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ORIGINAL PAPER

Brevibacillus fortis NRS-1210 produces edeines that inhibit the in vitro growth of conidia and chlamydospores of the onion pathogen *Fusarium oxysporum* f. sp. *cepae*

Eric T. Johnson · Michael J. Bowman · Christopher A. Dunlap

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Abstract Onions can be damaged by Fusarium basal rot caused by the soilborne fungus *Fusarium oxysporum* f. sp. *cepae* (FOC). Control of this pathogen is challenging since there is limited genetic resistance in onion. The identification of molecules that inhibit this pathogen is needed. Antagonism screening showed *Brevibacillus fortis* NRS-1210 secreted antifungal compounds into growth medium. The spent growth medium, diluted 1:1, inhibited growth of FOC conidia after seven hours and killed 67–91% of conidia after 11 h. The spent medium also inhibited growth of propagules from *F. graminearum, F. proliferatum*, *F. verticillioides* and *Galactomyces citri-aurantii*. Full strength spent growth medium did not effectively kill FOC conidia and chlamydospores inoculated into a sand cornmeal mixture. In silico analysis of the *B. fortis* NRS-1210 genome indicated the biosynthetic clusters of several antibiotics. Fractionation of spent medium followed by reverse-phase liquid chromatography with tandem mass spectrometry analysis found that fractions with the most antifungal activity contained a combination of edeines A, B and F and no other recognized antibiotics. ^{1}H NMR signals of the active fraction corresponded to edeine, a pentapeptide with broad spectrum antimicrobial activity which blocks translation in both prokaryotes and eukaryotes. Comparative genomics of *Brevibacillus* genomes shows edeine producers form a clade which consists of: *Brevibacillus brevis, Brevibacillus formosus, 'Brevibacillus antibioticus', Brevibacillus schisleri, Brevibacillus fortis*, and *Brevibacillus porteri*. This observation suggests edeine played an important role in the evolution and speciation of the *Brevibacillus* genus.

Keywords Divergence · Secondary metabolite · Genome mining · Biocontrol · Peptide · Translation inhibitor · Cluster · Dereplicate

Introduction

Onions (*Allium cepa*) are an important vegetable crop in the United States and are susceptible to numerous fungal pathogens that reduce yield and quality. FBR is a root and bulb onion disease in temperate and subtropical climates (Brayford 1996). FBR, caused by FOC, can also damage shallots, Welsh onion, and

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E. T. Johnson (✉) · C. A. Dunlap
Crop Bioprotection Research Unit, USDA ARS, 1815 N. University St., Peoria, IL 61604, USA
e-mail: eric.johnson2@usda.gov

M. J. Bowman
Bioenergy Research Unit, USDA ARS, 1815 N. University St., Peoria, IL 61604, USA

chives (Cramer 2000). The first sign of FOC disease in mature onion plants is chlorosis of all leaves. Disease within the onion basal plate leads to root death and root abscission. FOC also causes damping-off disease and can delay emergence of onion seedlings (Taylor et al. 2013). Onion growers in the United Kingdom report that FBR is becoming more prevalent in their country and may be a greater problem in the future with changing climates, as the disease is favored by warm temperatures (Taylor et al. 2013). Although some onion lines with resistance to FBR have been identified (Rout et al. 2016; Taylor et al. 2013), FBR continues to be a problem for onion growers in the USA (Christopher Cramer, personal communication). Almost all regions of USA onion production are prone to FBR to various degrees based on diverse factors that influence this pathogen (Lindsey du Toit, personal communication).

Management of FBR is possible through chemicals, but it would be preferable to develop alternatives to chemical pesticides. In addition, FOC can survive in the soil for several years as chlamydospores. After a field becomes contaminated with FOC it is very difficult to produce disease free onions (Brayford 1996). Biological control using microbes could provide effective management of the disease, be nontoxic to humans, and is biodegradable. Also, some biological control organisms can promote growth of plants.

Previous studies have indicated that FOC damage of onions can be reduced through the use of biological control organisms (Coşkuntuna and Özer 2008; Dabire et al. 2016; Malathi 2015). In all of these studies, *Trichoderma* fungi provided effective control of FOC. In addition, several *Pseudomonas* strains and one *Bacillus subtilis* strain were able to reduce FOC mycelial growth (Malathi 2015; Rajendran and Ranganathan 1996). Three biological control products are recommended to mitigate FBR in the Pacific Northwest of the USA, but their efficacy is not known (Ocamb and Gent 2019). Moreover, two of the products utilize recently registered *Bacillus amyloliquefaciens* F727 (Dunlap 2019), while the active ingredient in the third product is *Gliocladium catenulatum*. Discovery of additional biological control organisms (or identification of novel antifungal compounds) may help onion growers reduce losses due to FBR and reduce the opportunity for FOC to develop resistance against commercial biological control products. A variety of *Fusarium* based plant diseases have been successfully treated with biological control strains (Dunlap et al. 2015; Pallazzini et al. 2016; Schisler et al. 2014, 2015). *Brevibacillus* has been previously shown to produce secondary metabolites that are inhibitory to both bacteria and fungi (Chen et al. 2017; Jiang et al. 2015; Jianmei et al. 2015; Joo et al. 2015; Song et al. 2012). Some of these metabolites are secreted into media of laboratory cultures, and include peptides (e.g. tostadin, bogorol B) as well as low molecular weight biochemicals (e.g. ethylparaben). Our laboratory culture collection (Northern Regional Research Laboratory, NRRL) contains 25 *Brevibacillus* strains that were screened for secreted molecules with in vitro inhibitory activity of FOC conidia growth (data not shown). This paper reports on a *Brevibacillus* strain that produces extracellular compound(s), identified as edeines, that inhibit FOC growth.

Materials and methods

Organisms

FOC 36342 was obtained from the NRRL culture collection; this isolate caused disease in potted onions when inoculated into the soil. FOC New Mexico 9 was isolated from onions with FBR symptoms. Both FOC strains were cultured in the dark on potato dextrose agar (PDA). Genomic DNA was isolated from both FOC strains using a previously described method (Johnson and Dowd 2016). A portion of the *CRX1* gene was amplified by PCR with previously described primers (Taylor et al. 2016); the *CRX1* gene is present in most FOC isolates that cause FBR in onions and was detected in isolates 36342 and New Mexico 9 (data not shown). FOC 36342 chlamydospores were prepared as described (Nguyen et al. 2019) except that Supersoil potting soil (Rod McLellan Co., Marysville, OH) was used and a 2 mm mesh sieve was utilized to remove large soil particles in preparing the medium. *Fusarium oxysporum* (accessions 22870 and 25891, cultured on PDA), *Fusarium proliferatum* (accession 13569, cultured on V8 juice medium (Johnson et al. 2018), *Rhizopus stolonifera* (accessions 1483 and 66454, cultured on PDA), *Colletotrichum gloeosporioides* (accession 45420, cultured on PDA) and *Galactomyces citri-aurantii* (accessions Y-17913 and Y-

17923, cultured on YM medium for yeasts [NRRL medium No. 6]) were obtained from the NRRL culture collection. *Fusarium graminearum* (strain III-B), originally isolated from a diseased wheat head, was cultured on clarified V8 juice medium (Johnson et al. 2015). *Fusarium verticillioides* (strain AMR F-4), originally isolated from symptomless maize kernels, was cultured on V8 juice medium. All fungi, except *F. graminearum*, were grown at room temperature in the dark. *F. graminearum* was grown in an incubator at 28° C with 13 h of fluorescent lighting and 11 h of darkness. *B. fortis* NRS-1210, as well as *Brevibacillus parabrevis* NRS-780, were obtained from the NRRL Culture Collection. The initial screening of *Brevibacillus* strains determined that NRS-780 produced no antifungal compounds and was used as a negative control in several experiments.

