Antibiotic and genetic profiling of field-collected and laboratory wild-type strains reveals seven novel Bacillus spizizenii strains that produce the lanthipeptide subtilin

Markus Helfrich
Goethe-Universitat Frankfurt am Main Fachbereich 15 Biowissenschaften
Karl-Dieter Entian
Goethe-Universitat Frankfurt am Main Fachbereich 15 Biowissenschaften
Torsten Helge Stein (Torsten.Stein@hs-aalen.de)
Aalen University of Applied Sciences: Hochschule Aalen https://orcid.org/0000-0001-5749-8215

Research Article

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Abstract

Two dozen field-collected *Bacillus* and a dozen laboratory *Bacillus spizizenii* wild-type strains were selected on the basis of their antagonistic properties against the Gram-positive strain *Micrococcus luteus*. Their genetic and antibiotic profiles were characterized (subtilin encoding *spaS* gene sequences, mass spectrometric and quantitative reversed phase rpHPLC analyses, as well as presence of the lanthionin cyclase protein SpaC by western blotting). We identified seven novel producer of the lanthipeptide subtilin. Phylogenetic analyses of the field collected (HS and N5) and laboratory (DSM 618, 1087, 6395, 6405, and 8439) subtilin producer strains on the basis of their 16S rDNA encoding *rrn* sequences showed that all seven strains can be classified to *B. spizizenii*. To the best of our knowledge, all *B. spizizenii* strains described so far are characterized by the fact that they can produce a lanthipeptide from the subtilin family. Furthermore, the potential of the analyzed strains with regard to the coproduction of the sactipeptide subtilosin and the lipopeptides surfactin and fengycin were determined.

Introduction

Gram-positive spore-forming bacteria of the genus *Bacillus* are among the most widespread bacteria worldwide; they can be found on the one hand in the soil, in fresh or sea water, as well as in the air (Ferrari et al. 1993; Moszer et al. 2002), on the other hand ind the gastrointestinal tract of ruminants and humans (Hong et al. 2005; Cutting 2011). Some *B. subtilis* strains share long history of safe use in fermented foods such as Nattō (*B. subtilis natto*, Japanese fermented soybeans) (Sun et al. 2016) and Doenjang (*B. subtilis* together with *Aspergillus oryzae*, Korean soybean paste Yue et al. 2021). For this reason, *B. subtilis* is generally classified as safe (GRAS). According to the currently accepted definition, probiotics are “live microorganisms which provide health benefits when consumed", for example by improving or restoring the gut flora (Hill et al. 2014). Very recently, it has been shown that lanthipeptide producing commensal strains of the human gastrointestinal tract reduce vancomycin-resistant *Enterococcus faecium* (VRE) colonization and represent potential probiotic agents (Kim et al. 2019). Lanthipeptides are gene-encoded small peptides (19-38 amino acids in length) that possess the unusual bridge-forming sulfur-containing amino acids meso-lanthionine and 3-methyl-lanthionine (Freund and Jung 1992; Stein 2005; Chatterjee et al. 2005; Arnison 2013; Letzel et al. 2014).

B. subtilis as well as very closely related *B.spizizenii* strains are able to produce more than two dozen antibiotics with an amazing variety of structures (Stein 2005). Peptide antibiotics represent the predominant class, among them lanthipeptides and non-ribosomal biosynthesized lipopeptide antibiotics from the surfactin, iturin, fengycin-class (Stein 2005; Zhao et al. 2018; Caulier et al. 2019). One of the one of the earliest described lanthipeptides is subtilin, a 32-amino-acid peptideproduced by a *Bacillus* strain (see Fig. 1a for a structure representation) that has been originally described 1944 (Jansen and Hirschmann 1944). The subtilin producing strain was originally isolated 1911-1912 by Karl Kellerman (Kellerman et al. 1912) and deposited as *B. subtilis* strain No. 6633 into the American Type Culture Collection (ATCC) by the “Bureau of Plant Industry, Soils, and Agricultural Engineering of the United States Department of Agriculture” (Garibaldi and Feeney 1949). Furthermore, several *B. subtilis* wild-type strains

produce the macrocyclic sactipeptide subtilosin A (Fig. 1b; Zheng et al. 1999; Stein et al. 2004) with a series of unusual intramolecular thioether linkages (Marx et al. 2001; Kawulka et al. 2003; Stein 2020). The lipoheptapeptide lactone surfactin (Fig. 1c) is a powerful surfactant with potentantimicrobial activities of surfactin arebased on its detergent-like action on biological membranes (Heerklotz und Seelig 2001; Carrillo et al. 2003). Surfactin is distinguished by its exceptional emulsifying, foaming, antiviral, antitumor, antimycoplasma, and hypocholesterolemic activities (Peypoux et al. 1999; Kaspar et al. 2019).

