

Genome annotation and antimicrobial properties of *Bacillus toyonensis* VU-DES13, isolated from the *Folsomia candida* gut

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Abstract

Antibiotic resistance necessitates the search for new bioactive compounds with novel mechanisms of action. Natural products derived from bacteria and fungi are widely used in the field of medicine and new environments can be explored as sources of antimicrobials. Bacteria associated with springtails have shown high inhibitory activity against pathogens. Here, we characterized a bacterial strain with high potential for antimicrobial activity, isolated from the gut of the springtail *Folsomia candida* Willem (Collembola: Isotomidae). The strain was characterized using the ‘analytical profile index’ and the ‘minimal inhibitory concentration’ assay to test for antibiotic resistance. Agar overlay and agar disk diffusion assays were used to test the inhibitory activity of the strain and its extract against a variety of pathogens, and reporter assays were used to investigate the mode of action. High-performance liquid chromatography was used to analyze and fractionate the extract of bacterial culture, followed by additional assays on the fractions. The genome of the strain was screened for presence of antibiotic resistance genes and secondary metabolite gene clusters. The isolate was identified as *Bacillus toyonensis* Jiménez et al., but it displayed differences in metabolic profile when compared to the type species. The isolate was highly resistant to penicillin and inhibited the growth of a variety of pathogenic microorganisms. Genome analysis revealed an enrichment of resistance genes for β -lactam antibiotics compared to the type isolate. Also, secondary metabolite clusters involved in the production of siderophores, bacteriocins, and nonribosomal peptide synthetases were identified. In conclusion, a unique *Bacillus* strain was isolated from the gut of *F. candida*, for which we provide evidence of inhibitory activity against an array of pathogens. This, coupled with high resistance to penicillin as substantiated by the presence of resistance genes, points to the potential of *B. toyonensis* VU-DES13 to provide a new source of antimicrobial compounds.

Introduction

Symbiotic microorganisms have a variety of functions in their arthropod host. They play an important role in

nutrition, by providing essential nutrients and helping with digestion (Akman Gündüz & Douglas, 2009), and they contribute to protection from pathogens through multiple mechanisms, for example, by competitive exclusion and the production of defense compounds (Kaltenpoth & Engl, 2014). Various antibiotics have been isolated from insect symbionts (Fredenhagen et al., 1987;

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Flórez et al., 2017). These and other molecules with antimicrobial activity have potential applications in the food and in the pharmaceutical industry.

Recently, we demonstrated that several bacteria isolated from the gut of the springtail *Folsomia candida* Willem (Collembola: Isotomidae) display antagonistic potential against pathogenic microorganisms (Agamennone et al., 2018) and we hypothesized that this activity results from the production of antimicrobial compounds. Here, we apply specific bioassays to a *Bacillus* species isolated from the gut of *F. candida*, to guide the discovery of new bioactive compounds. *Folsomia candida* is an established model in ecotoxicology and ecogenomics, and it is known to be resistant to and feed on entomopathogens (Broza et al., 2001). Its genome contains secondary metabolite biosynthetic gene clusters, such as for β -lactam compounds (Roelofs et al., 2013), which are specifically induced in the gut epithelium upon adverse conditions (Nota et al., 2008). In the past, amplicon-sequencing studies revealed a prominent presence of *Bacillus* sp. in the microbial community of this springtail (Czarnetzki & Tebbe, 2004; Agamennone et al., 2015).

Bacteria of the *Bacillus* group and their products are used in a variety of applications: as biopesticides and biofertilizers in agricultural practice (Pérez-García et al., 2011), as probiotics for both animals and humans (Lodemann et al., 2008; Cutting, 2011), and as a source of antibacterial and antifungal compounds (Gebhardt et al., 2002; Raaijmakers et al., 2010). Furthermore, they have been evaluated as biocontrol agent to reduce healthcare-associated infections (Vandini et al., 2014). Such an application relies on the colonization of non-pathogenic probiotic *Bacillus* strains on hard surfaces of hospitals, counteracting the proliferation of pathogenic strains.

Bacillus cereus Frankland & Frankland (sensu lato) is a group of Gram-positive, spore-forming, facultative anaerobic bacteria that display a wide ecological diversity and variety of lifestyles. Members of this group, such as *B. toyonensis*, are commonly found in soils, where they live as saprophytes (Vilain et al., 2006). They are also known to establish symbioses with plants and soil insects, and act as pathogens in various animal hosts (Swiecicka, 2008; Ceupens et al., 2013). It has been suggested that all members of the *B. cereus* group go through a life cycle during which they establish a symbiotic relationship with an appropriate invertebrate host (Jensen et al., 2003). Filamentous spore-forming bacteria belonging to the *B. cereus* cluster were found in the guts of soil-dwelling arthropods, and their life cycle was described as possibly symbiotic (Margulis et al., 1998). *Bacillus* spores in the soil are ingested by arthropods and when present in their guts, a moist and nutrient-rich environment, they germinate. There, they grow in their

filamentous form and attach to the intestinal epithelium. Cells are then released from the distal end of the filaments and return to the soil with the animal's feces (Margulis et al., 1998).

In this study we present the *B. toyonensis* strain VU-DES13 isolated from the gut of the soil-dwelling springtail *F. candida*. This bacterium was selected among others isolated from the springtail because of its high activity against pathogens (Agamennone et al., 2018). We provide the initial description of this isolate, which belongs to the *B. cereus* clade, focusing on antibiotic resistance and antimicrobial production. The potential for the production of inhibitory substances was assessed using growth inhibition assays targeted against a variety of pathogenic microorganisms. Reporter assays were then applied to elucidate mechanisms of action. We also describe the genome of this strain, with an emphasis on the presence of secondary metabolite biosynthetic gene clusters that could represent a genetic basis for antibiotic activity. Finally, we discuss the ecological relevance of our findings in the context of resistance of the springtail *F. candida* against colonization by entomopathogenic fungi.

Materials and methods

Isolation procedure

Folsomia candida was reared in plastic boxes with a bottom layer consisting of plaster of Paris. The animals were kept in a climate room under stable conditions (20 °C, 75% r.h., and L12:D12 photoperiod), feeding on dried baker's yeast (Dr. Oetker, Bielefeld, Germany). Guts were dissected from 10 adult springtails and crushed in sterile phosphate buffer saline using a plastic pestle. Ten-fold dilutions of the extract were prepared and 100 μ l of each dilution was spread on nutrient agar [NA; 15.0 g peptone, 3.0 g yeast extract, 6.0 g NaCl, 1.0 g D(+)-glucose, 15.0 g agar]. Plates were incubated at 30 °C and bacterial growth was checked regularly. Colonies with different morphologies were transferred to fresh plates and kept at 4 °C (Agamennone et al., 2018).

Phenotypic characterization with the API system

Two analytical profile index (API) identification kits – the API 20E (first 12 tests) and the API 50CH (bioMérieux, Marcy-l'Étoile, France) – were used to determine the physiological profile of *Bacillus* VU-DES13. The method is based on the detection of metabolization of substrates by the microorganisms, revealed by a color change due to the precipitation or enzymatic conversion. Alternatively fermentation is detected by a drop in pH. The biochemical panel of the API 20E test measures the metabolism of

various carbohydrates and amino acids and detects the presence of specific enzymes, such as gelatinase and β -galactosidase. The API 50CH test provides a detailed carbohydrate fermentation profile.

Bacteria were grown overnight on NA and harvested in 2 ml normal saline (0.85% NaCl, wt/vol). Two suspensions were then prepared, one in normal saline for the API 20E test, and one in API 50 CHB/E medium for the API 50CH test. The wells of the strips were inoculated with 120 μ l of microbial dilution. The test was conducted in duplicate. The strips were incubated at 34 °C for 48 h and checked at 24 and 48 h. The last eight tests of the API20E strips were not used, as the pH indicator is not suitable for measuring the fermentation of carbohydrates by *Bacillus* (Logan & Berkeley, 1981).

