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Genomic analysis of *Bacillus subtilis* OH 131.1 and co-culturing with *Cryptococcus flavescens* for control of Fusarium head blight

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ABSTRACT

Bacillus subtilis OH 131.1 is a bacterial antagonist of *Fusarium graminearum*, a plant pathogen which causes Fusarium head blight in wheat. The genome of *B. subtilis* OH 131.1 was sequenced, annotated and analyzed to understand its potential to produce bioactive metabolites. The analysis identified 6 synthetic clusters for metabolites that could impact biocontrol efficacy. Five of the clusters were confirmed functional with HPLC–MS/MS of the culture supernatant. The analysis also provided the data to determine the phylogeny of the subject strain. The phylogeny results characterize the strain as *B. subtilis* subsp. *subtilis*. Comparative genomics was used to compare the genome of *B. subtilis* OH 131.1 with closely related strains. Previously, the culture supernatant of *B. subtilis* OH 131.1 showed no antifungal activity, which suggested that it may be a suitable candidate to combine with a yeast antagonist (*Cryptococcus flavescens* OH 182.9) of Fusarium head blight for possible enhanced biocontrol efficacy. Co-cultures of *B. subtilis* OH 131.1 and *C. flavescens* OH 182.9 were produced with varying ratios of the starting inoculum of the two strains. The strains alone and the various ratios were evaluated for biocontrol activity in greenhouse assays. Co-cultures of *B. subtilis* OH 131.1 and *C. flavescens* OH 182.9 initially inoculated at a ratio of 1:100 or 1:10, respectively, were the most effective in reducing disease symptoms compared to the control. The most effective co-cultures were not statistically more efficacious than individual strains. This study reports the first co-culturing of bacterial and yeast biocontrol antagonists and their efficacy in greenhouse assays.

1. Introduction

A large variety of bacteria and fungi that can antagonize plant pathogens has been identified. These microbes utilize a wide variety of modes-of-action to inhibit plant pathogens (Santoyo et al., 2012). Such observations have prompted several researchers to explore mixtures of strains for possible improved biocontrol consistency and efficacy (Pierson and Weller, 1994; de Boer et al., 1999; Guetsky et al., 2001; Schisler et al., 2005; Slininger et al., 2010). While many mixtures showed improved efficacy, some have resulted in a decrease in biocontrol efficacy compared to component strains tested individually (Vanneste and Yu, 1996; Stockwell et al., 2010). In these cases, the mode-of-action of the component strains may be incompatible and results in some mixtures exhibiting less than expected levels of efficacy in reducing disease (Stockwell et al., 2011).

The use of a combination of strains also generates additional logistic considerations, such as, should component strains be produced independently and combined or can they be cultured together; the latter approach having been only recently reported in the biological control literature (Slininger et al., 2010; Schisler et al., 2011). Capital costs for producing a single co-cultured product would likely be less than costs associated with mixing two or more independently produced microbial products given the savings in development, production and formulation of biomass, registration and quality control costs for the co-cultured product (Slininger et al., 2010). In the biological control of post-harvest maladies on potatoes, a co-cultured product containing three different isolates of *Pseudomonas fluorescens* provided greater control than a mixture of the same three strains produced separately demonstrating an additional potential advantage of co-culturing strains (Slininger et al., 2010). Whether co-cultures of microbial strains from different taxonomic Kingdoms can be consistently produced and whether such co-cultures would have efficacy in reducing a plant disease have not been reported.

Our laboratory has previously identified several microbes that inhibit plant pathogens. These include, *Cryptococcus flavescens* (formerly *Cryptococcus nodaensis*) OH 182.9 (NRRL Y-30216), which is a basidiomycetous yeast that reduces Fusarium head blight (FHB) in greenhouse and field studies on wheat (Schisler et al., 2002b; Khan et al., 2004). A fungicide tolerant variant of this strain also reduces FHB (Schisler and Boehm, 2012) and was used in this study. *C. flavescens* is thought to inhibit the primary causal agent of FHB on wheat in North America,

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Gibberella zeae (anamorph: *Fusarium graminearum*), through competitive exclusion (Schisler et al., 2014), since no antifungal activity has been observed in culture supernatant or direct competition assays (Schisler, unpublished). In addition, *Bacillus subtilis* OH 131.1, a gram-positive bacterium was isolated as a potential antagonist of FHB (Khan et al., 2001). This strain was originally isolated from newly emerged anthers of field grown winter wheat and was subsequently shown to be effective in reducing FHB incidence and severity in wheat (Khan et al., 2001; Schisler et al., 2002a, 2002b; Khan et al., 2004). *B. subtilis* OH 131.1 does not produce antifungal compounds in culture supernatant or exhibit antibiosis of *G. zeae* in direct competition assays (Dunlap et al., 2011). The lack of antifungal compounds suggests that *B. subtilis* OH 131.1 maybe a compatible candidate for co-culturing with *C. flavescens* OH 182.9 to produce a biocontrol product with enhanced efficacy.

The goal of the current study was to sequence, assemble, and annotate the genome of *B. subtilis* OH 131.1 and subsequently determine its potential to produce secondary metabolites that impart desirable biocontrol activities (e.g., the production of antifungal compounds) or impact the ability of the strain to grow in co-culture with *C. flavescens* OH 182.9. The genomic data will then be used to conduct phylogenetic analysis of the strain. An additional goal was to assess the ability of *B. subtilis* OH 131.1 to be co-cultured with the yeast *C. flavescens* OH 182.9 and to determine the biocontrol efficacy of the co-cultured product against FHB in a greenhouse study.