The aim of this study was to analyze the potential of *Bacillus* strains to produce the lanthipeptide subtilin, the sactipeptide subtilosin, and the lipopeptide surfactin. Both, laboratory and field collected *Bacillus* strains were taxonomically classified by gene sequencing (SSU 16SrRNA encoding *rrn* and subtilin encoding *spaS* alleles). The antibiotic profiles were characterized under conditions optimal for the production of lanthi- and sactipeptides; MALDI mass spectrometry and reversed phase rpHPLC were used for qualitative and quantitative verification of the produced peptides. Furthermore, the presence of subtilin modifying enzymes (SpaC, the subtilin cyclase, for example) was verified by immunoblotting.

Materials And Methods

Bacterial strains and media

The *Bacillus* strains (for a complete list see Table 1) are from culture collections ATCC (American Type Culture Collection) and DSMZ (German Collection of Microorganisms and Cell Cultures; strain collections for microorganisms and links to them can be found in the Tab. S1, Supplement) or natural isolates (see below). For antibiotic production liquid Landy medium cultures were routinely grown aerobically at 37°C (Landy et al. 1948) supplemented with 0.5% yeast extract (Heinzmann et al. 2006; Stein 2020). Solid media contained TY agar (0.8% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) or Luria-Bertani (LB) agar (the commercial supplyer of the media components was Gibco, Neu-Isenburg, Germany), standard incubation conditions were overnight (15-20 h) at 37°C.

Table 1
B. subtilis trains used in this study and their profiles in subtilin (Sub), subtilosin (Sbo), and surfactin (Srf) production

***Bacillus* strain**	**Synonyms[a], properties**	**Sub[b,c,d,e]**	**Sbo[b,c,e]**	**Srf[e]**	**Reference**
spizizenii					
ATCC 6633	DSM 347, NRS 231 (safe), IAM 1069, $Sub^+/Sbo^+/Srf^+$ - reference strain	+	+	+	Garibaldi and Feeney (1946) Heinzmann et al. (2006)
DSM 618	Test strain for the detection of antibiotics in meat	+	+	+	this work
DSM 1087	W23	+	+	+	this work
DSM 6395	W23 2A2	+	+	+	this work
DSM 6405	mutant of W23 SR	+	+	+	this work
DSM 8439^T	W23, IAM 12021	+	+	+	this work
N5	soil isolate	+	+	+	this work
HS	soil isolate	+	+	+	this work
subtilis					
168[f,g]	DSM 402, ATCC 23857 Sub^- / Sbo^+ / Srf^- - reference	-	+	-	Stein et al. (2004)
DSM 10^T	ATCC 6051^T, NCIB 3610	-	+	+	this work; Fuchs et al. (2011)
IP	soil isolate	-	+	+	this work

[a] description an links to the strain collections are given in the Supplement (Tab. S1); [b] PCR, gene sequencing; [c] rp-HPLC

[d] Western blotting (SpaC/SpaB protein); [e] MALDI-TOF MS; [f] genome sequence (NC_000964.3; Kunst et al. 1997)

[g] *B. subtilis* 168 can be converted to a surfactin producer after introduction of the *sfp* gene (Lambalot et al. 1996)

Bacillus strain	Synonyms[a], properties	Sub[b,c,d,e]	Sbo[b,c,e]	Srf[e]	Reference
DSM 2109	ATCC 11774, NCTC 8236	-	+	+	this work
others					
DSM 2109	ATCC 11774, NCTC 8236	-	+	+	this work
DSM 3256	IAM 1213	-	+	+	this work
DSM 3258	IAM 1260	-	-	+	this work
A1/3	ericin A/S	(+)	-	+	(Stein et al. 2002; Hofemeister et al. 2004)
HI-1	soil isolate	-	-	+	this work
DSM 15029	TU-B-10, entianin	(+)	+	+	Fuchs et al. (2011)
DSM 1088	*B. natto*	-	+	+	Stein et al. (2004)
DSM 2277	*B. atrophaeus* ATCC 51189, NCTC 10073	-	+	+	Fritze and Pukall (2001) Stein et al. (2004)

[a] description an links to the strain collections are given in the Supplement (Tab. S1); [b] PCR, gene sequencing; [c] rp-HPLC

[d] Western blotting (SpaC/SpaB protein); [e] MALDI-TOF MS; [f] genome sequence (NC_000964.3; Kunst et al. 1997)

[g] *B. subtilis* 168 can be converted to a surfactin producer after introduction of the *sfp* gene (Lambalot et al. 1996)

Isolation of subtilin producing Bacilli

Soil samples with different nutrient content were isolated from nutrient-rich arable land (N, 50° 11'19”N; 08 ° 47'54"E) and forest (IP, 50° 08'22"N; 09° 16'33”E) in Hessen, Germany, and from alpine surroundings (HI, 47° 21'13”N; 10° 06’01”'E and HS, 47° 19’36”'N; 10° 11’02"E; both approx. 2000 m, Vorarlberg, Austria). Twenty five grams of soil were suspended in 100 mL of sterile water, pasteurized (10 min at 80 ° C) in order to accumulate spore-forming bacteria, diluted 1: 100, plated out on TY plates and incubated at 37°C. for 15 h. To monitor the antimicrobial activities, individual colonies were replica plated on TY agar

plates in Petri dishes with cams in order to obtain optimal aerobic conditions for antibiotic production (Sarstedt, Nümbrecht, Germany) with *Micrococcus luteus* ATCC 9341 as the test organism (Stein 2004).