Antibiotic resistance

We tested the susceptibility of *Bacillus* VU-DES13 to penicillin by using the broth microdilution method described by Wiegand et al. (2008) to determine the minimal inhibitory concentration (MIC). Bacteria were grown overnight in nutrient broth (NB) (Sigma-Aldrich, St. Louis, MO, USA). The optical density (OD) of liquid bacterial cultures at 600 nm was measured on a Smart-Spec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). The cultures were then diluted to $OD_{600} = 0.1$, corresponding approximately to 5×10^6 colony-forming units (CFU) ml^{-1} (Biesta-Peters et al., 2010) to perform the test. We tested a range of 10 penicillin G (Sigma-Aldrich) concentrations, each increasing approximately two-fold from 1.5 up to 800 μ g ml^{-1} . *Micrococcus luteus* (Schroeter) Cohn, a gram-positive bacterium highly sensitive to β -lactam antibiotics, was used as a positive control. The test was performed in a 96-well microtiter plate. For each row of the plate, 10 wells were used to test the different concentrations of antibiotic, one well was used to test bacterial growth and one well was used to control for contamination. To minimize evaporation during the incubation, the test was conducted in a volume of 200 μ l per well. The test wells were inoculated with 100 μ l antibiotic dilution + 100 μ l bacterial culture, the growth control with 100 μ l bacterial culture + 100 μ l NB, and the sterility control wells with 100 μ l NB. The plate was incubated overnight at 30 °C in a THERMOstar microplate incubator (BMG Labtech, Ortenberg, Germany) and OD was measured the next day on a Spark 10M microplate reader (Tecan, Männedorf, Switzerland).

Pathogen inhibition

Bacillus VU-DES13 was initially screened for inhibitory activity against 10 pathogens: the bacteria *Staphylococcus*

aureus Rosenbach (Firmicutes), *Escherichia coli* (Migula) Castellani & Chalmers (Proteobacteria), and *Bacillus subtilis* (Ehrenberg) Cohn (Firmicutes), and the fungi *Rhizoctonia solani* JG Kühn (Basidiomycota), *Fusarium solani* (Mart.) Sacc., *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen, and *Beauveria bassiana* (Bals.-Criv.) Vuill. (all Ascomycota), and *Pythium ultimum* Trow, *Saprolegnia diclina* Humphrey, and *Phytophthora capsici* Leonian (all Oomycota). The strains and growing conditions for each of these microorganisms are specified in Table S1.

To test *Bacillus* VU-DES13 against the bacteria, we used the agar overlay method. The agar plug method was used to test antimicrobial activity against fungi and oomycetes. For the agar overlay method, the isolate was grown overnight in tryptic soy broth (Sigma-Aldrich), at 30 °C with shaking at 400 r.p.m. Subsequently, 5 μ l of the liquid culture of each isolate was transferred to solid medium. *Pseudomonas protegens* Ramette et al. Pf5, a broad-range biocontrol model strain, was used as a positive control on each plate. The plates were incubated overnight at 28 °C. On the same day, liquid cultures of the bacterial pathogens were prepared by inoculating colonies in 4 ml of LB broth base (Lennox L Broth Base; Thermo Fisher Scientific, Bleiswijk, The Netherlands) and growing them overnight at 30 °C with shaking at 400 r.p.m. The following day, growth of VU-DES13 on the agar plates was observed, and the pathogens grown during the night were prepared for the overlay. A total of 100 μ l of liquid culture of the pathogen was inoculated in 2 ml top agar, mixed well by vortexing, and poured over the plate to completely cover colonies of VU-DES13. The plates were then incubated overnight at the appropriate temperature for the pathogens (Table S1) and the next day the presence of inhibition zones was recorded. For the agar plug method, agar plugs covered in mycelium of the pathogens were placed on the plates between VU-DES13 colonies, after growth on the plates. The plates were sealed with paraffin film (Parafilm; Bemis, Neenah, WI, USA) and incubated at the appropriate temperature for the pathogens (Table S1) until growth allowed the observation of clear inhibition zones. For both the agar overlay and the agar plug method, the screening was conducted on three media: 1/10 tryptic soy agar (1/10 TSA), potato dextrose agar (PDA), and NA (Figure S2).

Pathogen inhibition by culture extracts

Solid phase extraction. *Bacillus* VU-DES13 was grown overnight in 10 ml NB at 30 °C with 225 r.p.m. shaking. Two-milliliter of the culture was inoculated in 250 ml potato dextrose broth (PDB) or NB and incubated for 3 days with shaking at 150–200 r.p.m. The culture was then centrifuged for 20 min at 3024 g and the supernatant was extracted by solid phase extraction (SPE). In order to

capture and purify a broad range of secondary metabolites with unknown chemistry, the ABN Evolute Express columns (Biotage AB, Uppsala, Sweden) for acidic, basic, and neutral analytes were used. To that end, 6 ml column volume with 200 mg of the above mentioned sorbent mass were used to extract *Bacillus* VU-DES13 cultures grown in PDB and NB media. Columns with 500 mg sorbent mass were used in bioassay-guided screening. Before applying the samples, the columns were conditioned with 6 ml methanol and equilibrated with 6 ml water. Afterwards, the columns were washed with 6 ml of water and finally eluted with 6 ml of methanol to collect the analytes. The extracts obtained with SPE from liquid *Bacillus* VU-DES13 cultures were used for (1) agar diffusion assays, (2) high-performance liquid chromatography (HPLC) analysis, (3) reporter assays, and (4) HPLC fractionation for bioassay-guided screening. For both the HPLC analysis and the reporter assays, 3 ml of the extract resulting from SPE was used. These volumes were completely evaporated in autosampler point vials using two nitrogen evaporators (Dionex ASE 500 and Pierce Reacti-Therm III & Reacti-Vap III; Thermo Fisher Scientific). The sample to be used for HPLC was dissolved in 1 ml methanol, and the one used for the reporter assays was dissolved in 50 μ l dimethyl sulfoxide (DMSO).

Inhibition from SPE extracts. Agar diffusion assays were performed to investigate the antimicrobial properties of extracts of *Bacillus* VU-DES13 grown in NB and PDB for 3 days. The assays were performed against the bacteria *E. coli*, *M. luteus*, *Pseudomonas syringae* van Hall, *B. subtilis*, and *S. aureus*, and the yeast *Candida albicans* (CP Robin) Berkhout (Table S1). The assays were performed in triplicate with slight modification of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Disk Diffusion Manual v.5.0, January 2015). Liquid cultures of the pathogens in LB were diluted to McFarland standard 0.5, with the exception of cultures of *C. albicans* that were diluted to McFarland standard 1.0, and the dilutions were used to evenly seed plates of Müller-Hinton (MH) agar with a sterile cotton swab. The plates were rimmed and left to dry for a few minutes, then wells were made in the agar using a sterile cork borer. Fifty-microliters of *Bacillus* VU-DES13 extract, medium only extract, and 30 μ g of ketoconazole or kanamycine as a positive control for *C. albicans* or bacteria, respectively, were applied to the wells. The plates were then incubated at the appropriate temperature for the pathogen (Table S1). After one or more days, inhibition zones were recorded.

Bacterial reporter strain construction

The reporter constructs in bacteria were made by cloning the respective promoters, and in some cases the associated transcription factor, at the *Bam*HI/*Xho*I restriction sites of the medium copy number plasmid pCS26Pac (Bjarnason et al., 2003; Table 1). The target fragments were amplified with PCR primers (Table 1) which contained the appropriate restriction sites in the 5'-end, by making use of the Phusion High Fidelity polymerase (cat. nr. M0530L; New England BioLabs, Ipswich, MA, USA). All cloning steps were performed in *E. coli* DH5 α . The redox-cycling reporter plasmid pPHZlux-1 was constructed by amplifying the monooxygenase PA14_35160 promoter and the *soxR* gene from the *P. aeruginosa* PA14 genome with primers 1 and 2. This monooxygenase promoter was described in Dietrich et al. (2006) as part of a phenazine signaling pathway in the *P. aeruginosa* quorum sensing system. This reporter plasmid was used in the bioassays with *E. coli* DH5 α as a host. The cell wall stress-inducible pBLAlux-2 was based on the *ampC* (PF_4054) promoter of *P. protegens* Pf-5 and included also the divergently oriented *ampR* regulator gene (PF_4053). It was amplified with primers 3 and 4 and cloned in pCS26Pac to result in pBLAlux-1. Because of the higher transcriptional induction in an *E. coli* Δ *ampD* genetic background (Langae et al., 2000), which was previously created by a kanamycin resistance-bearing transposon cassette by Baba et al. (2006), an alternative antibiotic resistance gene was needed on the reporter plasmid. Therefore, a different backbone was amplified from a chloramphenicol resistance-containing plasmid, pBAD33 (Guzman et al., 1995) with the primers 5 and 6, containing a 5'-tail with *Pac*I restriction sites. This PCR fragment was subsequently ligated to the transcriptional regulatory unit and *luxCDABE* operon digested from pBLAlux-1 with *Pac*I.