2. Material and methods

2.1. Strain culturing

B. subtilis strain OH 131.1 was obtained from the ARS (NRRL) Patent Culture Collection (http://nrrl.ncaur.usda.gov) as NRRL B-30212. The strain was subsequently produced in a semi-defined complete liquid culture medium (SDCL) (Slininger et al., 2007) for all assays. Samples of bacterial cultures frozen at −80 °C in 10% glycerol were streaked for purity onto one-fifth strength tryptic soy broth agar (TSBA/5), pH 6.8 (Difco Laboratories, Detroit, MI). After 24 h incubation at 28 °C, cells were removed from the surface of colonized plates using sterile cotton swabs and utilized to initiate liquid starter cultures of the strain. Ten milliliters of SDCL in 50 ml Erlenmeyer flasks were inoculated to an optical density of approximately 0.2 at 620 nm wavelength light (A_{620}) for liquid starter cultures. The starter cultures were then incubated in a shaker incubator (Innova 4230, New Brunswick Scientific, Edison, NJ) at 25 °C with a throw of 2.5 cm and 250 rpm for 24 h. Afterwards, the starter cultures were used to inoculate liquid test cultures composed of 50 ml of SDCL in 250 ml Erlenmeyer flasks to an optical density of 0.1 (A_{620}). Test cultures were incubated as described for liquid starter cultures and harvested at 9, 12, 24, and 48 h of culture growth as needed for conducting analyses and bioassays.

2.2. Genome sequencing

B. subtilis strain OH 131.1 was cultured as described above to early stationary phase (~24 h) and harvested by centrifugation. DNA extraction was performed on the pelleted bacterial biomass using the Epicentre Masterpure DNA Purification kit (Illumina Inc., Madison, WI). The total genomic DNA extraction was subsequently fragmented to 200 bp and size-selected using an E-gel apparatus (Life Technologies Inc., Grand Island, NY). Sequencing adapters were ligated using an Ion Express™ Plus Fragment Library kit (Life Technologies Inc., Grand Island, NY). Emulsion PCR to incorporate the DNA fragment library to the sequencing beads was performed using the Ion OneTouch™ instrument with an Ion OneTouch™ System Template kit (Life Technologies Inc., Grand Island, NY). The library sample was finally sequenced on an Ion Torrent Personal Genome Machine using an Ion 316 chip and the Ion PGM™ 200 sequencing kit (Life Technologies Inc., Grand Island, NY) following the manufacturer's suggested protocols. The resulting reads were quality trimmed to the Q20 confidence level. The genome was assembled using both CLCbio 6.1 (Qiagen Inc., Cambridge, MA) and SeqMan NGen® 4.1 (DNAstar Inc., Madison, WI) using default parameters. The gaps were closed using primer walking with standard PCR. The sequence was deposited in NCBI GenBank under accession number CP007409.

2.3. Culture production and sample preparation of secondary metabolites

B. subtilis strain OH 131.1 was cultured as described above and the culture supernatant was collected during the exponential growth phase (9 h and 12 h), early stationary phase (24 h) and late stationary phase (48 h). The cells were removed by centrifugation, and the cell-free supernatant was partitioned into fractions using a C_{18} syringe cartridge (Maxi-clean SPE 300 mg C_{18}, Grace Inc., Deerfield, IL). Four milliliters of cell-free supernatant was loaded on to the cartridge and washed with 5 ml of water, then two fractions were eluted using 1 ml of 70% and 100% methanol. In addition to the above method, a second method was utilized after initial attempts to identify subtilomycin were unsuccessful. This method was based on a previous method used to isolate subtilomycin (Phelan et al., 2013). Briefly, 25 ml of supernatant was precipitated with ammonium sulfate in steps of 10% saturation up to 40% at 4 °C. The precipitate from the 40% saturation step was collected by centrifugation at 16,000 *g* for 20 min and decanting the supernatant. The precipitate was resuspended in 1 ml HPLC grade water and analyzed using RP-LC–MS/MS.

2.4. Reverse-phase liquid chromatography tandem mass spectrometry (RP-LC–MS/MS)

The C_{18} fractionated supernatant mixtures from above were analyzed by RP-LC–MS/MS (20 μl injections; Agilent 1100 HPLC, equipped with a PDA monitoring at λ 280 nm) through a C_{18} column (3.0 mm × 15 cm, 3 μm particle size; Inertsil, GL Sciences, Inc., Torrance, CA) running a gradient elution of 95% A:5% B (buffer A 0.1% formic acid, buffer B 100% acetonitrile) to 5% A:95% B over 60 min at a flow rate of 300 μl/min, followed by a 5 minute B washout and 15 minute re-equilibration, while maintaining a constant column temperature of 30 °C. Electrospray positive mode ionization data were collected with a Q-oTOF (quadrupole-orthogonal time-of-flight) mass spectrometer (Applied Biosystems/MDS Sciex Qstar/Elite) via turbo spray ionization in the positive mode. Fragmentation data was attained using collision energy (CAD = 5, CE = 45). Fragmentation energies were held at a level that maintained a large percentage of the parent ion peak in order to minimize the generation of secondary fragments. Tandem mass spectra were collected isolating the 2^+ charge state for subtilosin and 3^+ charge state for subtilomycin, as these were the most abundant charge states.

2.5. Phylogenetic analysis

In order to determine the taxonomic affiliation of strain OH 131.1, the phylogenetic framework developed by Rooney et al. (2009) for the *B. subtilis* species complex was used. The dataset encompasses a concatenation (5550 bp) of six genes including 928 bp from *gyrA*, 964 bp from *rpoB*, 875 bp from *purH*, 777 bp from *polC*, 835 bp from *groEL*, and 1171 bp from 16S rRNA taken from strains deposited in the ARS Culture Collection (http://nrrl.ncaur.usda.gov). The homologous sequence data from strain OH 131.1 was added to this dataset, along with corresponding sequence data from strains QB928 (Yu et al., 2012) and BSn5 (Deng et al., 2011). Phylogenetic analyses of gene sequence data were conducted using the neighbor-joining (NJ) method (Saitou and Nei, 1987). The computer program MEGA 6.06 (Kumar et al., 2004; Tamura et al., 2013) was used to reconstruct NJ trees from Tamura–Nei gamma distances (Tamura and Nei, 1993). The gamma shape

parameter used was 0.22, which was estimated using MEGA 6.06. The reliability of internal branches was assessed from 1500 bootstrap pseudoreplicates.