Measurements of FOC growth inhibition

Bacterial strains NRS-780 and NRS-1210 were grown in liquid BEPSY medium (5 g beef extract, 3 g peptone, 5 g yeast extract, 10 g sucrose, pH 7.2–7.4 in 1 l) at 30 °C, 250 RPM for 3 d. FOC New Mexico 9 conidia were harvested from PDA plates using sterile water and passed through Miracloth (EMD Millipore Corporation, Billerica, MA). The number of conidia in the suspension was calculated using a hemocytometer. Conidia were diluted in potato dextrose broth (PDB) to 1×10^5 conidia ml^{-1} and incubated at room temperature for 16 h. Conidia germination images were taken (labeled as 0 h) using the EVOS fl. auto imaging system (Thermo Fisher Scientific, Waltham, MA) and the conidium length measured using the provided software. Germinated conidia were diluted 1:1 with filter sterilized (0.2 µm pore size) bacterial culture spent medium. After 7 h at room temperature, conidia were imaged and their length measured using the EVOS system.

Time course measurements of FOC conidia death

Bacterial strains NRS-780 and NRS-1210 were grown as described above. FOC New Mexico 9 conidia were harvested from plates as described above and diluted in PDB to 1×10^5 conidia ml^{-1}. The conidia were diluted 1:1 with filter sterilized (0.2 µm pore size) bacterial culture spent medium and incubated at room temperature. At various time points, 10 µl of the mixture was diluted 1:10 in sterile Ringer's solution (123 mM NaCl, 1.5 mM $CaCl_2$, 4.9 mM KCl, pH 7.3–7.4). Ten µl of the 1:10 dilution was placed on a PDA plate; 90 µl of sterile Ringer's solution was added to the plate and the mixture spread throughout the plate using a sterile cell spreader. Colony forming units were counted after 2–4 d.

Bioassays on fungal propagules

Strain NRS-1210 was grown as described above. FOC, as well as the other fungi, were grown for 5–10 days on plates and the propagules (conidia or arthroconidia) were harvested and counted as described above. The propagules were diluted using PDB to 1×10^5 cells ml^{-1}. Media from the NRS-1210 cultures was filter sterilized (0.2 µm pore size). Propagule growth with 50% medium was screened initially; if active against the fungus at 50%, medium was diluted to a final concentration of 12.5, 6.3, 3.1, 1.3, and 0% with the propagule-PDB mixture in wells of a 96 well plate. Triplicate wells (final volume of 160 µl) were filled for each concentration. The bioassays with FOC chlamydospores were slightly different; the harvested propagules were a mixture of conidia and chlamydospores (see Supplementary Fig. 1). Each well contained 80 µl of FOC chlamydospore-conidia mixture (approximately 8000 chlamydospores and 12,800 conidia), 30 µl of PDB and dilutions of spent bacterial medium as described above, with a final volume of 160 µl. Optical density of the filled wells were measured at an absorbance of 550 nm using a SpectraMax 250 plate reader (Molecular Devices, Sunnyvale, CA) or an Epoch 2 plate reader (BioTek, Winooski, VT) immediately after loading all of the samples and after 5 d at room temperature in the dark. Some bioassays were stopped after 2 d as no growth inhibition was observed. The lethal dose was the concentration of spent medium at which the mean absorbance of the treated propagules was 10% (or lower) of the mean absorbance of the non-treated propagules (abbreviated as LD90).

Bioassays using heat treated spent media

Strain NRS-1210 was grown as described above. Aliquots of the spent media were filter sterilized (0.2 µm pore size). The spent media was incubated at 50, 75, and 100 °C for 1 h in a heat block; room temperature incubation served as the control. FOC

conidia were harvested as described above and diluted using PDB to 1 × 10^5 cells ml^{-1}. Temperature treated spent media were diluted 50% with the conidia-PDB mixture into wells of a 96 well plate. Absorbance values were recorded as described above.

FOC bioassays on sand matrix

Play sand (Rocks etc., Lockport, IL) was mixed with yellow corn meal mix (Hodgson Mill Effingham, IL) at a ratio of 8:1 as recommended (Türkkan and Erper 2014). Fifteen g of the sand mixture was dispensed into a 5 cm diameter glass Petri dish. The dishes were sterilized in an oven at 130 °C for 5.5 h and cooled. Filter sterilized (0.45 µm pore size) supernatant (4 ml) from a 3 d old culture of NRS-780 or NRS-1210 (grown as described above) was added to the sterile sand mixture and stirred with a sterile inoculating loop until all the sand mixture was wetted (5 plates for each spent medium). The FOC chlamydospore-conidia mixture (described above) was diluted 1:10 with sterile water and 40 µl (approximately 400 chlamydospores and 640 conidia) was added to 3 plates while 2 plates were not inoculated. All the plates were kept at room temperature in the dark. Measurements of the largest possible straight line across the fungal mass in each plate was completed after 3 and 4 d using a ruler. The filter sterilized supernatants (60 µl) were also tested for inhibitory activity with 40 µl of the FOC chlamydospore-conidia mixture and 20 µl of PDB added in triplicate wells of a 96 well plate; sterile water served as a control for the supernatants. Absorbance readings at 550 nm were taken with an Epoch 2 plate reader at the start of the assay and after 5 d.

Genome mining for secondary metabolites

The genome of *B. fortis* NRS-1210 (NCBI accession PXZM01) was screened for antibiotic biosynthetic gene clusters using the anti-SMASH algorithm (Blin et al. 2017) and by the BLAST algorithm at NCBI. Genome mining for the edeine cluster in all available *Brevibacillus* genomes (as of 1/1/2020) was conducted using BLAST (McGinnis and Madden 2004). Genome comparisons and alignments for phylogenetic trees were made using BIGSdb software (Jolley and Maiden 2010). The phylogenetic trees were constructed using MEGA 7.09 software (Kumar et al. 2016) and are based on a 6 gene multilocus sequence alignment (MLSA) previously described (Rooney et al. 2009). Neighbor-joining trees were reconstructed using the Tamura-Nei model (Tamura and Nei 1993) with a gamma correction (alpha value = 0.5) with complete deletion. This model was chosen on the basis of the likelihood test implemented in MEGA 7.09. Measures of bootstrap support for internal branches were obtained from 1500 pseudoreplicates.