The pSOSlux-2 plasmid of the DNA damage reporter was created by introducing the colicin D promoter, subcloned from the pJAMA8-cda plasmid (Tecon et al., 2010) with primers 7 and 8 in the pCS26Pac plasmid by using ClonTech In-Fusion HD Cloning Kit (cat. nr. 639650; Clontech Laboratories, Mountain View, CA, USA). Because of the need for a wild-type *recA* gene in the genetic background, for the SOS response to take place, the wild-type MG1655 was used as host strain. Finally, a non-specific toxicity reporter was made by using a high-level constitutive promoter (nr. 20 from Braatsch et al., 2008), which was made by annealing the complementary oligopeptides 9 and 10 at 1 pmole μ l⁻¹ in annealing buffer (10 mM Tris/HCl, pH = 7.5, 1 mM EDTA and 50 mM NaCl) following 5 min of boiling, gradual cooling to room temperature and subsequent ligation in *Bam*HI/*Xho*I

Table 1 Phenotypic characteristics of *Bacillus* VU-DES13, compared to closely related strains in the *Bacillus* genus, as revealed by selected tests from the API 20E and API 50CH (carbohydrate fermentation) systems

	<i>B. toyonensis</i> VU-DES13	<i>B. toyonensis</i> BCT-7112 ^T	<i>B. cereus</i> (CECT 148)	<i>B. thuringiensis</i> (CECT 197)	<i>B. anthracis</i>
<i>API 50CH</i>					
Anaerobic growth	+	+	+	+	–
L-Arginine dihydrolase	–	+	+	+	+
Citrate utilization	–	+	+	+	–
Acetoin production	–	+	+	+	+
Gelatinase	+	+	+	+	?
<i>API 20E</i>					
Glycerol	±	–	–	–	–
D-Ribose	±	+	+	±	+
D-Mannose	–	–	–	+	–
Methyl- α -D-glucopyranoside	–	+	–	–	–
Amygdalin	±	±	±	–	–
Arbutin	+	+	+	+	?
Salicin	+	+	+	+	–
D-Cellobiose	±	–	+	+	–
D-Saccharose	–	+	+	+	+
D-Trehalose	+	+	+	+	+
Starch	+	+	+	+	+
Glycogen	+	+	+	+	+
D-Turanose	–	+	–	–	–

± indicates a weak positive result. ? indicates that different strains gave different results. Data obtained from Jiménez et al. (2013b).

linearized pCS26Pac. PBPlux-2 was created by subcloning the *PacI* restriction fragment with the transcriptional reporter unit of pBPLux-1 in pBAD33, analogous to the procedure of pBLAlux-2 construction. All bacterial strains and plasmids used for bioreporter construction are listed in Table 2.

Bacterial luciferase reporter assays for antimicrobial mode of action

The glycerol batches of the bacterial reporter strains were diluted 20-fold in MOPS-buffered minimal medium (8.5 mM NaCl, 18.7 mM NH₄Cl, 47 mM MOPS, 0.3 mM Na₂HPO₄·2H₂O, 0.3 mM KH₂PO₄, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.2% glucose, pH = 7) and exposed at 1% vol/vol DMSO in a final volume of 150 µl in white polystyrene 96-wells plates (Greiner Bio-One, Kremsmünster, Austria). For the assay, the evaporated extracts were dissolved in DMSO, and for application in the exposure assays dilution series were made. DMSO is an amphipathic exposure vehicle for efficient uptake by cells from the medium in bioassays. The exposures of pBLAlux-2 (cell wall stress), pPHZlux-1 (redox cycling), and pSOSlux-2 (DNA damage) were incubated at 37 °C at 400 r.p.m. for 6, 1, and 1 h, respectively. In every plate, a standard curve of a reference compound dissolved in DMSO was taken along (penicillin G, pyocyanine, or mitomycin D, respectively). After

the exposure, the autonomous luminescence was measured in every well for 4 s with a TriStar luminometer (Berthold, Vilvoorde, Belgium). Samples with luminescence levels above the detection limit, i.e., average (RLU_{blank}) + 3*SD (RLU_{blank}) (RLU, relative luminescence unit; SD, standard deviation), were normalized for the cytotoxicity as measured by reduction of luminescence by the control reporter measurements with the same plasmid backbone (pBPlux-1, or -2), analogous to Leedjäv et al. (2006).

HPLC analysis of SPE extract

The total eluate volume of 6 ml resulting from the SPE of *Bacillus* VU-DES13 grown in PDB was used to perform HPLC fractionation (1260 Infinity Binary LC System; Agilent Technologies, Amstelveen, The Netherlands). The extract was evaporated to dryness and dissolved in 1 ml methanol. Ten-microliters of the extract was injected for UV diode array detector (DAD) HPLC fractionation along with the extract of a PDB blank. This extract was separated on a Phenomenex Kinetex 2.6u Biphenyl 100 Å 150 × 4.6 mm column and eluted with a 3–30% gradient of acetonitrile in water. During the complete time of elution a steady flowrate of 1.5 ml per min was used at a maximum pressure of 600 bar and the absorbance in the 200–900 nm spectral range was recorded by DAD.

Table 2 Growth inhibition of target pathogens by *Bacillus* VU-DES13

	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Pythium ultimum</i>	<i>Phytophthora capsici</i>	<i>Saprolegnia diclina</i>	<i>Fusarium oxysporum</i>	<i>Fusarium solani</i>	<i>Rhizoctonia solani</i>	<i>Beauveria bassiana</i>
1/10 TSA	–	–	–	–	x	++	++	+/++	++	–
PDA	–	–	–	+	+	–	–	–	–/+++	+
NA	–	–	–	x	x	–	–	–	x	+

TSA, tryptic soy agar; PDA, potato dextrose agar; NA, nutrient agar; x, no growth of pathogen; –, no inhibition; +, growth reduction; ++, <5 mm inhibition; +++, >5 mm inhibition.

Bioassay-guided fractionation of SPE extract

Half of the 6 ml SPE extract from a 3-day culture in PDB was dissolved in 1 ml of methanol. From this, 100 µl aliquots were injected in a similar HPLC setup as described above, but adapted to make use of a semi-prep HPLC column of the same chemistry, i.e., a Kinetex 5u Biphenyl 250 × 10 mm column. The adjusted flow speed conditions were 5 ml per min and 100 µl injection (of consecutive samples). Fractions of the extract were collected during the time of elution in windows of 1 min using a Teledyne ISCO Foxy 200 (Lincoln, NE, USA) fraction collector for further investigation of their antimicrobial properties and mode of action. The absorbance in the 200–900 nm spectral range was recorded by DAD. The fractions were dried under vacuum (Genevac Rocket Evaporator, Ipswich, UK) and subsequently dissolved in 100 µl DMSO. They were then used for reporter assays (conducted at 1% exposure volume of 10-fold diluted fractions) and agar well diffusion assays (conducted with 50 µl of undiluted fractions).

De novo genome shotgun sequencing, assembly, and annotation

Genomic DNA sequencing was performed on an Ion Torrent PGM, making use of the Ion PGM Hi-Q sequencing kit (ThermoFisher Scientific, Bleiswijk, The Netherlands). Genome assembly was performed using Spades (Bankevich et al., 2012). All details on sequencing and assembly are described in Janssens et al. (2017). The genome was annotated automatically using the Prokka pipeline (Seemann, 2014), extended with the analysis for carbohydrate active enzymes, CAZY (Lombard et al., 2014), and for secondary metabolism biosynthetic gene clusters, anti-SMASH (v.3.0.5) (Weber et al., 2015). Additionally, BAGEL3 (van Heel et al., 2013) was applied in order to confirm the bacteriocin clusters. The tRNA genes were predicted using the package ARAGORN (Laslett & Canback, 2004).

Blastn was applied to screen for similarities with the plasmids and associated virulence genes from *Bacillus anthracis* Cohn (plasmid pXO1 with associated genes *cya*, *lef pagA*, and *repX*, and plasmid pXO2 with *capA*, *capB*, *capC*, *capD*, *capE*, and *repS*). The assembly was also checked for similarities to virulence plasmids from *Bacillus thuringiensis* Berliner pAW63 and pBT9727 (Van der Auwera et al., 2005). Delta-endotoxins, related to the Bt-toxin, were searched by checking the Pfam family output from the Prokka pipeline for the following families: PF03945, PF00555, PF09131, PF03944, PF05431, PF01338, PF12495, and PF03538 (Finn et al., 2016).