2.6. Bioinformatics and comparative genomics

The determination of open reading frames (ORFs) and annotation was carried out using both RAST server (Aziz et al., 2008) and NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) (Angiuoli et al., 2008). The annotations were manually refined by direct comparison to closely related completed genomes. The comparative genomics and core genome analysis was performed using CMG-Biotools (Vesth et al., 2013). To simplify comparisons, automated gene prediction was performed on all of the genomes using Prodigal (Hyatt et al., 2010), to eliminate differences in gene calling. Strains and GenBank files used in comparisons were: *B. subtilis* subsp. *subtilis* str. BSn5 (CP002468), *B. subtilis* subsp. *subtilis* str. QB946 (CP003783), *B. subtilis* subsp. *subtilis* str. 168 (AL009126), *B. subtilis* subsp. *spizizenii* str. W23 (CP002183), *B. subtilis* subsp. *inaquosorum* KCTC 13429 (AMXN01000001–AMXN01000024) and *Bacillus amyloliquefaciens* subsp. *plantarum* str. FZB42 (CP000560). In the proteome comparisons, homologs and paralogs were based on 50% homology with 50% coverage.

2.7. Co-culture of B. subtilis OH 131.1 and C. flavescens OH 182.9 3C

Cultures of *C. flavescens* OH 182.9 3C (NRRL Y-30216) and *B. subtilis* OH 131.1 were initiated on 1/5 strength tryptic soy broth agar (TSBA, Difco Laboratories, Detroit, MI) from 10% glycerol stocks of the strains stored at −80 °C. Twenty-four hour liquid starter cultures of each strain were produced as described earlier and used to inoculate liquid test cultures composed of 50 ml of SDCL in 250 ml Erlenmeyer flasks. A total of 1 ml of starter inoculum was used to inoculate two starter cultures per treatment. The starter inoculum used were OH 131.1 alone, OH 182.9 3C alone, and various ratios of the two strains including 1:100, 1:10, 1:1, 10:1, and 100:1 respectively. Flask cultures were incubated as described earlier and harvested at 48 h, and colony forming units (cfu) per ml of OH 131.1 and OH 182.9 were determined by serial dilution onto TSBA/5 and TSBA/5 + 100 ppm streptomycin, respectively. The experiment was conducted three times. Harvested cultures were used at 25% full strength for tests of biocontrol efficacy against FHB on wheat.

2.8. Efficacy of co-cultured inoculum against Fusarium head blight in a greenhouse bioassay

Wheat plant production and bioassays against FHB were as described previously (Schisler et al., 2011) with minor exceptions. Briefly, wheat plants (hard red spring cultivar Norm) were grown 3 per 19-cm-diameter plastic containing air-steam pasteurized (60 °C for 30 min) potting mix (Terra-lite Redi-earth mix, W.R. Grace, Cambridge, MA). Plants were grown in a growth chamber (23 °C day, 18 °C night, 14-h photoperiod, 600 μmol/[m^2/s]) for 6–8 weeks prior to transfer to greenhouse benches. Experiments to determine the efficacy of co-cultures and the individual microbial strains were conducted in a climate-controlled greenhouse. Conidial inoculum of *G. zeae* Z-3639 was produced on clarified V8 juice agar (CV8 agar) in Petri plates under a regime of 12 h/day fluorescent light for seven days at 24 °C. Conidia were then harvested from plates flooded with a weak PO_4 buffer (Schisler et al., 2014) to obtain conidial suspensions of the pathogen (Schisler et al., 2002b). Approximately one week after transferring plants to the greenhouse, wheat heads were inoculated after plants had entered anthesis (Feekes 10.5.1, (Large, 1954)) by spraying 25% freshly harvested, 48 h liquid test cultures of individual antagonist strains and co-cultures PO_4 buffer and a final concentration of 0.036% Tween 80 (Sigma Chemical Co., St. Louis, MO). Antagonist cells were applied in the greenhouse at a field equivalent rate of approximating 375 l/ha. For each treatment application, 40 ml of 25% test culture was used to treat six plants representing a total of 12 to 16 heads. Heads were then challenged immediately by spraying 12 ml of a conidial suspension of *G. zeae* (2.5×10^4 conidia/ml) in PO_4 buffer with 0.036% Tween® 80. Wheat heads treated with buffer and Tween® 80 only, followed by a suspension of *G. zeae* served as a disease control and untreated pots were used to insure pathogen inoculum was not spreading between treatments. Treated plants were misted lightly with distilled water and incubated in a plastic humidity chamber at 17 °C to 20 °C at night and 25 °C to 28 °C during the day for 3 days before being transferred to greenhouse benches. Treatments were arranged in a completely randomized design. Fusarium head blight severity was visually estimated using a 0 to 100% scale (Stack and McMullen, 1995) at 16 days after inoculation when disease symptoms had reached a maximum value. All greenhouse experiments were conducted 3 times. Disease severity data from repeated experiments were combined and analyzed using one-way analysis of variance (ANOVA) after preliminary analysis revealed that experiment by treatment interactions was not significant ($P \leq 0.05$). Means were separated using Fisher's protected LSD test (FPLSD, $P \leq 0.05$).