Identification of antifungal compounds in spent medium

B. fortis NRS-1210 was grown as described above. The culture medium was centrifuged at 13,000*g* for 10 min and the spent medium was removed followed by sterile filtration with a 0.2 µm membrane. Mass spectrometry of the spent medium samples (5 µl injection) were analysed by LC-MS (Thermo Acella HPLC) through a narrow-bore (2.1 µm × 150 mm, 3 µm particle size) C18 column (Inertsil, GL Sciences, Inc., Torrance, CA) running a gradient elution of 95% A:5% B (eluent A: 18 MΩ water/0.1% formic acid, eluent B: 100% methanol/0.1% formic acid) to 5% A:95% B over 65 min at a flow rate of 250 µl min^{-1}, followed by a 5 min B washout and 10 min re-equilibration, while maintaining a constant column temperature of 30 °C. Electrospray positive mode ionisation data were collected with a linear ion trap-Orbitrap mass spectrometer (Thermo LTQ-Orbitrap Discovery) under Xcalibur 2.1 control. Prior to LC-MS^n experiments the instrument was tuned and calibrated using the LTQ tune mix. Tandem mass spectral data was collected using collision-induced dissociation [CID, collision energy (CE) = 25 and 35] in the LTQ and Higher-energy collision dissociation (HCD, CE = 25 and 35) in the Orbitrap analyzer. Ions for fragmentation were selected based on likely antibiotics present in *B. fortis* NRS-1210.

Enrichment and characterisation of active component

Culture supernatant was centrifuged at 4 °C and 16,000 *g* for 10 min, followed by filtration through a 0.22 µm filter to remove any remaining cells. The supernatant (300 ml) was fractionated by ultrafiltration (Amicon (MilliporeSigma, Burlington, MA) 1000 Da molecular cutoff regenerated cellulose membrane,

76 mm diameter, at a flow rate of $\sim$ 1 ml min^{-1}). A portion (20 ml) of the $<$ 1000 Da medium flow through was bound in triplicate Sephadex C-25 weak cation exchange (WCX, Sigma, St. Louis, MO) resin columns (2 g resin, 15 ml of resin slurry each). The resin had been preequilibrated with ammonium acetate (pH 5.0), followed by extensive washing with 18 MΩ water until the pH returned to neutral using gravity flow. After loading, the columns were washed with 30 ml water, followed by 30 ml eluents in a step-gradient containing 50 mM; 250 mM; and 500 mM ammonium hydroxide (Westman et al. 2013). Samples were frozen, lyophilised, and resuspended on 20 ml 18 MΩ water 3 times to remove ammonia. Samples were resuspended in 10 ml 18 MΩ water (2× media concentration). Prior to testing inhibition on fungal conidia, dilutions of the samples were prepared at 1:4; 1:20; and 1:100 fraction volume:18 MΩ water. FOC New Mexico 9 was grown for 5–10 days on PDA plates and the conidia harvested and counted as described above. The conidia were diluted using PDB to 1 $\times$ 10^5 cells ml^{-1}. The dilution series of the WCX fractions were added to a final 50% volume to the conidia-PDB mixture in wells of a 96 well plate. Triplicate wells of each dilution were filled for each concentration (25% media equivalent; 5% media equivalent, 1% media equivalent, $<$ 1000 Da ultrafiltration fraction starting material, media positive control, water negative control). Absorbance values of the filled wells were measured at 550 nm using an Epoch2 plate reader immediately after loading all of the samples and after 5 d at room temperature in the dark. Data was plotted as 5% initial media equivalent versus % inhibition.

The bioactive fraction was analysed by NMR in 0.75 ml D_2O ($\sim$ 25× concentration). ^1H NMR spectra (1024 scans) were collected using a Bruker Avance 500 MHz NMR spectrometer (Bruker BioSpin Corp., Billerica, MA) operating at 27 °C using a 5-mm z-gradient BBO probe. The pulse sequences used were those supplied by Bruker, and processing was done with SpinWorks v1.3 pl8 software.

Determination of edeine content

Analytical separation of edeine polyamines was accomplished by LC-UV-LTQ-MS (Thermo Ultimate 3000 UPLC coupled to a linear ion trap (Thermo LTQ) under Xcalibur 2.1 control) on a SeQuant® ZIC®-cHILIC 100 $\times$ 2.1 mm column (Millipore, Billerica, MA) operated at 50 °C with a flow rate of 0.2 ml min^{-1}. Effluent was monitored at 272 nm prior to electrospray introduction into the mass spectrometer using positive mode ionisation to confirm peak identities. Mobile phases were A: acetonitrile with 1% (w/w) formic acid and B: 100 mM ammonium acetate with 3% (w/w) formic acid. Isocratic elution at 50%A:50%B provided the separation. A response curve was constructed using tyramine, a structurally similar compound with absorbance at 272 nm, using 1 μg ml^{-1}, 4 μg ml^{-1}, 10 μg ml^{-1}, and 100 μg ml^{-1} concentrations; these values were converted to molar quantities and the response curve was applied to the absorbance of edeine components to estimate the amount present in bioassayed media and WCX fractions.

Results

Measurements of FOC growth inhibition

B. fortis NRS-1210 secreted compound(s) into the bacterial growth medium that consistently inhibited FOC conidia growth. Because this strain could produce one or many antifungal compounds, we will refer to the inhibitory activity due to "compounds" throughout this manuscript. Conidia of FOC New Mexico 9 were germinated in potato dextrose broth for 16 h and then imaged using an inverted light microscope (Fig. 1a). The germinated conidia were diluted 1:1 in a number of different sterilised media and then incubated. After 7 h, the conidia were imaged again. Germinated conidia incubated in fresh BEPSY medium or spent BEPSY medium from a culture of *B. parabrevis* NRS-780 appeared larger than before (Fig. 1b, c). The germinated conidia did not grow well during the 7 h incubation in spent medium of a *B. fortis* NRS-1210 culture (Fig. 1d). The conidia had grown almost three times in length during the 7 h incubation period when diluted with fresh BEPSY medium or spent medium of a *B. fortis* NRS-780 culture (Table 1). The conidia did not grow at all during the 7 h incubation period when diluted with spent medium of *B. fortis* NRS-1210 (Table 1). A second experiment produced similar results: the conidia growing in the spent medium of *B. parabrevis* NRS-780 grew five times in size after 7 h while

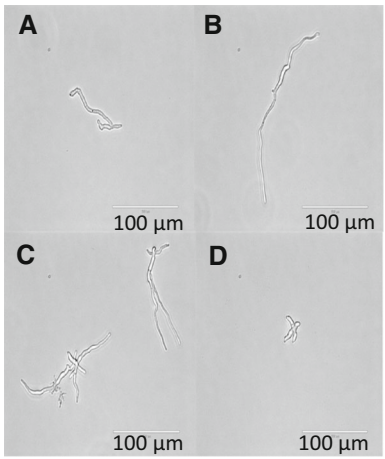

Fig. 1 Spent bacterial medium inhibits growth of FOC New Mexico 9 germinated conidia. Filter sterilized bacterial medium was diluted 50% with germinated conidia and imaged (**a**). After 7 h, images were taken of conidia incubated in NRS-780 medium (**b**), BEPSY medium (**c**), and NRS-1210 medium (**d**). Ruler (100 µm) indicates the approximate size of the conidia in each panel

conidia growing in the spent medium of *B. fortis* NRS-1210 grew only 1.5 times in size during this period. These data indicate that *B. fortis* NRS-1210 secretes compounds into the medium that inhibited the growth of FOC.