Antibiotic resistance genes were detected by applying hidden Markov models (HMMs) scanning of the predicted amino acid sequences against the curated Res-fams v.1.2 database of protein families and ontologies

associated with verified antibiotic resistances. The core database was trained with CARD (Comprehensive Antibiotic Resistance Database), LacED (Lactamase Engineering Database), and Jacoby's and Bush's collection, whereas the full database was supplemented with verified accessory functions from protein families that contribute to the resistance (Gibson et al., 2015). In the case of multiple hits, the hit with the lowest E-value was reported. For the detection of plasmids, the coverage of mapped reads on the respective contigs, as mapped by the 'bwa mem' algorithm (Li, 2013), and as implemented in Unipro UGENE (Golosova et al., 2014) was calculated. The PlasmidFinder v.1.3 tool (Carattoli et al., 2014) was deployed in order to find replication elements of Gram-positive plasmids. In order to retrieve prophage signatures from the genomes of VU-DES13 and BCT-7112, the PHASTER tool was used (Arndt et al., 2016). Fasta files of the respective genomes were uploaded via a web request of the PHASTER API.

Phylogenetic analysis

Two methods of whole-genome phylogeny were conducted. First, the novel genome was compared to other *Bacillus* genomes using feature frequency profiling (FFP), a method based on counting the features of a particular length that occur in a genome (Sims et al., 2009; Wang & Ash, 2015). The frequency of features was determined for the novel strain and for 30 additional strains representative of the phylogenetic clusters of the *B. cereus* clade (Zwick et al., 2012; Liu et al., 2016), taking *B. subtilis* strain 168 as an outgroup. Prior to the calculation of the distance matrix, the optimal feature length was determined in the newly sequenced genome. One thousand replicated neighbor-joining trees were constructed, based on a Jensen-Shannon divergence distance matrix calculated from the normalized feature frequency profiles. Subsequently, a consensus tree was made with PHYLIP (Felsenstein, 2002) and plotted with FigTree v.1.4.1 (Rambaut, 2014). The second approach used for whole-genome phylogenetic positioning was the Genome BLAST Distance Phylogeny, which allows to perform digital DNA:DNA hybridization (dDDH) (Meier-Kolthoff et al., 2013) to obtain whole-genome sequence similarity scores. This approach was implemented using a webtool (available on <http://ggdc.dsmz.de/>) and according to the recommendations by the authors. The method uses a species threshold level of 70% dDDH, and is robust for the analysis of incomplete genome sequences.

Comparative genomics

The genome rearrangements, synteny, and gene content were compared with the type strain *B. toyonensis* BCT-

7112^T (Jiménez et al., 2013a,b), by making use of the progressiveMauve alignment algorithm in Mauve (Darling et al., 2010). Differential genes compared to the two other *B. toyonensis* genomes were detected by performing a comparison with Panseq (Laing et al., 2010). The respective gene ontology (GO) terms associated with these protein coding genes were compared and summarized with the redundancy reducing and semantic analysis offered by Revigo (Supek et al., 2011).

Results

Description and phenotypic characterization of the isolate

The genus *Bacillus* comprises Gram-positive, aerobic endospore-forming rods. *Bacillus* VU-DES13 cells have rod-shape, observe singularly, in pairs or in filaments, and produce endospores. They also grew in anaerobic conditions. Colonies appeared flat, milky-white, and slightly granulated (Figure S1). We used API 20E and API 50CH systems to characterize the phenotype of *Bacillus* VU-DES13. The API system is an efficient method to discriminate *Bacillus* based on their physiological profile (Logan & Berkeley, 1984). The phenotypic characters of *Bacillus* VU-DES13, in comparison with *B. toyonensis* BCT-7112, *B. cereus* CECT-148, *B. thuringiensis* CECT-197, and *B. anthracis* (as reported by Jiménez et al., 2013a,b), indicate that *Bacillus* VU-DES13 has a physiological profile distinct from closely related *Bacillus* species, including the previously described *B. toyonensis* BCT-7112^T (Table 1). Relative to the other *B. cereus* clade species in the table, *Bacillus* VU-DES13 was unable to ferment saccharose, and relative to the type strain *B. toyonensis* BCT-7112^T it was unable to ferment methyl- α -D-glucopyranoside and D-turanose in the API 50CH test. The API 20E test indicated that *Bacillus* VU-DES13 cannot utilize citrate as substrate nor produce acetoin. Furthermore, *Bacillus* VU-DES13 does not show L-arginine dehydro-lase activity when compared to the other *Bacillus* strains.

Antibiotic resistance

Genome annotation and β -lactam ELISA analysis suggest that the *F. candida* host is able to synthesize a penicillin-like antibiotic (Suring et al., 2017). Thus, colonization of *F. candida*'s gut by *Bacillus* VU-DES13 will only be successful, if the strain can withstand β -lactam (penicillin-like) antibiotic exposure from the host. Previous studies provided initial evidence that most *Bacillus* strains or species are resistant to the antibiotic penicillin, except for *B. anthracis* (Turnbull et al., 2004). The broth microdilution method to determine the minimum inhibitory concentration of penicillin indicates that *Bacillus* VU-DES13 is indeed not susceptible to penicillin, even at the highest concentration tested (Figure 1). Bacterial cultures of

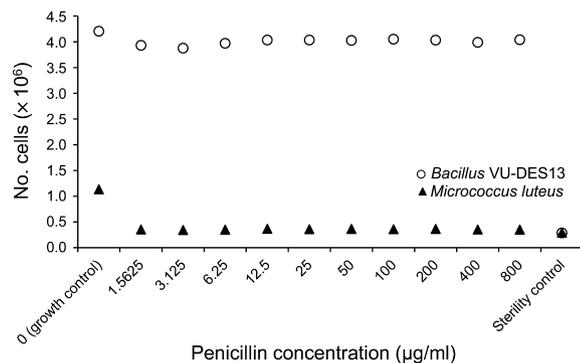


Figure 1 Cell density of *Bacillus VU-DES13* (open circles) and *Micrococcus luteus* (triangles) cultures grown overnight at 30 °C at increasing concentrations of penicillin. The cell density was inferred from optical density (OD) measurements at 600 nm (assuming that OD₆₀₀ = 0.1 corresponds to 5 × 10⁶ cfu ml⁻¹). The starting OD₆₀₀ for both bacterial cultures was 0.1. The sterility control contained only broth.

Bacillus VU-DES13 did not show any significant difference in OD₆₀₀ growth between the control conditions (mean ± SD = 0.84 ± 0.02) and the exceptionally high exposure level of 800 µg ml⁻¹ of antibiotic (0.81 ± 0.02). In contrast, *M. luteus* growth was inhibited by penicillin already at the lowest concentration. The OD₆₀₀ bacterial cultures of *M. luteus* grown in control conditions (0.23 ± 0.02) significantly differed from bacterial cultures grown in any of the concentrations of penicillin.

Pathogen inhibition by isolate

We tested the ability of *Bacillus VU-DES13* to inhibit the growth of several microbial pathogens. The growth inhibition assay indicates that *Bacillus VU-DES13* did not inhibit bacterial pathogens, but it inhibited both oomycetes and fungi, with the strongest effect observed against fungi

under poor media conditions (1/10 TSA and PDA plates; Table 2). An exception to this was constituted by *B. bassiana*, which was inhibited by *Bacillus VU-DES13* also on NA (Figure S2A). On 1/10 TSA, *Bacillus VU-DES13* induced a kind of fragmentation/sporulation of *B. bassiana* (Figure S2B). On PDA, the growth of *Bacillus VU-DES13* was retarded in the presence of *B. bassiana* (Figure S2C), whereas the growth of *Bacillus* was normal in the absence of the fungus.

Pathogen inhibition by SPE extracts

We also exposed *Bacillus VU-DES13* PDB and NB extracts to six non-filamentous pathogens: the bacteria *E. coli*, *M. luteus*, *P. syringae*, *B. subtilis*, and *S. aureus*, and the yeast *C. albicans*. The PDB extract inhibited the growth of *C. albicans* and *M. luteus*, and the NB extract inhibited the growth of all tested pathogens, except *E. coli* (Table 3, Figure S3). Although extracts from growth in NB were effective against a wider variety of pathogens, the inhibitory effect from PDB extract were much stronger and more specific. Therefore, we decided to assess the mode of action of the PDB extract using reporter assays.