3. Results

3.1. B. subtilis strain OH 131.1 genome sequencing

B. subtilis strain OH 131.1 was sequenced to an average coverage depth of 49×. The assembly and subsequent gap closure yielded one scaffold with a length of 4,039,155 bp. A summary of the results is provided in Table 1. Potential secondary metabolite clusters were screened using an automated genome mining algorithm, antiSMASH (Medema et al., 2011) or direct searching with BLAST for gene clusters known to be present in *B. subtilis* strains. The automated genome mining algorithm was useful in identifying the two common types of secondary metabolite gene clusters, non-ribosomal peptide synthetases and polyketide synthases. A summary of the gene clusters identified is provided in Table 2. The results identify four potential antifungal compounds, surfactin, plipastatin, subtilomycin and subtilosin. In addition, it identified two other compounds that could impact biocontrol efficacy; a siderophore (bacillibactin), and an antibacterial (bacillaene). In addition to these synthetic clusters, a 31 kD protein (TasA) with reported antibacterial activity was found (Stöver and Driks, 1999) and the ytpA protein which was reported to synthesize bacilysocin, a phospholipid based antibiotic (Tamehiro et al., 2002).

3.2. HPLC–MS/MS

To confirm that the synthetic clusters were functional, we screened culture supernatants during different growth stages for the presence of the metabolites. We screened samples from the exponential growth phase, early stationary phase and the late stationary phase. The results show that subtilosin (3399 Da, Fig. 1) (Kawulka et al., 2004) was produced in significant amounts and increases with culture time and was one of the most prominent metabolites produced. Subtilosin ion fragmentation occurred primarily in the proline-containing macrocyclic ring and the adjacent larger alanine- and glycine-rich macrocycle. Due to the high number of glycine and alanine components of the ring,

Table 1
Genome statistics of *B. subtilis* subsp. *subtilis* OH 131.1.

Size	4,039,155 bp
Number of genes	4061
Number of proteins	3885
tRNAs	80
rRNAs	24
GC%	43.85%
Average coverage	49×

Table 2
Secondary metabolite synthetase clusters in *B. subtilis* OH 131.1.

Compound	Synthetase type	Genes	Size (kb)	Location
Surfactin	NRPS	srfAA,AB,AC,AD	26.1	364980…391127
Plipastatin	NRPS	ppsA,B,C,D,E	34.1	1988046…1953885
Bacillibactin	NRPS	besA,dhbA,C,E,B,F	12.8	3093546…3106386
Bacillaene	PKS	baeB,C,D,E,acpK baeG,H,I,J,L,M,N,R,S	77.0	1725401…1802496
Subtilomycin	Lantibiotic	subA,P,B,C,I,T	8.1	203076…211144
Subtilosin	Bacteriocin	sboA, sboX, albA,B,C,D,E,F,G	6.9	3657524…3664461

fragment ions could be assigned from multiple sites; therefore, brackets are used to indicate the positions where fragmentation was observed. The two lipopeptides, surfactin and plipastatin, as well as siderophore bacillibactin, were observed in small amounts, while bacillaene was not detected under these growth conditions. Subtilomycin (3234 Da, Fig. 1) (Phelan et al., 2013) was observed during the exponential growth phase (9 and 12 h), followed by a diminishing signal during the stationary phase (24 and 48 h). Tandem mass spectra of subtilomycin yielded a small number of fragments due to the cyclic components of the lantibiotic class; however fragments containing the N-terminus were observed, as indicated by arrows in Fig. 1. Specifically, the N-terminal 2-oxobutyrate with the adjacent tryptophan (271.08); its adjacent ala (342.19); then fragmenting at the 4th (Ile) residue (538.32); 7th (Abu) residue (806.54); and the 14th (Arg) residue (1404.12) are consistent with the subtilomycin structure. The ammonium sulfate precipitation used to isolate the products led to ammonium adducts of the products being observed in the mass spectra; tandem mass spectra of these peaks contained almost exclusively the neutral loss of ammonia without informative fragmentation.

3.3. Phylogenetic analysis

The phylogenetic placement of OH 131.1 is shown in Fig. 2. The strain is a member of the *B. subtilis* subsp. *subtilis* clade and closely related to three strains (NRRL BD-586, NRRL BD-587 and NRRL BD-588) isolated from soil collected in Greenland and a strain (NRRL BD-566) isolated from sand collected in Ghana (isolate source data obtained from the ARS Culture Collection database).

3.4. Comparison to related strains

The genome of *B. subtilis* subsp. *subtilis* OH 131.1 had two large additions (>10 kbp) not found in closely related strains (*B. subtilis* subsp. *subtilis* 168, *B. subtilis* subsp. *subtilis* BSn5, and *B. subtilis* subsp. *subtilis* QB946). Both were identified as prophages and identified using phage searching software (Zhou et al., 2011). The first was identified as a complete intact prophage spanning 37.4 kb at position (1258892…1296305). The second was questionable on completeness and spanned 62.9 kb at position (18163370…1879343). The genome of *B. subtilis* subsp. *subtilis* OH 131.1 had two deletions of known antibiotics that are found in their close relatives, sublancin (Paik et al., 1998) and subtilin (Klein et al., 1992).