Time course measurements of FOC conidia death

The next experiments sought to determine if the viability of FOC ungerminated conidia was altered by the compounds produced by *B. fortis* NRS-1210. The results of two independent experiments indicated that spent *B. fortis* NRS-1210 medium killed many FOC conidia by 11.5 h (Table 2). Most of the control conidia could be seen after 2 d on the plates, but the *B. fortis* NRS-1210 medium-treated conidia grew slower and were counted after 3–4 d, suggesting that some residual inhibitory compounds were transferred to the plates.

Bioassays on fungal propagules

In the experiments described above, growth and viability of conidia was inhibited with a 50% dilution of the spent medium from *B. fortis* NRS-1210. Bioassays using ungerminated propagules were conducted to determine the relative antifungal activity of the spent medium on FOC and other fungal species. FOC strain NRRL 36342 conidia, as well as chlamydospores, were killed (LD90) with 3.1% of spent medium from NRS-1210 (Table 3). FOC strain New Mexico 9 conidia were killed with 1.3–3.1% of NRS-1210 spent medium. Additional *Fusarium* species were tested with results similar to that of the FOC strains. The spent medium from *B. fortis* NRS-1210 did not inhibit growth of *R. stolonifera* or *C. gloeosporioides* propagules very well. *G. citri-aurantii* propagule growth was sensitive to the spent medium at approximately the same levels for the *Fusarium* species.

Table 1 Spent bacteria media inhibit FOC New Mexico 9 conidium growth

Sample	Mean conidium length, µm ± SE (N)
Experiment 1	
0 h	40 ± 6 (11) a
7 h NRS-1210 spent medium	32 ± 2 (15) a
7 h NRS-780 spent medium	116 ± 11 (12) b
7 h fresh medium	109 ± 7 (16) b
Experiment 2	
0 h	33 ± 2 (31) a
7 h NRS-1210 spent medium	48 ± 5 (24) b
7 h NRS-780 spent medium	170 ± 13 (35) c

FOC New Mexico 9 conidia were incubated in PDB for 16 h. Germinated conidia were imaged at 0 h and then diluted 1: 1 with sterilized spent medium from bacterial cultures or fresh medium. After 7 h at room temperature, conidia were imaged. All conidia were measured using the EVOS system. For each experiment, means followed by different letters were significantly different ($P < 0.05$) by ANOVA

Table 2 NRS-1210 spent media rapidly kill FOC New Mexico 9 conidia

Time	NRS-780 spent medium	NRS-1210 #1 spent medium	NRS-1210 #2 spent medium
Experiment 1	FOC mean cfu $\mu l^{-1} \pm$ SD (N)	FOC mean cfu $\mu l^{-1} \pm$ SD (N)	FOC mean cfu $\mu l^{-1} \pm$ SD (N)
6.5 h	25 ± 6 (6) a	12 ± 3 (3) b	5 ± 2 (3) b
11.5 h	43 ± 4 (6) a	4 ± 1 (3) b	4 ± 3 (3) b
Experiment 2			
6.5 h	7 ± 2 (6) a	5 ± 3 (3) a	1 ± 1 (3) b
11.5 h	21 ± 9 (6) a	5 ± 2 (3) b	7 ± 2 (3) b

Conidia in PDB were diluted 1:1 with sterilized spent media. At the indicated time, an aliquot of the mixture was diluted and spread onto a PDA plate. Colony forming units (cfu) were counted after 2 d (NRS-780) or 3 d (NRS-1210). For each experiment, different letters in the same row indicate that the means are significantly different ($P < 0.05$) by ANOVA

Table 3 Spent bacteria medium kills propagules of several fungi

Fungal strain	Concentration of NRS-1210 spent medium resulting in LD90
FOC NRRL 36342	3.1%, 3.1%
FOC NRRL 36342 conidia and chlamydospores	3.1%, 3.1%
FOC New Mexico 9	3.1%, 1.3%
Fusarium oxysporum NRRL 22870	3.1%, 3.1%
Fusarium oxysporum NRRL 25891	3.1%, 1.3%
Fusarium proliferatum NRRL 13569	3.1%, 3.1%
Fusarium graminearum III-B	3.1%, 1.3%
Fusarium verticillioides AMR F-4	3.1%, 3.1%
Rhizopus stolonifer NRRL 1483	50%, 50%
Rhizopus stolonifer NRRL 66454	> 50%, > 50%
Colletotrichum gloeosporioides NRRL 45420	> 50%, > 50%
Galactomyces citri-aurantii NRRL Y-17913	6.3%, 6.3%
Galactomyces citri-aurantii NRRL Y-17923	6.3%, 3.1%

Two independent bacteria cultures were tested and listed

Bioassays using heat treated spent medium

Additional experiments were completed to determine the thermal stability of the antifungal compounds secreted by *B. fortis* NRS-1210. Most of the inhibitory activity of the spent medium, diluted 50% with FOC conidia, remained after a 1 h incubation at 50 °C, but declined after 1 h at 75 °C (Table 4). All inhibitory activity of FOC was lost after 1 h at 100 °C.