Reporter assay: mode of action

The strongest inhibitory effect of *Bacillus VU-DES13* was observed with the extract grown for 3 days in PDB. We used the bacterial reporters pPHZlux-1 (DH5α), pSO-Slux-2 (MG1655), and pBLAlux-2 Δ*ampD* to detect redox cycling activity, DNA damage (SOS response), and cell wall stress, respectively, as these are common mode of actions of antimicrobials. Furthermore, the general toxicity of the samples was monitored by using the extract to expose the reporter and pBPlux-2 Δ*ampD*.

The signals of all redox cycling and DNA damage assays were below the detection limit relative to the DMSO blank, indicating that these two stress responses

Table 3 Results of Müller-Hinton (MH) agar diffusion assays performed with extracts of *Bacillus VU-DES13* grown in potato dextrose broth (PDB) and nutrient broth (NB) medium against various pathogens. Ketoconazole (30 µg) was used as a positive control against *Candida albicans*, and kanamycin (30 µg) against the other pathogens. The values indicate the mean (± SD; n = 3) size of the inhibition zones (cm diameter). ‘–’ indicates that no inhibition zone was observed

Pathogen	PDB		NB	
	<i>Bacillus VU-DES13</i> extract	Positive control	<i>Bacillus VU-DES13</i> extract	Positive control
<i>Candida albicans</i> BSMY 212 (DSMZ 10697)	2.3 ± 0.1	3.6 ± 0.1	0.9 ± 0.3	3.5 ± 0.3
<i>Escherichia coli</i> WA321 (DSMZ 4509)	–	2.9 ± 0.1	–	2.6 ± 0.1
<i>Staphylococcus aureus</i> 533R4 (DSMZ 20231)	–	2.2 ± 0.1	1.3 ± 0.1	2.4 ± 0.1
<i>Pseudomonas syringae</i> DC3000	–	3.0 ± 0.1	1.6 ± 0.4	3.0 ± 0.1
<i>Micrococcus luteus</i> (strain ATCC 49732)	2.2 ± 0.1	3.8 ± 0.2	1.3 ± 0.2	3.8 ± 0.1
<i>Bacillus subtilis</i> strain 168	–	3.2 ± 0.1	1.0 ± 0.3	3.2 ± 0.1

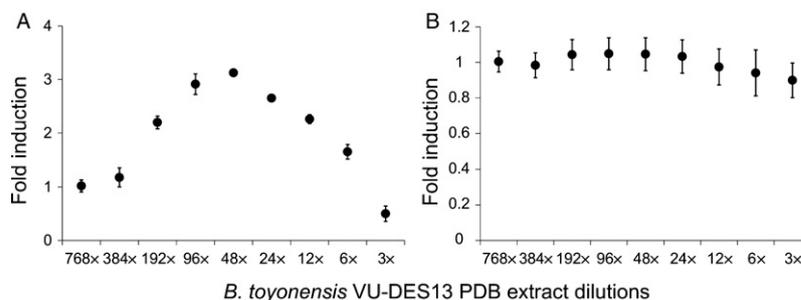


Figure 2 Mean (\pm SD; $n = 3$) fold induction [relative to dimethyl sulfoxide (DMSO) blank] of the (A) pBLAlux-2 $\Delta ampD$ and (B) pBPlux-2 $\Delta ampD$ bacterial luciferase reporters exposed to dilutions of *Bacillus* VU-DES PDB extract. The calibration curve for induction of the pBLAlux-2 $\Delta ampD$ by the reference compound (penicillin G) is given in Figure S4.

were not induced by the VU-DES13 PDB extract. In contrast, the pBLAlux-2 $\Delta ampD$ reporter assay revealed that the *Bacillus* VU-DES13 PDB extract induces cell wall stress. The signal indicating cell wall stress increased with further dilutions of *Bacillus* VU-DES13 PDB extract (Figure 2A), and was highest at dilution factor 48, where reporter activity increased three-fold relative to the DMSO control. Subsequent increasing dilutions produced weaker signals, which could not be explained by general toxicity (Figure 2B). Moreover, the general stress reporter induction also did not indicate toxicity of less diluted extracts (Figure 2B). The absolute luminescence signal from the 48-fold diluted extract was 27% of the maximum induction by penicillin G, used as positive control for the performance of the assay (compare Figure 2A to Figure S4).

HPLC analysis and bioassay-guided fractionation

Gradient HPLC was used to separate the PDB extracts of *Bacillus* VU-DES13. Absorption peaks were detected at various wavelengths. Differential absorption peaks between the bacterial extract and the sterile medium were evident at 250 nm. In order to effectively separate the *Bacillus* VU-DES13 extracts and collect the fractions, a Kinetex 5u Biphenyl 250 \times 10 mm semi-prep column was used with an adjusted flow rate. This changed the peak profile compared to the analytical column. Fractions were collected at 1-min intervals and were used to perform agar well diffusion assays to test inhibition of *C. albicans* (Figure 3A) and to study cell wall stress-induced bioactivity and general toxicity by using the pBLAlux-2 ($\Delta ampD$) and pBPlux-2 ($\Delta ampD$) reporter assays (Figure 3B). For the reporter assays, 10-fold dilutions of each of the fractions were used.

Fourteen out of the 26 fractions inhibited the growth of *C. albicans* with various intensities, with fraction 21 showing the strongest inhibitory effect (Figure 3A). This

fraction also clearly induced cell wall stress in the *E. coli* reporter assays (Figure 3B), suggesting that fraction 21 comprises a dual function in potential pathogen defense: induction of bacterial cell wall stress detected by reporter pBLAlux-2 $\Delta ampD$, and inhibition of the pathogenic fungus *C. albicans* (most probably via a different, but yet unknown mechanism).

Genome assembly and annotation

After assembly of the raw reads with SPADES and post-assembly treatment by MeGAMerge, a draft genome was made available for *Bacillus* VU-DES13 (Janssens et al., 2017). The total genome size was estimated to be 5.45 Mbp, corresponding to 40 contigs, with an N50 of more than 25 kbp and a GC content of 35%. A detailed description of assembly and initial annotation is published (Janssens et al., 2017). According to the distribution of the coverage of the mapped reads on the contigs, no evidence for the presence of a plasmid was found. Moreover, the PlasmidFinder tool failed to indicate any plasmid elements in our dataset. Here, we report a phylogenetic analysis with related *B. cereus* and *B. toyonensis* strains, as well as a comparative analysis of VU-DES13 with the related *B. toyonensis* type strain.

Phylogenetic analysis

Feature frequency profiling is based on the frequency of sequence measures across different genomes (Wang & Ash, 2015). The optimal feature length, i.e., the length which accounts for the highest number of different features, was estimated to be 11 bp in the newly sequenced genome. This method was applied to genomes representative of every cluster of the *B. cereus* clade and other *B. toyonensis* isolates, resulting in a divergence distance matrix. This matrix was then used to produce 1 000 bootstrapping trees, on which a consensus tree was based, rooted using *B. subtilis* 168. The second method to perform a whole-