The protein-coding regions of *B. subtilis* subsp. *subtilis* OH 131.1, a closely related biocontrol strain (*B. subtilis* subsp. *subtilis* BSn5), a closely related strain with the complete genome available (*B. subtilis* subsp. *subtilis* QB946), the *B. subtilis* subsp. type strains (*B. subtilis* subsp. *subtilis* 168, *B. subtilis* subsp. *spizizenii* W23, *B. subtilis* subsp. *inaquosorum* KCTC 13429) and the type strain of a common *Bacillus* biocontrol species (*B. amyloliquefaciens* subsp. *plantarum* strain FZB42) were compared using BLAST matrix. The results (Fig. 3) confirm that the phylogenetic analysis, which showed the closest relative to *B. subtilis* subsp. *subtilis* OH 131.1 is the biocontrol strain *B. subtilis* subsp. *subtilis* BSn5. The strains share 90.0% of their protein-coding regions with each other,

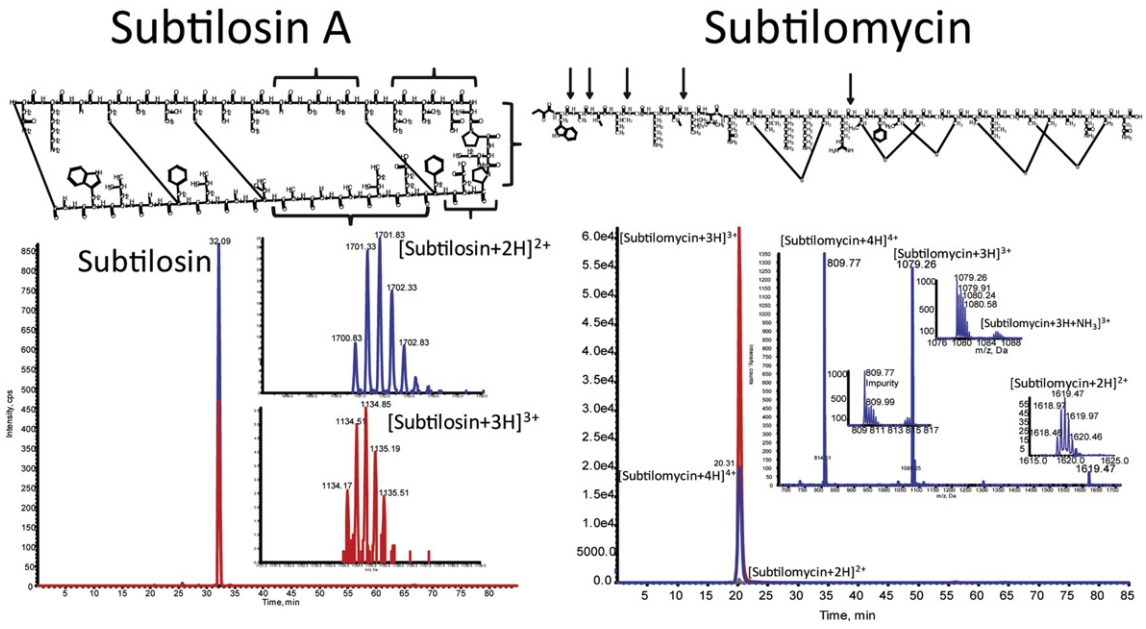

Fig. 1. A) Subtilosin at 48 h. EIC blue 2^+ ion, red 3^+ ion. Brackets indicate locations of ion fragmentations. B) Subtilomycin at 9 h. EIC gray 2^+ ion, red 3^+ ion, blue 4^+ ion (excluding intense impurity peak). Arrows indicate locations of ion fragmentations.

