re-extracted from TLC plate.

Based on R_f values and activity against *Forl*, spot ***z*** could be iturin. We used mutant strains of FZB42 which do not produce bacillomycin D and fengycin, to see which FZB42 spots, and possibly HC8 spots, correspond with these antibiotics. The results (Fig 3a) showed that the fengycin-deficient mutant lacks two spots, ***t*** and ***w***, both of which are present in both HC8 and FZB42. These spots are also still present in the fengycin producing mutant strain Δ*bmy*A indicating that compounds in positions ***t*** and ***w*** represent (derivatives of) fengycin. The bacillomycin D lacking mutant Δ*bmy*A does

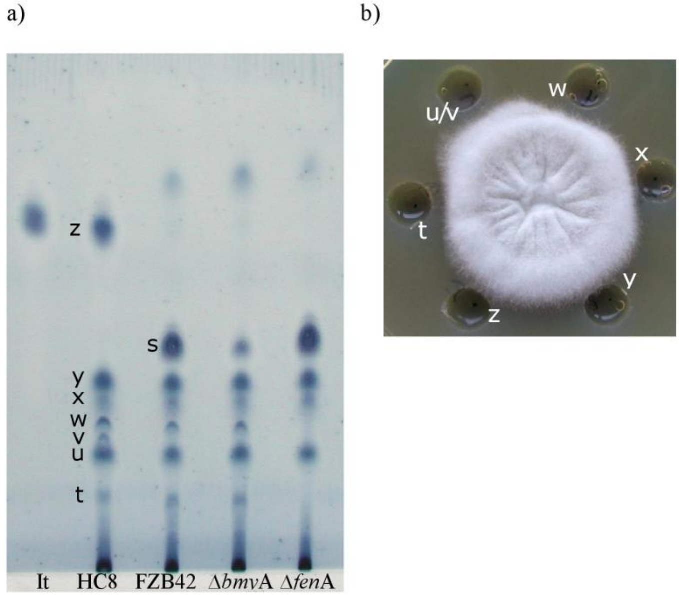

Fig. 3. Evaluation of antifungal metabolites produced by *B. subtilis* HC8 and B. *amyloliquefaciens* FZB42.

a) TLC analysis of methanol extract of the supernatant fluids of *Bacillus* strains. The plate was developed in chloroform/methanol/water 65:25:4 (v/v/v) for 2,5 hours. For visualization, the developed plate was stained in iodine followed by dipping in 1% aqueous starch. Pure iturin A (It) was used as a reference. HC8, endophytic strain *B. subtilis* HC8; FZB42, *Bacillus amyloliquefaciens* FZB42; Δ*bmy*A, mutant of FZB42 unable to produce bacillomycin D; Δ*fen*A, mutant of FZB42 unable to produce fengycin; ***t***-***z***, major spots of the HC8 crude extract; ***z***, likely correspond to iturin; ***s***, fraction likely to contain bacillomycin D; ***w*** and ***t*** likely to contain fengycin.

b) Antifungal activity of individual fractions of crude extract from *B. subtilis* HC8 towards *Forl in vitro.* ***t***-***z***, major fractions corresponding to spots in a).

produce spot ***s*** but in lower amounts than its wild type strain FZB42, therefore this spot probably contains bacillomycin D. No information on the identity of spots ***u***, ***x***, and ***y*** from HC8 was generated.

The antibiotics iturin and bacillomycin D belong to the same family of cyclic lipopeptides which comprises iturins A, C, D and E, bacillomycin D, F and L, bacillopeptin and mycosubtilin (Moyne et al., 2004). Iturins interact with the cytoplasmic membrane of the target cells forming ion-conducting pores (Magnet-Dana and Peypoux, 1994). These antifungals appeared to work synergistically with other lipopeptides, such as surfactins and fengycins. For example, Chen et al. (2009) report that the fungicidal activity of FZB42 is due to synergistic action of bacillomycin D and fengycin since without fengycin the antifungal effect of this strain is less profound. This may explain why the iturin and bacillomycin D fractions almost lack biological activity in our experiments.

Taking together all these data suggest that *B. subtilis* HC8 produces several (lipo)peptide antibiotics, some of them are different from FZB42 and may be important for antifungal and biocontrol activity of HC8.

Do endophytes play a role in the growth of the giant hogweed?

In this study we have isolated the novel biocontrol and plant growth promoting strain *B. subtilis* HC8 from the giant hogweed *H. sosnowskyi.* This plant can grow in low nutritional environments while reaching a high biomass. This observation has led us to speculate that microbes colonizing the inner plant tissues of *Heracleum* have beneficial traits which may contribute to its enormous growth. Strain HC8 appears to have the ability to produce a large variety of bioactive compounds that might play a role in biocontrol and plant growth promotion mediated by this strain. Although it was isolated from *H. sosnowskyi*, it is able to promote the growth of radish and reduce TFRR in tomato plants. This will facilitate its application as a bioinoculant. Endophytes from the plant *Heracleum* have never been isolated previously. It may be interesting to evaluate the entire endophytic microbial content of *Heracleum* in more detail.

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