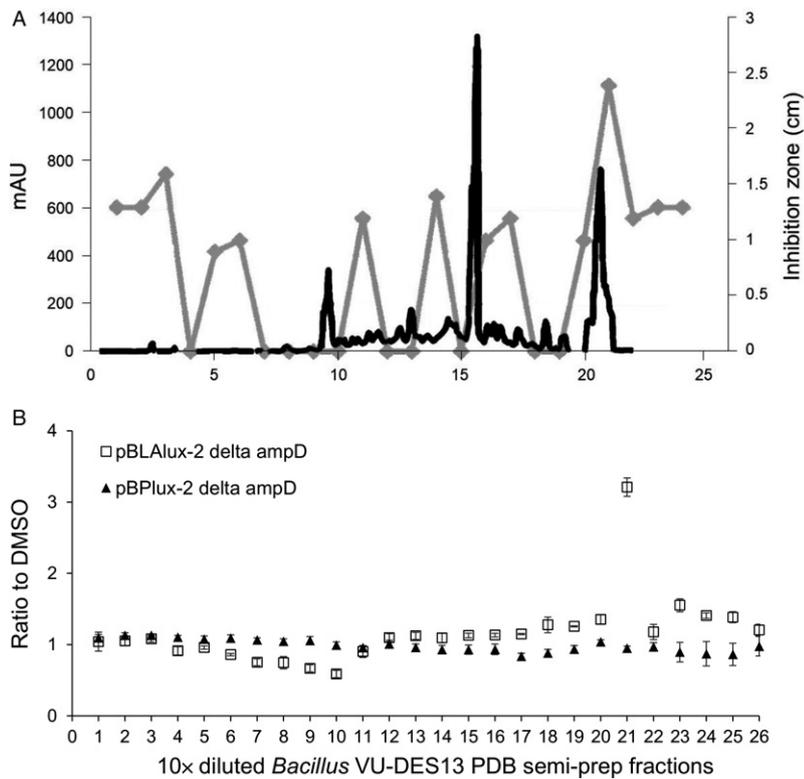


Figure 3 Fraction analysis for the 25 *Bacillus* VU-DES13 1-min fractions represented on the x-axis: (A) UV-VIS DAD chromatogram at 250 nm absorbance of the *Bacillus* VU-DES13 PDB extract separated on the Kinetex 5u Biphenyl 250 × 10 mm HPLC column (milli-absorbance units, mAU; black line), and inhibition of *Candida albicans*, as indicated by the size of inhibition zones (cm diameter; gray line), and (B) induction [expressed as mean (\pm SD; $n = 3$, vertical bars) ratio of the dimethyl sulfoxide (DMSO) blank] of the pBLAlux-2 $\Delta ampD$ reporter (squares) and the pBPux-2 $\Delta ampD$ reporter (triangles).

genome phylogeny was based on digital DNA-DNA hybridization (dDDH) measures, which were performed on the same genomes as used for the FFP method. The FFP method positioned *Bacillus* VU-DES13 in between the other *B. toyonensis* strains (BCT-7112^T and BAG60-1), and dDDH values higher than 70% on the whole genome alignment supported this result, indicating that *Bacillus* VU-DES13, BCT-7112^T, and BAG60-1 belong to the same species (Figure 4).

Comparative genomics to type strain

Initial analysis by the progressiveMauve aligner, allowed us to confirm syntenic regions between the two genomes of *Bacillus* VU-DES13 and BCT-7112^T (data not shown). First, the length of the consensus of both genomes differs with proximally 400 kbp, suggesting substantial genome sequence divergence. Also, *Bacillus* VU-DES13 lacks the two plasmids (pBCT8 and pBCT77) that were identified in BCT-7112^T. Subsequent analysis by Panseq revealed additional differential stretches of DNA between the strains. The length of these stretches amounted to ca. 672 kbp (159 fragments) for *Bacillus* VU-DES13 and ca. 260 kbp (144 fragments) for BCT-7112^T. These fragments contained 690 and 336 ORFs, respectively. By comparing the annotations and GO terms associated with biological processes for these fragments, we interpreted the putative

biological differences between both isolates. In *Bacillus* VU-DES13, the processes encoded by the differential gene content are related to oxidation-reduction, transport of metabolites across membranes, and spore germination. Pathogenesis factors and biosynthetic gene clusters were also detected, as well as genes for recombination and integration mechanisms and signal transduction proteins. In BCT-7112^T, the most prominent biological processes represented in the differential gene content are DNA recombination, phosphorelay transduction systems, metabolism, and gluconeogenesis, as well as spore germination. Comparative analysis to identify prophages revealed that the VU-DES13 genome contained one intact and one incomplete prophage in contig 18. In contrast, three incomplete prophage regions were identified in the BCT-7112 genome (see supplementary file SI_PHASTER), which could not be linked to any regions in the VU-DES13 genome. This confirms that both strains are phylogenetically related, but have diverged substantially.

Virulence-related genes

The well-known human pathogen *B. anthracis* confers its virulence by the presence of the virulence plasmids pXO1 and pXO2, essential for pathogenicity. Related plasmids pAW65 and pBT9727 have also been detected in *B. thuringiensis*. Sequences of these plasmids were used to