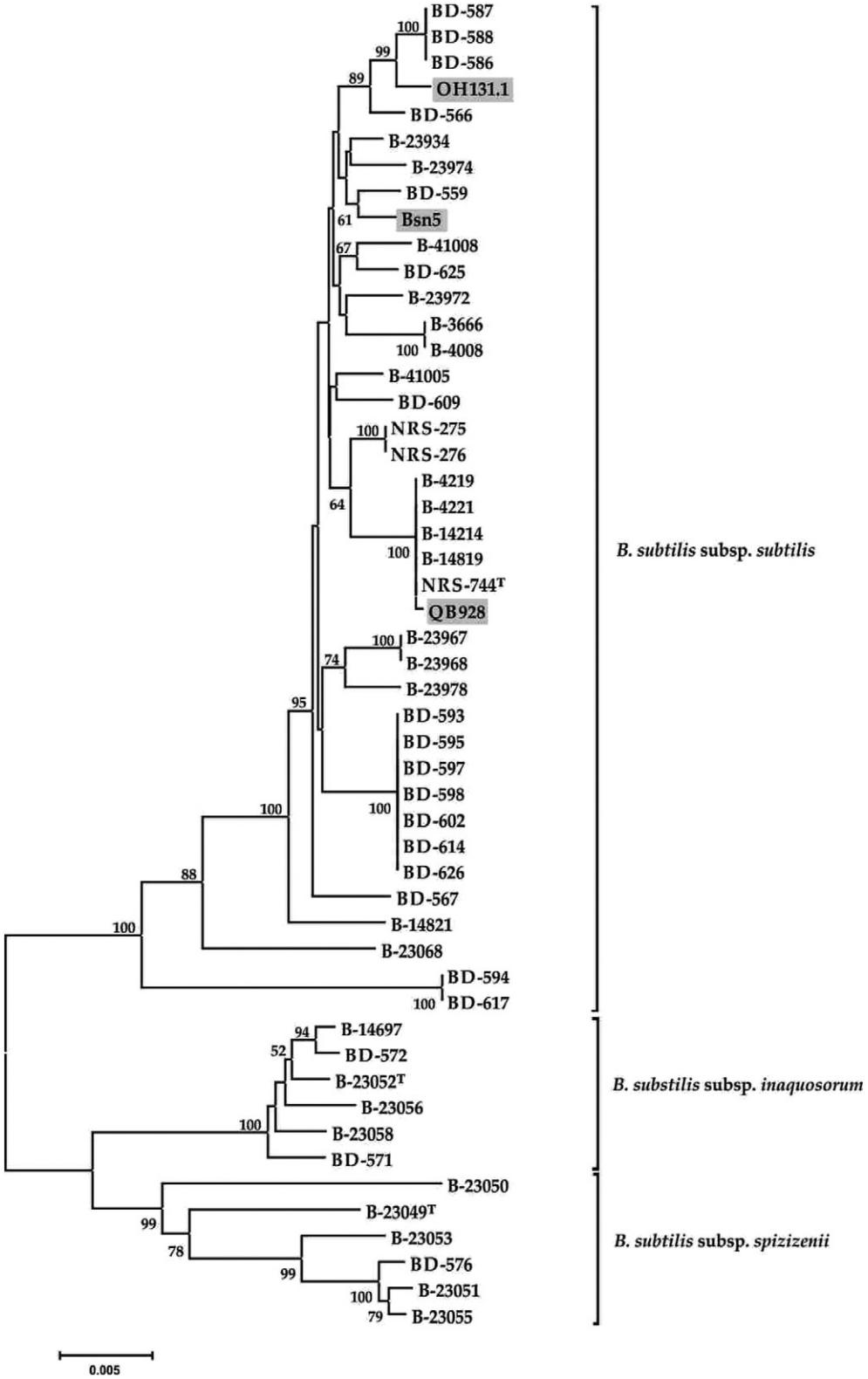

Fig. 2. Phylogenetic placement of OH 131.1 among other strains of *B. subtilis*. The tree was reconstructed from Tamura–Nei gamma distances using the NJ method; numbers at nodes represent bootstrap percentages based on 1500 pseudoreplicates (only values greater than 50% are shown). Strains for which complete genome data were analyzed in this study are highlighted in gray.

while the subject strain shares 85.8% of the protein-coding regions with the type strain for the subspecies (*B. subtilis* subsp. *subtilis* str. 168). The protein-coding region comparisons were used to determine if the two biocontrol strains (OH 131.1 and BSn5) had any genes not found in the other nonbiocontrol *B. subtilis* strains. The results identified 17 unique genes shared by the biocontrol strains (Supplemental Table 1). The set contained the 6 genes that make up the subtilomycin cluster, a serine/threonine kinase, comX pheromone precursor protein, a transcriptional regulator (merR family) and 8 hypothetical proteins.

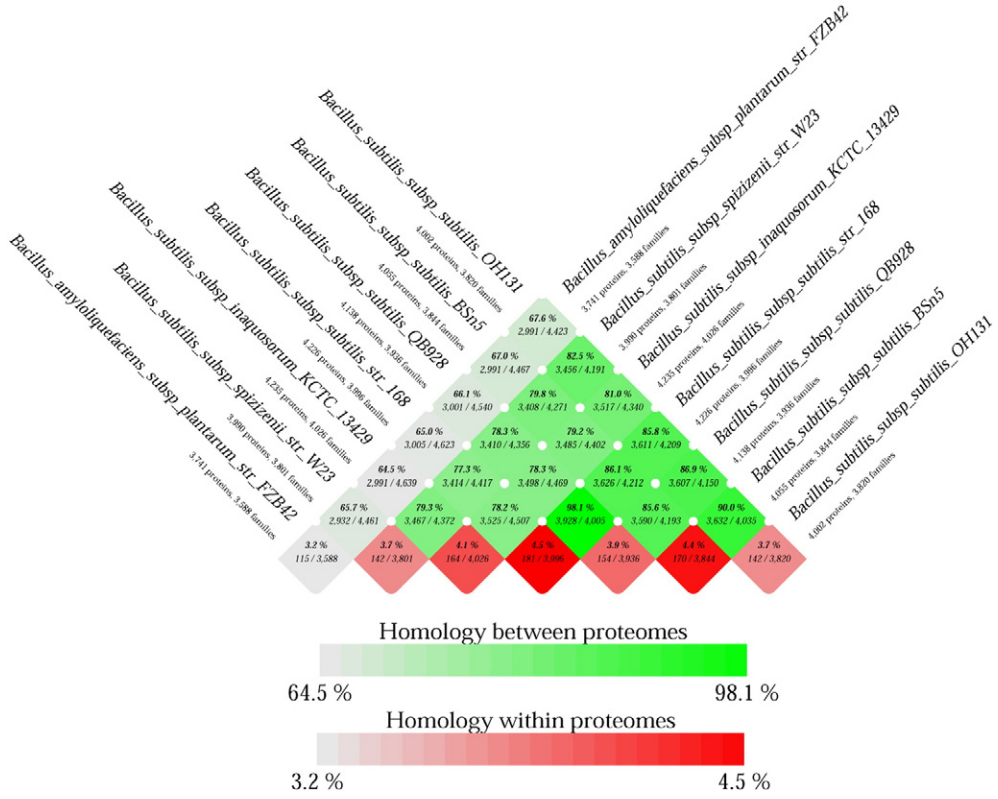

Fig. 3. Protein-coding region comparisons between *B. subtilis* subsp. *subtilis* str. OH 131.1 and related strains using BLASTmatrix.

3.5. Co-culture of Bacillus sp. OH 131.1 and C. flavescens OH 182.9 3C

When cultured alone, populations (log_{10} cfu/ml) of *B. subtilis* OH 131.1 and *C. flavescens* OH 182.9 reached 8.64 and 8.24, respectively, after 48 h (Table 3). Populations of each component strain in the 1:100 OH 131.1:OH 182.9 co-culture were within 0.50 log unit of their respective populations reached when cultured alone (Table 3). While populations of OH 131.1 in the co-cultures inoculated at all the different ratios of the two component strains which were never more than 0.6 log units different from that of OH 131.1 cultured alone, OH 182.9 populations dropped consistently in co-cultures as the inoculation ratio of OH 182.9 ranged from being 100× that of OH 131.1 to being 1/100 that of OH 131.1. In the 100:1 OH 131.1:OH 182.9 co-culture, the population of OH 182.9 was 4.87 (log_{10} cfu/ml), more than 3 log units lower than when grown alone or in the 1:100 OH 131.1:OH 182.9 co-culture (Table 3).