FOC bioassays on sand matrix

Sterilized spent medium from cultures of *B. parabrevis* NRS-780 or *B. fortis* NRS-1210 were added to a sterilized sand and cornmeal mixture. FOC chlamydospores and conidia were inoculated into the wetted sand mixtures. After 3 d, the average size of the fungal mass in each plate was 1.6 cm (SD = 0.5, N = 3) growing in *B. parabrevis* NRS-780 spent medium and 1.0 cm (SD = 0.1, N = 3) growing in *B. fortis* NRS-1210 spent medium; the means were not statistically different. After 4 d, the fungal masses were larger, and there were no significant differences in the average size between the *B. parabrevis* NRS-780 and *B. fortis* NRS-1210 treated wetted sand mixtures. No fungal growth was found in the non-inoculated wetted sand mixtures. The FOC chlamydospores and conidia did not grow in wells of a 96 well plate containing 50% of the NRS-1210 spent medium used for the sand mixture experiments; abundant

Table 4 Effect of temperature on stability of NRS-1210 inhibitory molecules

FOC + NRS-1210 spent medium	Percent inhibition at indicated temperature			
	RT	50 °C	75 °C	100 °C
FOC New Mexico 9	98 ± 0 (3)	99 ± 0 (2)	80 ± 8.8 (3)	0 (2)
FOC NRRL 36342	97 ± 0.3 (3)	99 ± 0 (2)	93 ± 3.3 (3)	0 (2)

Mean values ± SE are percent inhibition compared to controls using sterile water. Independent biological replicates are noted in parentheses

growth of FOC chlamydospores and conidia was found in wells with 50% of the *B. parabrevis* NRS-780 spent medium (or 50% water) used for the sand mixture experiments. These experiments suggest that the antifungal compounds of *B. fortis* NRS-1210 in the wetted sand mixtures were too dilute to slow the growth of the FOC chlamydospores and conidia.

Genome sequencing and identification of antifungal compounds in spent medium.

Genome sequencing of a number of *Brevibacillus* species from the NRRL culture collection, including *B. fortis* NRS-1210, was previously completed (Johnson and Dunlap 2019). The genome of *B. fortis* NRS-1210 was analysed for putative antibiotic and secondary metabolite clusters using anti-SMASH 4.0 (Blin et al. 2017). Biosynthetic gene clusters for 6 antibiotics were identified: tyrocidine, gramicidin, edeine, bacteriocin (thiazole/oxazole modified microcin, thiopeptide), bacteriocin and lantipepetide #1. In addition, the biosynthetic cluster for the siderophore, petrobactin was identified. A number of *trans*-acyltransferase type I polyketide synthase-nonribosomal peptide synthetase and nonribosomal peptide synthetase biosynthetic gene clusters were found in this analysis but the enzymes nor the final compounds of these clusters have been characterised. This in silico analysis indicates that *B. fortis* NRS-1210 could produce a variety of antifungal compounds.

Spent medium from an *B. fortis* NRS-1210 culture was analyzed by reverse-phase C18 LC-high-resolution MS to confirm the presence of secondary metabolites identified from the organism's genome. Reverse-phase chromatography was used due to the wide diversity of secondary metabolites that can be separated on this matrix. Aside from ions corresponding to edeine (Fig. 2a), no additional putative antibiotics were observed; this was confirmed by a disk assay with an edeine-resistant *Brevibacillus* isolate (see Supplementary Fig. 2). Edeines exist as four major types: edeine A (Fig. 2a) contains a β-tyrosine on one end and spermidine (polyamine) on the other; edeine B contains a β-tyrosine on one end and guanyl-spermidine on the other; edeine D contains a β-phenylalanine on one end and spermidine (polyamine) on the other; and edeine F contains a β-phenylalanine on one end and guanyl-spermidine on the other. These four types have distinct masses that can be discerned by mass spectrometry. The presence of three variations of edeine was confirmed by accurate mass (Orbitrap-MS) where the spectra (Fig. 2b) showed the analytes to predominately ionize at the 2^+ charge state. Edeine A ($[M + 2H]^{2+}$ m/z = 378.2245) was less than 1 ppm error from the calculated mass; similarly edeine B ($[M + 2H]^{2+}$ m/z = 399.2354; possessing a guanidinium group on the polyamine tail) and edeine F ($[M + 2H]^{2+}$ m/z = 390.7221; possessing a guanidinium group on the polyamine tail and lacking the hydroxyl group on the aromatic ring) were initially identified by mass. Due to the hydrophilic nature of the edeine molecules, the analytes were poorly retained on the reverse-phase C18 column and eluted early during the chromatographic runs (Fig. 2c). The identity of edeines A, B, and F were further confirmed by tandem mass spectrometry. The fragmentation of edeine A is dominated by the loss of water and amines using CID in the ion trap instrument; however, using HCD with Orbitrap detection allowed for more informative spectra (Fig. 2d). The two largest ions from this method also correspond to loss of water and loss of two waters ($[M + 2H]^{2+}$ ions m/z = 360.71 and 369.71) followed by m/z 147.04 corresponding to the polyamine tail of edeine A. Additionally, lower abundance fragment ions allow for positive