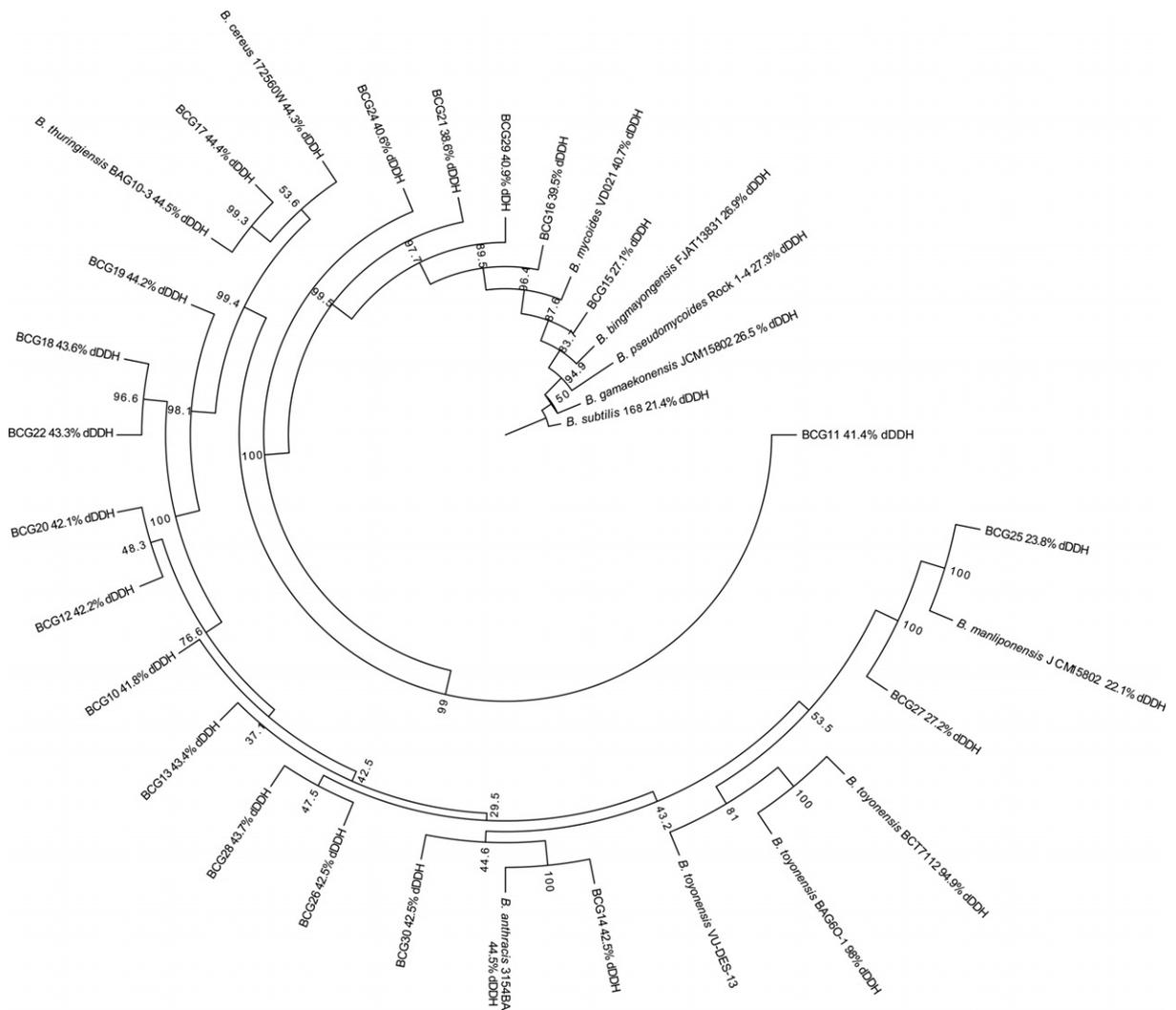


Figure 4 Consensus bootstrap (n = 1000) neighbor-joining (NJ) tree based on the feature frequency profiles of 11 bp among representative genomes from the respective *Bacillus cereus* clades (Zwick et al., 2012; Liu et al., 2015). The tip labels contain the % dDDH as calculated on the whole genome alignment of the respective accessions with *B. toyonensis* VU-DES13. *Bacillus anthracis* 3154, NZ_ANFF01000000; *B. mycooides* VD021, NZ_AHES01000000; *B. cereus* 172560W, NZ_CM000717.1; *B. thuringiensis* BAG10-3, NZ_AHCP01000000; *Bacillus pseudomycooides* Rock 1-4, NZ_CM000743.1; *Bacillus gaemokensis* Jung et al. JCM15801, JOTM00000000; *Bacillus manliponensis* Jung et al. JCM15802, JOTN00000000; *Bacillus cytotoxicus* Guinebretière et al. NVH 391-98, NC_009674.1; *B. toyonensis* BCT-7112^T, NC_022781.1; BAG6O-1, GCF_000293525.1; BCG 10, NC_003909.8; BCG 11, NZ_CM000740.1; BCG 12, NC_011658.1; BCG 13, NZ_AHDA01000000; BCG 14, NZ_CM000721.1; BCG 15, NZ_AHFC01000000; BCG 16, NZ_AHDJ01000000; BCG 17, NC_011772.1; BCG 18, NZ_AHFW01000000; BCG 19, NZ_AHCZ01000000; BCG 20, NC_017200.1; BCG 21, NZ_AHEX01000000; BCG 22, NZ_AHDL01000000; *Bacillus bingmayongensis* Liu et al. FJAT-13831, AKCS01000000; BCG 24, NZ_AHDP01000000; BCG 25, ARSX01000000.; BCG 26, NZ_CM000720.1; BCG 27, NZ_CM000733.1; BCG 28, NZ_CM000718.1; BCG 29, NZ_AHEA01000000; BCG 30, NZ_CM000739.1; *B. subtilis* subsp. *subtilis* ATCC 6051, NC_000964.3.

query the *Bacillus* VU-DES13 genome using blastn. None of these plasmids showed similarity to sequences in the *Bacillus* VU-DES13 genome. Some relatively short blastn hits pointed to similarities with the backbone sequences, mobile elements, or encoding non-virulence genes. This

was supported by the tblastx analysis of the anthrax-related virulence factors, which pointed to a short stretch of homology to the *capA* capsular biosynthesis gene, but failed to indicate that anthrax-related virulence genes were present in the assembly. This was also confirmed by Pfam

Table 4 Summary of the Resfam analysis on the occurrence of antibiotic resistance genes in *Bacillus* VU-DES13 (details in Table S3)

Antibiotic mechanism	<i>Bacillus</i> VU-DES-13	<i>B. toyonensis</i> BCT-7112 ^T (Khatri et al., 2016)
ABC transporter	124	123
Acetyltransferase	124	113
Aminotransferase	36	30
Beta-lactamase	54	41
Chloramphenicol resistance	1	4
D-ala ligase	10	8
Gene modulating resistance	43	72
Glycopeptide resistance	12	106
Methyltransferase	36	41
MFS transporter	73	73
Other	24	28
Other efflux	9	17
Quinolone resistance	0	4
RND antibiotic efflux	4	4
rRNA methyltransferase	0	2
Stress response	3	3
Target redundancy/ overexpression	21	15
Tetracycline MFS efflux	2	3
Tetracycline ribosomal protection	0	3
Total	576	693

family annotations related to various classes of Bt-toxin in the Prokka annotation output. Finally, the *B. cereus* toxin cereulide encoded and synthesized by a specific nonribosomal peptide synthetases (NRPS) was not detected in the antiSMASH analysis (see ‘Secondary metabolism’ below), although the annotation pointed to four hemolytic enterotoxin genes.

Antibiotic resistance

The predicted amino acid sequences of the assembled genome were analyzed for the occurrence of antibiotic resistance genes, by comparing them to the 166 HMMs of the full Resfam database (Table 4; all details in Table S3). Resfam analysis indicates that the *Bacillus* VU-DES13 isolate is resistant to β -lactams, tetracyclin, chloramphenicol, and various glycopeptide antibiotics. The total number of antibiotic resistance-related genes is slightly lower in *Bacillus* VU-DES13 compared to the type isolate BCT-7112^T, and it is shifted toward the resistance to β -lactam compounds (54 in *Bacillus* VU-DES13 compared to 41 in BCT-7112^T). Furthermore, the type isolate BCT-7112^T contains almost 10 \times more resistance genes against

glycopeptide antibiotics, and contains additional tetracycline and quinolone resistance genes.

Secondary metabolism

AntiSmash 3.0 identified 10 secondary metabolite-related clusters (Table 5; details in Table S4). Five gene clusters were involved in the production of bacteriocins (of which three were confirmed by the BAGEL3 package), including one lantipeptide and one lassopeptide. Furthermore, two NRPSs were identified, of which one cluster encodes the machinery for the biosynthesis of a siderophore, and one cluster is involved in the production of a terpene (Table 5). Four clusters showed similarity hits with the MIBiG (minimum information on biosynthetic gene clusters) database (Table S4). One of these clusters showed 83% similarity to a petrobactin (siderophore) and another showed 83% similarity to a thuricin (lantipeptide) biosynthetic gene cluster.

Discussion

In this study we have described a putatively important member of the *F. candida* microbiome, *B. toyonensis* VU-DES13. A previous study based on cloning and 16S rRNA gene sequencing isolated 95 clones from the springtail, 83 of which (87%) were identified as *Bacillus weihenstephaniensis* Lechner et al. (Czarnetzki & Tebbe, 2004). Agamennone et al. (2015) also indicated that members of the *B. cereus* group are among the dominant groups in *F. candida* microbiome, representing 4% of the reads in a natural population of springtails. However, the limited resolution of 16S rRNA in this clade did not allow for a more specific identification within this clade. Just like the life cycle stage-specific association of *B. anthracis* and *B. thuringiensis* with ungulates and insects, the data presented in this study as well as by Czarnetzki & Tebbe (2004) suggest that there might be an association of other *B. cereus* clade members with animals (Margulis et al., 1998; Jensen et al., 2003). *Bacillus cereus* is abundant in soils and has been associated with some cases of food poisoning, because of the rare occurrence of the emetic toxin cereulide (Altayar & Sutherland, 2006). To more precisely identify our *Bacillus* VU-DES13 isolate, we used the large-scale phylogeny on 224 complete genomes of *B. cereus* strains from Liu et al. (2015). Their results indicated the presence of 30 clusters within this clade. The role of horizontal gene transfer in the transfer of virulence genes and plasmids between these strains is significant, yet the presence of these sequences showed little correlation with the phylogenetic position within the clade (Liu et al., 2015). By using representative elements from this phylogeny, we demonstrate here that the genome sequence of *Bacillus* VU-

Table 5 Results of the antiSMASH analysis of the *Bacillus toyonensis* VU-DES-13 assembly, including information on the nature of the biosynthetic gene cluster (BGC) and the presence in the conspecific strains *B. toyonensis* BCT-7112^T and BAG6O-1 (details in Table S4)

Type	Most similar known cluster	Detected by BAGEL3	Present in BCT-7112 ^T	Present in BAG6O-1
Terpene	Molybdenum cofactor biosynthetic gene cluster (11% of genes show similarity)	No	Yes	Yes
Lasso peptide	–	Yes	Yes	Yes
Nrps	Bacillibactin biosynthetic gene cluster (38% of genes show similarity)	No	Yes	Yes
Bacteriocin	–	No	No	Yes
Nrps	–	No	Yes	Yes
Siderophore	Petrobactin biosynthetic gene cluster (83% of genes show similarity)	No	Yes	Yes
Other	–	No	Yes	Yes
Bacteriocin	–	Yes	Yes	Yes
Bacteriocin	–	No	No	Yes
Lantipeptide	Thuricin biosynthetic gene cluster (83% of genes show similarity)	Yes	No	No

DES13 clusters with the clade comprising *B. toyonensis* BCT-7112^T and BAG6O-1, two isolates originating from soil ecosystems. Interestingly, BCT-7112^T is currently used as a probiotic strain in the commercial formulation Toyocerin, a feed additive to improve feed efficiency and growth of livestock animals (Zwick et al., 2012). Another study reported high abundance of this species in the gut of fly larvae (Sánchez-Galván et al., 2017). The authors suggested that *B. toyonensis* is ingested with the breeding medium along with other inhabitants of the larval gut. They speculated that *B. toyonensis* may play a role in the digestion of organic material, although no data were presented to support this hypothesis (Sánchez-Galván et al., 2017).