Table 3
Influence of co-cultures of yeast antagonist *C. flavescens* OH 182.9 3C and bacterial strain *B. subtilis* OH 131.1 inoculated at different cell ratios on Fusarium head blight in greenhouse trials.[x,y]

Treatment[z]	Log_{10} cfu/ml of treatment culture at 48 h harvest (OH 131.1/OH 182.9)	Wheat cultivar		
		Norm		
		DS (%)	DI (%)	100 Kwt (g)
Untreated control	NA	50^{A}	63^{A}	1.51^{A}
1:100 (OH 131.1: OH 182.9)	8.93/8.11	18^{CD}	31^{D}	1.78^{A}
1:10	9.20/7.89	17^{D}	32^{CD}	1.65^{A}
1:1	8.84/6.98	35^{ABC}	55^{AB}	1.57^{A}
10:1	8.39/4.84	38^{AB}	52^{ABC}	1.66^{A}
100:1	8.61/4.87	24^{BCD}	37^{BCD}	1.66^{A}
OH 182.9 3C	8.24	24^{BCD}	40^{BCD}	1.74^{A}
OH 131.1	8.64	34^{BCD}	47^{ABCD}	1.67^{A}
P value		0.001	0.005	0.72

[x] Within a column, means not followed by the same letter are significantly different ($P \leq 0.05$, Fisher's protected LSD).

[y] DS = disease severity (=average % of individual head visually diseased), DI = disease incidence, 100 Kwt = one hundred kernel weight.

[z] Ratios (vol:vol) of different seed inocula, composed of a mixture of 24 h log growth cultures of OH 131.1 and OH 182.9 3C, respectively, used to inoculate test cultures are indicated. Test cultures were harvested after 48 h, log_{10} cfu/ml of each component strain determined (representative values in the adjacent column), and the harvested cultures applied to wheat plant heads at a rate of 25% of full strength.

3.6. Efficacy of co-cultures against Fusarium head blight

Cells of OH 131.1 and OH 182.9 cultured alone reduced FHB disease severity and OH 182.9 cultured alone also reduced FHB incidence compared to the control ($P \leq 0.05$, FPLSD, Table 3). All co-culture treatments arithmetically reduced disease severity and all treatments significantly reduced disease severity except for the 1:1 and 10:1 co-cultures ($P \leq 0.05$, FPLSD, Table 3). The 1:100 and 1:10 co-cultures of OH 131.1:OH 182.9 reduced disease severity by more than any other treatment (~65%) arithmetically, but were not significantly more efficacious than the cultures of each strain grown individually. Single strain and co-culture treatments also reduced disease incidence with all but the 1:1, 10:1 and OH 131.1 alone treatments reducing incidence significantly ($P \leq 0.05$, Fisher's protected LSD, Table 3). Though all treatments resulted in higher 100 kernel weights than the control, none were statistically significant (Table 3).

4. Discussion

The completed genome of *B. subtilis* subsp. *subtilis* OH 131.1 provided new insights into the potential metabolites it is capable of producing as well as its phylogeny. The genome size, gene complement, and GC content are consistent with those of previously published *B. subtilis* genomes (Becher et al., 2011; Earl et al., 2012). The analysis suggests that the subject strain has the potential to make two antifungal molecules

(plipastatin and subtilomycin) which may inhibit *G. zeae*. Of the potential other antimicrobial compounds, surfactin does not have activity against the causal agent of FHB (*G. zeae*) (Dunlap et al., 2011). The spectrum of antimicrobial activity of subtilosin is primarily limited to bacteria with strong activity against *Listeria* sp. (Shelburne et al., 2007). Similarly, bacillaene is an antibiotic that is active against a broad spectrum of bacteria (Patel et al., 1995).

The three clusters (surfactin, plipastatin, bacillibactin) that produced trace amounts of metabolites and the single cluster (bacillaene) from which we could not detect the metabolite are all dependent on the *sfp* gene for activity. All polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) require a posttranslational modification to become catalytically active (Lambalot et al., 1996). The *sfp* gene is a phosphopantetheinyl transferase that transfers a 4′-phosphopantetheinyl moiety to a conserved serine in PKS and NRPS enzyme modules (Lambalot et al., 1996). A fatal frameshift mutation in *sfp* has been observed in the *B. subtilis* type strain 168, and it does not produce surfactin or plipastatin even though it has the NRPS clusters (Nakano et al., 1992). Analysis of the putative *sfp* gene in the subject strain shows that the gene should be functional. Analysis of 100 bp upstream of the gene start finds the region 100% homologous with 12 *B. subtilis* subsp. *subtilis* strain genomes (data not shown), which suggests that they all share the same promoter and transcription factor binding. It remains unclear why these *sfp*-dependent clusters are not more productive under the conditions tested. We have also seen sharp increases in the iron siderophore bacillibactin production under iron starvation conditions (data not shown) (Miethke et al., 2006), which suggests that the *sfp* gene is functional and regulated.

Plipastatin is a known antifungal, but it was only produced in trace amounts under these conditions and these concentrations are insufficient to inhibit *G. zeae* (Dunlap et al., 2011). It's important to note, plipastatin and fengycin have been recently reported to be the same molecule with a variety of potential alkyl tail isomers (Honma et al., 2012). Production of plipastatin requires the *pps* genes (*ppsABCDE*), the *sfp* gene and the pleiotropic regulator *degQ* (Tsuge et al., 2007). The *pps* genes are functional, since small quantities (sub-nanomolar concentrations) of plipastatin were detected. The *degQ* gene is present with high homology to other known producers. It remains unclear when this pathway is upregulated.