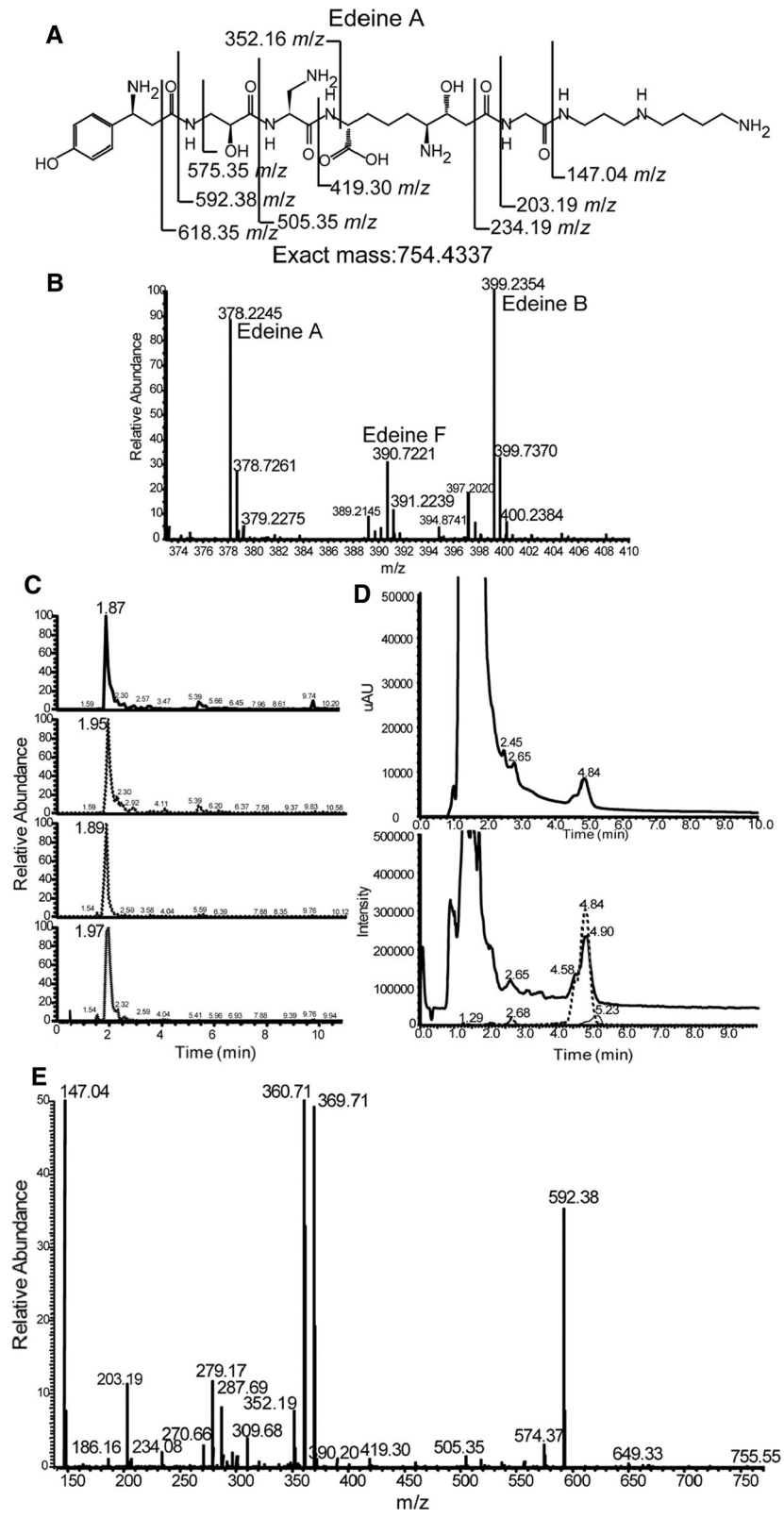

Fig. 2 **a** Structure and fragmentation assignments for edeine A. **b** Mass spectrum summed from 1.8–2.0 min of LC-Orbitrap-MS run zoomed on the masses corresponding to $[M + 2H]^{2+}$ for edeine A; edeine B; and edeine F. **c** Extracted ion-chromatograms from reverse-phase chromatography, from top to bottom, for: LTQ-ion trap m/z 755–756 (edeine A), black line; LTQ-ion trap m/z 797–798 (edeine B), dotted line; orbitrap m/z 378.1–378.3 (edeine A^{2+}), dotted line; LTQ-ion trap m/z 399.1–399.3 (edeine B^{2+}), dotted line. **d** ZIC-HILIC LC-UV-MSLTQ chromatograms. Top UV spectrum monitored at 272 nm; Bottom, positive mode ESI-MS, solid line, total ion current; dashed line, combined EIC for edeine F (2.68 min) and edeine B (4.84 min); dotted line, EIC for edeine A (5.23 min). **e** Orbitrap high-resolution MS/MS spectrum of parent ion *m/z* 378.2 corresponding to edeine A. HCD CE = 35

identification of edeine A (Fig. 2a, e). Edeine B fragmentation followed the same pattern with the addition of 42 Da in all fragments containing the polyamine tail due to the presence of the guanidinium group. Edeine F has a nearly identical fragmentation to edeine B due to the structural variation (β phenylalanine to β-tyrosine) occurring on the portion of the molecule that is part of the neutral losses in each fragment, i.e. all fragments contain the polyamine portion of the molecule as denoted by the fragmentation flags pointing to the right (Fig. 2a). Using an additional zwitterionic-hydrophilic interaction (ZIC-HILIC) chromatography matrix, that has been shown to separate aminoglycoside antibiotics, the amount of edeine A, B, and F in NRS-1210 spent medium could be estimated at 0.3 μg/ml, 6.0 μg/ml, and 0.4 μg/ml, respectively (Fig. 2d), based on a UV absorbance molar response curve of tyramine.

Fractionation studies of spent medium using reverse-phase C18 and ultrafiltration demonstrated that all fractions containing the edeine signals showed inhibition in the liquid culture bioassay. The use of C18 was not pursued due to the poor retention of the edeine components; however, others have reported successfully purifying edeines using reverse-phase separations (Westman et al. 2013). Therefore, media was fractionated by ultrafiltration (1000 Da molecular weight cutoff) into < 1000 Da fraction and > 1000 Da fractions. The low molecular weight fraction (< 1000 Da) from ultrafiltration contained all antifungal activity, therefore it was used for the cation exchange enrichment. To confirm that edeine was the active component of the media, cationic components were enriched using a weak cation exchange resin. The sample was fractionated using a step gradient of 0, 50 mM, 250 mM, and 500 mM ammonium hydroxide. Samples were repeatedly lyophilized to remove the ammonium, followed by resuspension and dilution to 1%, 5%, and 25% original media equivalents to test inhibition using the bioassay on fungal conidia. Nearly all of the antifungal activity was found in the 250 mM ammonium hydroxide fraction from the WCX column (Fig. 3a). Subsequent ^{1}H NMR analysis revealed that the major component of the 250 mM ammonium hydroxide fraction was edeine (Fig. 3b). ^{1}H NMR signals correspond to the assignments previously reported (Westman et al. 2013) with no extraneous peaks, with the exception of acetate from the preconditioning of the WCX column, demonstrating that the weak cation exchange fraction was primarily composed of edeines. Analysis of concentrated WCX fractions by ZIC-HILIC-LC-UV-MS had values consistent with the expected concentrated edeine A, B, and F values, indicating that the fractions contained the bulk of the edeine from the media.

Edeine producers in the *Brevibacillus* clade

Genome mining of all available *Brevibacillus* genomes shows the edeine biosynthetic clusters exists in a monophyletic clade of *Brevibacillus* species (Fig. 4). The monophyletic nature of the group suggests the cluster was inherited from a common ancestor. The results for the negative control, *B. parabrevis* NRS-780, are consistent with it not being an edeine producer.

Discussion

Several *Brevibacillus* species produce antibiotic compounds, including peptides, that the organism uses to compete against other neighbors in their ecological niche. Ethylparaben, a small phenolic compound produced by a *B. brevis* strain, inhibited the growth of seven species of bacteria and fungi (Jianmei et al. 2015). *Brevibacillus* produces a variety of linear and cyclic peptides using ribosomes and non-ribosomal peptide synthetases (Yang and Yousef 2018). Most of the peptides, such as gramicidin, tyrocidine, brevibacillin, and laterosporulin damage the cytoplasmic membrane, but edeine's mode of action is different than most *Brevibacillus* peptides. Edeine, a cationic, linear peptide produced by a peptide synthetase, was originally identified in *B. brevis* Vm4 (now *Brevibacillus schisleri*) several decades ago (Kurylo-Borowska 1959). At a concentration of 15 μg ml^{-1}, edeines slow bacteria growth and inhibit DNA synthesis (Kurylo-Borowska and Szer 1972). Higher concentrations of edeine (> 150 μg ml^{-1}) result in bacteria toxicity; edeines are universal inhibitors of translation, preventing the binding of fMet-tRNA to the P site of 30S ribosomes (Cozzarelli 1977; Dinos et al. 2004). Acetylation of edeine prevents the host from inhibiting its own translation machinery (Westman et al. 2013).