Physiological profiling with API tests, and additional phenotyping, is a standardized way to describe new species or isolates of *Bacillus* (Logan & Berkeley, 1981, 1984). The diversity of patterns within the genus is significant (Logan & De Vos, 2009). Here, we also reveal substantial differences even within the same species, among *Bacillus* VU-DES13 and *B. toyonensis* type strain BCT-7112^T. This variation may be linked to different ecological niches occupied by the two strains, which is also reflected by underlying genetic differences. An earlier study already suggested that most of the variation between *Bacillus* genomes occurred in genes involved in niche adaptation, reflecting specializations that allow these species to occupy different habitats (Alcaraz et al., 2010). By comparing the genome content of our isolate with the type strain, we found additional differences in the biosynthetic capacity of secondary metabolites, the presence of β -lactamases, and factors related to virulence and pathogenesis. We found no deviations in prophage occurrence between the two strains, confirming

that the two strains are highly related (Cleary et al., 2016). Finally, no known genes related to pathogenesis to humans were detected.

We observed a high resistance of the isolate *Bacillus* VU-DES13 to penicillin. Usually, a cutoff value between 16 and 32 $\mu\text{g ml}^{-1}$ is used in published MIC tests. For instance, Turnbull et al. (2004) applied a cutoff of 32 $\mu\text{g ml}^{-1}$ to assess antibiotic resistance to assess resistance for an array of *Bacillus* strains. They provided evidence that only *B. anthracis* is sensitive to penicillin. Here we applied up to 800 $\mu\text{g ml}^{-1}$ penicillin, and showed that our isolate is still not affected by this antibiotic, which strengthens the observation by Turnbull et al. (2004) that *Bacillus* strains are not susceptible to penicillin, except *B. anthracis*. Interestingly, from the analysis by the Resfam databases it became apparent that *Bacillus* VU-DES13 contains 13 additional β -lactams when compared to the BCT-7112^T genome, which may explain its high resistance to the β -lactam penicillin. This would support the genotypic cutoff concept, linking the distribution of resistance alleles to MIC (Tyson et al., 2017). Antibiotic producers can evolve mechanisms to protect themselves from the compounds they are producing (Hopwood, 2007). A study in the US Geological Survey has unraveled very strong correlations between heavy metal concentrations in soil and the resistance of *Bacillus* isolates to penicillin (Watterson et al., 1984). Extreme resistance was observed, with up to 5 mg ml^{-1} of penicillin, and the isolates belonging to the *B. cereus* clade were among the most resistant (Watterson et al., 1984). *Bacillus* VU-DES13 was isolated from *F. candida*, that is producing this class of secondary metabolites (Suring et al., 2016). This suggests that the resistance may

be directed toward the β -lactam compound produced by the host (Roelofs et al., 2013), providing a mechanism to (re-)colonize *Folsomia* guts and evolve as commensal.

Bacillus VU-DES13 and its extract inhibited the growth of bacterial and fungal pathogens in agar inhibition assays, especially on poorer media. The different efficacy of inhibition on different media suggests that the activity of *Bacillus* VU-DES13 is dependent on growth conditions. The mechanism of pathogen inhibition by *Bacillus* isolates may be different from the underlying inhibition by the extract. Growth inhibition by live microorganisms can be due to a variety of mechanisms leading to competitive exclusion, not only the production of substances such as siderophores, signaling molecules, or antimicrobials, but also limited nutrients or space resulting from the competitor's use of these resources. Inhibition by a bacterial extract is most likely due to the presence of specific compounds, that can be identified and whose mode of action can be elucidated by using bioassays such as the ones applied in this study. We have no explanation for the observation that fraction 21 exerts cell wall stress, but can also inhibit growth of the fungal pathogen *C. albicans*. Antifungal compounds usually act on ergosterol biosynthesis rather than cell wall integrity (Müller et al., 2017). In any case, the broad antimicrobial activity of fraction 21 seems highly promising to discover highly potent antimicrobials. Therefore, we plan to analyze this fraction, by using MALDI-TOF to link specific peptides to the inhibitory activity. Such peptides could be further investigated for their capacity to inhibit fungal pathogen growth and cause cell wall stress.

The annotation of the genome of *Bacillus* VU-DES13 with the packages antiSMASH and BAGEL identified 10 biosynthetic gene clusters. Three of them revealed a hit with the MIBiG database and seven were already known from the sequence of the type strain. The ribosomal antimicrobial proteins and the NRPSs were reported in a previous publication (Zhao & Kuipers, 2016). The siderophore biosynthetic cluster 6 identified in our analysis showed similarity to petrobactin, an NRPS-independent siderophore that depends on catecholate condensation (Lee et al., 2007). Cluster 10 exhibited similarity to thuricin, a bacteriocin described from *B. thuringiensis* (Favret & Yousten, 1989). As mentioned earlier, the type strain BCT7112^T is used commercially as a probiotic for application in feed for cattle and pigs. A recent study has shed light on a possible role of this probiotic strain in the inhibition of quorum sensing, resulting in reduced biofilm formation by enterotoxigenic *E. coli* (González-Ortiz et al., 2016). At this point the nature and identity of the antimicrobial agents in *Bacillus* VU-DES13 and BCT7112^T remain elusive.

From earlier work on *F. candida* it is known that this species is resistant against bacterial and fungal entomopathogens, and is even able to use such pathogens as food source, leading to increased growth and reproduction. This feeding behavior reduces the viability of remaining conidia after passage through the *Folsomia* gut (Broza et al., 2001). In light of the high level of penicillin resistance observed here, the presence of biosynthetic gene clusters for putative β -lactam compounds and for other secondary metabolites in the host's genome (Roelofs et al., 2013; Suring et al., 2016), together with the induction of these genes in conditions of stress (Nota et al., 2008), we suggest that these genes affect the growth and metabolism of specific members of the gut bacterial community. Possibly, host-derived β -lactams could modulate colonization resistance of the microbiome against pathogens. Antibiotics are known to increase fitness of the producer in a community by inhibiting sensitive competitors, but they have also been shown to function as signaling molecules (Yim et al., 2007) within and between species. Moreover, inter-kingdom signaling has been shown to have outstanding ecological relevance. Previous reports showed that such signaling processes can be highly instrumental with regard to plant biocontrol and human gut health (Williams, 2007; Fischbach & Segre, 2016). Specific elicitors (environmental conditions and chemical triggers) have been described that can explain the highly conditional and specific expression of biosynthetic gene cluster for secondary metabolites (Niu et al., 2016; Urem et al., 2016). These findings imply a highly complex interplay between host, microbiome, and environment, taking into account many variables that we have only started to unravel. With the experimental and genomic data from this study we have provided some elements to begin to understand the role of this dominant member of *F. candida*'s gut microbiome in the colonization resistance against entomopathogens. The discovery of active fractions that may contain novel antimicrobials constitutes a first step toward the development of new antifungal or antibacterial compounds.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supporting file SI_PHASTER Supporting file SI_PHASTER contains the results of prophage signatures from the genomes of VU-DES13 and BCT-7112 by uploading the Fasta files of the respective genomes via a web request of the PHASTER API and subsequent analysis using the PHASTER tool (Arndt et al., 2016).

Figure S1 Microphotographs (microscopic photographs) of *Bacillus toyonensis* VU-DES13

Figure S2 Pictures showing *Bacillus toyonensis* VU-DES13 inhibiting the growth of *Beauveria bassiana* on (A) nutrient agar (NA), (B) causing sporulation of *B. bassiana* on 1/10 tryptic soy agar (TSA), and (C) being inhibited by the fungus on potato dextrose agar (PDA).

Figure S3 Müller-Hinton (MH) agar diffusion assays with extract of *Bacillus* VU-DES13 grown in nutrient broth (NB) medium. The extract inhibited the growth of (A) *Bacillus subtilis*, (B) *Micrococcus luteus*, (C) *Pseudomonas syringae*, and (D) *Staphylococcus aureus*.

Figure S4 Calibration curve for induction of the pBLA-lux-2 *ΔampD* by penicillin G

Table S1 Pathogens used in the inhibition assays with *Bacillus* VU-DES13 isolate and extract. NA, not applicable; LB, Lennox L broth base; PDA, potato dextrose agar; Müller-Hinton, MH.

Table S2 *Escherichia coli* reporters used in this study

Table S3 Output of the Resfam analysis on the occurrence of antibiotic resistance genes

Table S4 Output of the antiSMASH analysis of the *Bacillus toyonensis* VU-DES-13 assembly, giving the nature, the position, and the database hits with known biosynthetic gene cluster (BGC), and the presence in the conspecific strains BCT-7112^T and BAG6O-1. MIBiG, minimum information on biosynthetic gene clusters database.