Subtilomycin is a recently described lantibiotic from *B. subtilis* strain MMA7, which was isolated from the marine sponge *Haliclona simulans* (Phelan et al., 2013). The *subA* gene, from the subtilomycin synthetic cluster, was previously detected in all *B. subtilis* strains isolated from different costal and deep water marine sponges (Phelan et al., 2013). While non-marine derived strains lacked the gene when screened by PCR or in silico genome analysis, the lone exception was *B. subtilis* strain BSn5, a biocontrol strain closely related to the subject strain that was isolated from *Amorphophallus konjac* (Deng et al., 2011). Discovering the cluster in the subject strain, which was isolated from a wheat anther, suggests that the cluster can also be found in terrestrial plant-associated *B. subtilis* strains, in addition to those isolated from marine sponges. A BLAST search of GenBank confirms that the cluster can only be found in the genomes *B. subtilis* strain BSn5 and OH 131.1. Subtilomycin production in the subject strain was temporally the same as the marine strain. Under these conditions, concentration peaked at 9 h and declined with time, suggesting a similar regulation.

The subtilosin cluster was found in all 12 completed genomes and 18 of 21 draft genomes for *B. subtilis* strains on GenBank. Subtilosin is a bacteriocin, one of the most common classes of antibacterials (Riley and Wertz, 2002). Bacteriocins are thought to mediate interactions in microbial communities that overlap ecological niches (Hawlena et al., 2012). This is based, in part, on bacteriocins often having activity against conspecific members of the population (Riley and Wertz, 2002). This should be an important consideration when choosing potential co-culture candidates, since they may also have been isolated from the same ecological niche.

The results of our phylogenetic analysis show that strain OH 131.1 is a member of *B. subtilis* subsp. *subtilis* and closely related to strains isolated from soil collected in Greenland and Ghana. Although this may seem surprising, it is not unexpected as *B. subtilis* and other members of *B. subtilis* species complex are distributed widely across the world (Rooney et al., 2009). Genome-level comparisons (Fig. 3) between OH 131.1 and other *B. subtilis* species complex strains whose complete genomes are available identified 17 protein-coding genes shared in common between, and which are unique to, OH 131.1 and BSn5, another biocontrol strain (Deng et al., 2011). Although BSn5 does not cluster with OH 131.1 at a level supported by high bootstrap values (less than 50%; Fig. 2), both strains share the subtilomycin cluster, which has a wide spectrum of antimicrobial activity (Phelan et al., 2013). Thus, it may be worthwhile to determine the biocontrol potential of strains that share a close phylogenetic affinity with Bsn5 and OH 131.1, as those strains might also possess the subtilomycin cluster and other homologs that may impart biocontrol activity to strains that possess them (strains: NRRL BD-566, NRRL BD-586, NRRL BD-587 and NRRL BD-588). The function of most of the remaining homologs is unknown, with 8 protein coding regions annotated as hypothetical protein and 2 belonging to a broad class of proteins, a serine/threonine kinase and a merR family transcriptional regulator. The merR family protein is part of a broad class of transcriptional factors that have been associated with the response to a variety of stresses, such as oxidative, heavy metals or antibiotics (Brown et al., 2003). A final homolog codes for the precursor of the comX pheromone. The comX pheromone is a quorum sensing molecule in *Bacillus* species, which regulates the genetic competence pathway (Grossman, 1995) and also contributes to the ability to form biofilms (Rooney et al., 2005); either or both of these traits could, in theory, contribute to biocontrol activity of OH 131.1 and BSn5, although experimental confirmation is needed to prove this theory.

Co-cultures of *B. subtilis* OH 131.1 and *C. flavescens* OH 182.9 initially inoculated at a ratio of 1:100 or 1:10, respectively, were the most effective in reducing FHB symptoms compared to the control and in some cases reduced disease to a greater extent than co-cultures inoculated at 1:1 or 10:1 respectively ($P \leq 0.05$, Fisher's protected LSD, Table 3). The sum of cfu/ml produced by each component strain was greatest in 1:100 and 1:10 co-cultures compared to other co-cultures or each strain cultivated alone which likely at least partially explains the increased efficacy of the 1:100 and 1:10 co-cultures. The high total cell number in these co-cultures could be due to the two organisms utilizing different portions of the casein hydrolysate that would be incompletely utilized when each strain is grown alone in the SDCL medium. Information gained in determining the whole genome sequence of *B. subtilis* OH 131.1 in this study provides a means in future studies to determine if the presence of high numbers of cells of *C. flavescens* OH 182.9 stimulates expression of genes likely involved in reducing disease such as subtilomycin production in OH 131.1 compared to co-cultures with lower levels of OH 182.9 cells.

Higher initial inoculum rates of OH 131.1 compared to OH 182.9 resulted in final co-cultures with greatly reduced populations of OH 182.9 while OH 131.1 populations at 48 h were within approximately 0.5 log units of OH 131.1 cultured alone for the various co-cultures regardless of the inoculum rates of OH 182.9 used (Table 3). In shake flasks with SDCL medium and adequate aeration, the doubling time for cells of OH 131.1 is approximately 1 h while the doubling time for cells of OH 182.9 cell populations takes twice as long (Schisler, unpublished). When OH 182.9 is inoculated at progressively lower ratios compared to OH 131.1, cells of OH 182.9 appear to be progressively less able to rapidly reproduce. Subtilomycin, shown in the current study to be produced by OH 131.1, has antifungal activity and may inhibit OH 182.9 to a greater extent in those co-cultures where OH 182.9 does not make up a large percentage of the initial co-culture inoculum. Interestingly, *B. subtilis* can produce quorum sensing peptides that trigger cell death in *Escherichia coli* (Kumar et al., 2013) and the basidiomycetous

yeast *Trichosporon loubieri* can degrade N-acyl-homoserine lactones involved in bacterial quorum sensing (Wong et al., 2013). With the genome of *B. subtilis* OH 131.1 characterized in the present study, future metabolomic and proteomic studies to characterize differential gene expression in co-cultures inoculated with varying ratios of OH 131.1 and OH 182.9 should clarify the reason for differences in final cell populations of each strain and levels of biocontrol efficacy achieved.

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