Secreted edeines produced by *B. fortis* NRS-1210 (medium diluted 50%) prevented or reduced

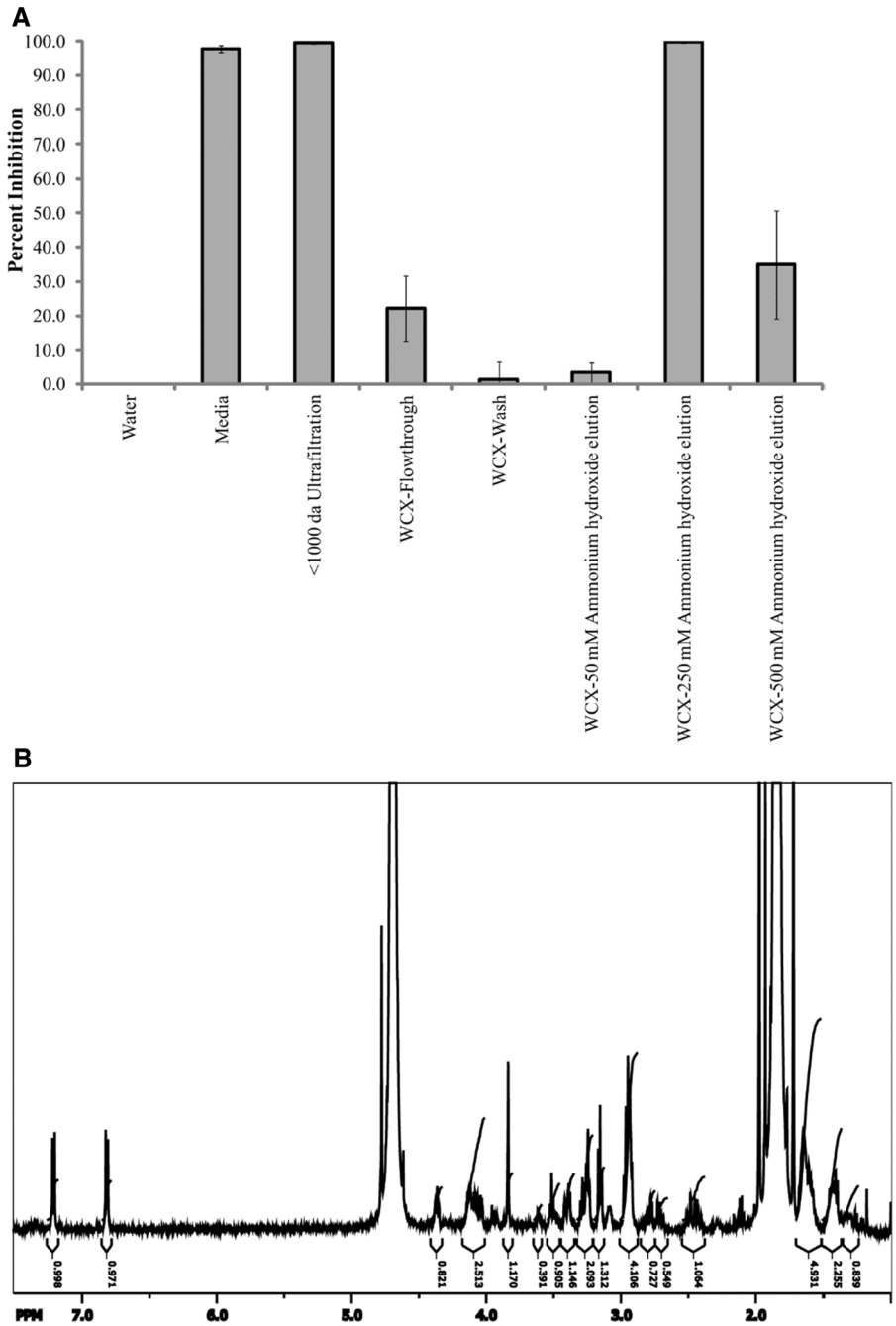

Fig. 3 **a** Liquid culture fungal conidia inhibition bioassay of weak cation exchange enrichment of bioactive ultrafiltration fractions expressed as percent inhibition of 5 percent medium equivalent. Each column represents the average percent inhibition of triplicate separations; standard deviations are shown at the top of the columns. **b** ^{1}H NMR of 250 mM ammonium hydroxide fraction containing edeines. Values under each peak correspond to the integrated area of the peak

additional growth of germinated FOC conidia within 7 h (Table 1). FOC conidia growth was not inhibited by spent growth medium of *B. parabrevis* NRS-780. The majority of screened *Brevibacillus* strains from the NRRL Culture Collection did not produce secreted compounds or did not produce sufficient quantities of

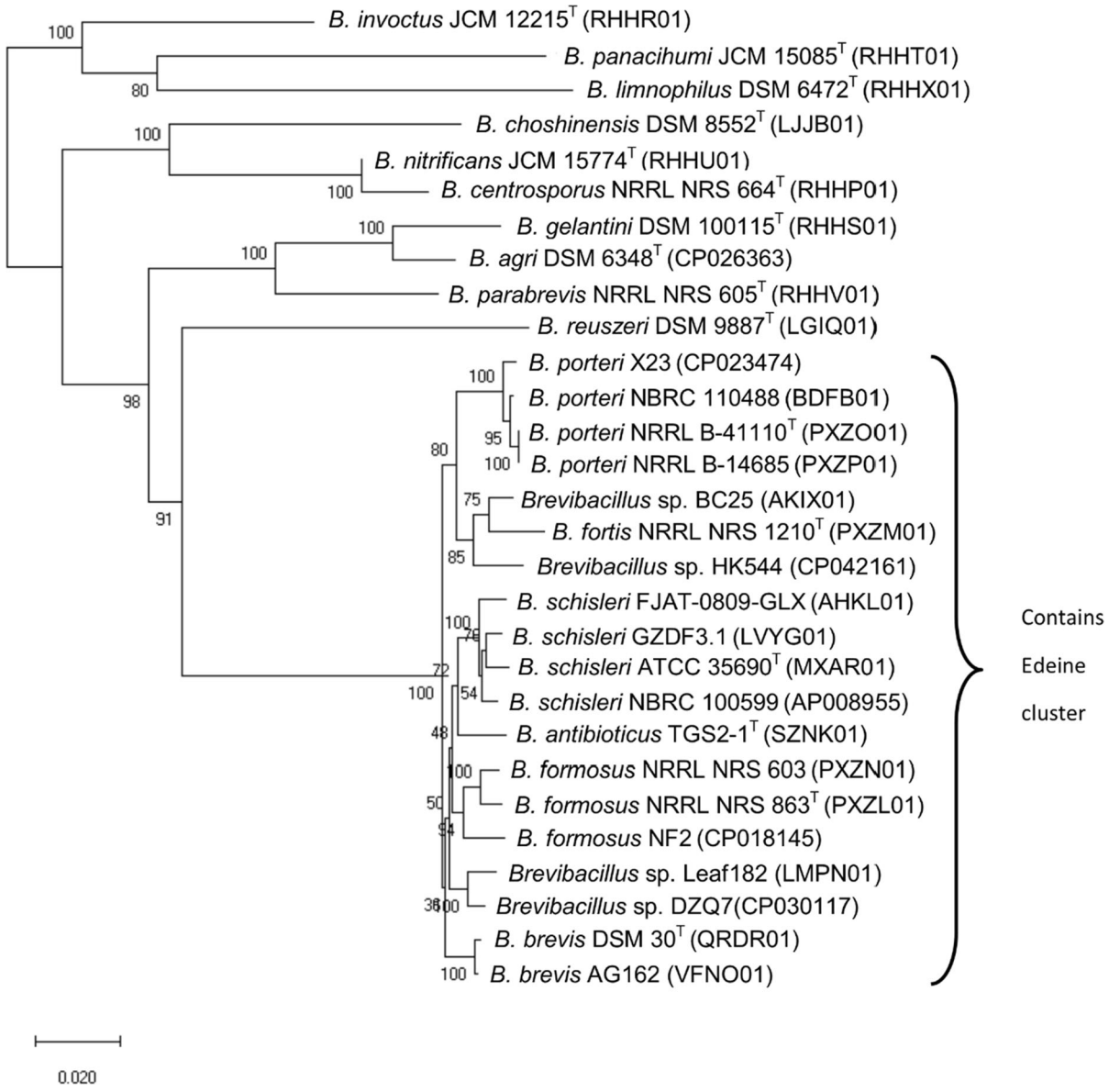

Fig. 4 Neighbor-joining phylogeny showing strains containing the edeine biosynthetic cluster across the *Brevibacillus* clade. The tree was reconstructed from a 6 gene MLSA previously described (Rooney et al. 2009). Bootstrap values > 70%, based on 1000 pseudoreplicates are indicated on branch points. The tree was rooted based on previous core genome phylogenies of the *Brevibacillus* genus (Johnson and Dunlap 2019). The scale bar corresponds to 0.02 nucleotide substitutions per site

secreted compounds that inhibited growth of FOC. In addition to arresting growth, the edeines in the spent growth medium were effective at killing ungerminated FOC conidia within 11.5 h (Table 2).

In the initial experiments, the antifungal activity was demonstrated with *B. fortis* NRS-1210 spent medium diluted by 50%. Serial dilution of the *B. fortis* NRS-1210 medium determined its relative antifungal activity (Table 3). FOC conidia germination and growth was completely inhibited using 1.3–3.1% of spent medium of *B. fortis* NRS-1210. Growth of a mixture of FOC conidia and chlamydospores was completely stopped by 3.1% of *B. fortis* NRS-1210 spent medium (Table 3). These results indicate that edeines can traverse the thickened walls of FOC

chlamydospores, which are the most difficult of FOC propagules to eliminate from soil (Brayford 1996).

Similar results were found with other *Fusarium* species, as well as *G. citri-aurantii*, a yeast that causes sour rot of citrus fruit (McKay et al. 2012). However, the growth of other fungi, *R. stolonifera* and *C. gloeosporioides*, was not affected by the spent medium of *B. fortis* NRS-1210. The differential activity of the spent medium among the fungi tested suggests the composition of the cell membrane plays an important role in susceptibility to edeines. The presence or absence of proteins, lipids or carbohydrates in the cell membrane could determine how much of the edeines are transported through the membrane. For example, *Candida albicans* was found to be eight times more sensitive to edeine B compared to *Aspergillus niger* (Czajgucki et al. 2006). Alternatively, it is possible that concentrating spent *B. fortis* NRS-1210 medium would result in greater inhibitory activity against *R. stolonifera* and *C. gloeosporioides* since edeine is considered a universal translation inhibitor.

The antifungal activity of the edeines in the culture medium was not affected by 50 °C treatment, diminished by 75 °C treatment, and completely abolished by 100 °C treatment (Table 4). Components in the spent culture medium may have protected the edeines from degradation at 50 and 75 °C. Alternatively, edeines could be inherently thermostable. Optimal edeine hydrolysis required treatment with 6N HCl for 36 h at 108 °C (Roncari et al. 1966). Edeines are also resistant to many proteolytic enzymes such as trypsin, chymotrypsin, pepsin and carboxypeptidase A (Hettinger and Craig 1970). These properties indicate that edeines are well suited to endure in harsh environments, such as soil. Our experiments utilising sand supplemented with cornmeal indicated that *B. fortis* NRS-1210 spent medium could not control the growth of FOC chlamydospores and conidia in this matrix. It is possible that edeines in the medium were too dilute, or became attached to sand or cornmeal particles, and were unavailable for entry into the FOC propagules. These results indicate that development of liquid formulations of edeines for soil drenching would need to be at higher levels than produced in the cultures of this study.

Our genome mining results show the edeine biosynthetic cluster is found in a monophyletic clade of *Brevibacillus* species. These results suggest the cluster was inherited from a shared ancestor and is at least partially responsible for the divergence of these species from the greater *Brevibacillus* genus. However, to our knowledge, there are few reported observations of edeine production by these bacteria. *Brevibacillus schisleri* ATCC 35690^{T} (previously reported as *Brevibacillus brevis* Vm4 (Johnson and Dunlap 2019) was the first strain reported to produce edeine (Kurylo-Borowska 1959) and was used extensively as a source of edeines for decades. *B. brevis* TT02-8 (Shimotohno et al. 1994) and *B. fortis* (this study) are the only other isolates known to secrete edeines into growth medium. It would be worthwhile to investigate edeine biosynthesis levels among the other strains containing the edeine biosynthetic cluster and what environmental conditions induce edeine production. These results are also consistent with recent studies in Bacillales showing the importance of secondary metabolites in the divergence and speciation of bacteria in this order (Dunlap et al. 2019, 2020).

The edeines are toxic to animals (Czajgucki et al. 2007) and inhibit protein synthesis in wheat germ (Hunter et al. 1977). Therefore, edeines are useful in studies of ribosome function but are unlikely to be utilised as antibiotics for pests of plants, humans or animals due to their toxicity. Edeines might be useful for killing soilborne pathogens of crops provided that the residual edeine in the soil could be removed or deactivated to levels that will not impact subsequently sown crops. Levels of atrazine, a toxic herbicide, were lowered in fields by adding killed recombinant *Escherichia coli* cells engineered to overproduce atrazine chlorohyrolase, which dechlorinates atrazine to non-toxic hydroxyatrazine (Strong et al. 2000). Levels of edeines in soil could be reduced in a similar manner with killed recombinant *E. coli* overproducing the edeine-modifying enzyme EdeQ, which inactivates edeines by *N*-acylation (Westman et al. 2013). Future studies will need to focus on the development of liquid formulations with enhanced levels of edeines for soil drenching.

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Author contributions Study conception: EJ. All three authors performed research, analyzed data, and contributed to the writing of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest in the manuscript.

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