Molecular biology techniques

Established protocols were followed for molecular biology techniques; *E. coli* plasmids were isolated by the rapid alkaline extraction procedure. DNA amplification using *Taq* DNA polymerase was performed according to the instruction of the commercial supplier (Boehringer GmbH; Mannheim, Germany) in a DNA Thermal cycler (Eppendorf; Hamburg, Germany). The SSU rRNA encoding gene was amplified by PCR using conserved primers 16S_forward 5'-GAGAGTTTGATCCTGGCTCAG-3' and 16S_reverse 5'-ACGACTTCACCCCAATCATC-3') according to Heyrman et al. (2001). The nucleotide sequences of *B. subtilis* DSM 6405, 3258, N5, HS1, IP, and HI has been deposited under the NCBI GeneBank records DQ452508-13, respectively, as well as DSM618: DQ529249 (for NCBI GeneBank links see the supporting informations. The structural gene of subtilin *spaS* including 250 bp of the upstream non-coding region has been PCR-amplified with primers SpaS_Seq1 (5'-CTATGAATCAATGGAAGGG-3') and SpaS_Seq2 (5'-CTTCATTTTCTTGTCCCG-3'); GeneBank records for *B. subtilis* DSM 618 (DQ452514: https://www.ncbi.nlm.nih.gov/nuccore/DQ452514), *B. subtilis* DSM 6405 (DQ452515: https://www.ncbi.nlm.nih.gov/nuccore/DQ452515), HS1 (DQ452516: https://www.ncbi.nlm.nih.gov/nuccore/DQ452516), and N5 (DQ452517:https://www.ncbi.nlm.nih.gov/nuccore/DQ452517). Subtilosin structural gene *sbo* sequencing has been performed with primers described previously (Stein 2004); GeneBank records for novel *sbo* sequences of the natural isolates *B. subtilis* HI-1, N5, and IP are deposited under accession numbers DQ452518-20, respectively. DNA cleavage and isolation was achieved with the QIAquick^{TM} purification kit (Qiagen GmbH; Hilden, Germany). Oligonucleotides were purchased from ARK (ARK Scientific GmbH Biosystems, Darmstadt, Germany). Sequencing was carried out by SRD (Scientific Research and Development, Oberursel, Germany); nucleotide sequences have determined at least two times for each DNA-strand.

DNA Sequence and phylogenetic analyses

Multiple sequence alignment (MSA) analyses were performed with Clustal Omega 1.2.4 that uses seeded guide trees and HMM profile-profile techniques to generate alignments (Waterhouse et al. 2009; Sievers et al. 2011) (https://www.ebi.ac.uk/Tools/msa/clustalo/) or MAFFT version 7 (Katoh et al. 2019; https://mafft.cbrc.jp/alignment/server/). Individual 16S rDNA sequences *rrnO*, *rrnA*, *rrnJ*, *rrnW*, *rrnI*, *rrnH*, *rrnG*, *rrnE*, *rrnD*, *rrnB* were extracted from the *B. subtilis* strains 168 (accession number NC_000964.3) and ATCC 6051 (NZ_CP020102.1). The SSU rDNA sequences of the *B spizizenii* strains ATCC 6633 (NZ_CP034943.1/CP034943.1), W23 (CP002183.1) and TU-B-10^{T} (CP002905.1) were extracted from Silva (https://www.arb-silva.de/) or manually. GC content calculation and GC profiling was performed with ENDMEMO (http://www.endmemo.com/bio/gc.php). Phylogenetic analyses were carried out by the neighborjoining method (Saitou and Nei 1987) using MAFFT (version 7; Kuraku et al. 2013) or Clustal Omega 1.2.4 software. The NCBI BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for homology searches.

MALDI-TOF mass spectrometry (MALDI-TOF MS)

Cell free aliquots of 500 µL culture supernatants of overnight grown

B. subtilis strains in Landy media were extracted with 500 µL 1-butanol, 400 µL of the organic phase was dried in a speed-vac evaporator, and extracted peptides were dissolved in 5 µL 50% acetonitrile and 1% trifluoroacetic acid (v/v in H_2O). 0.3-0.5 µL aliquots of the solutions were mixed directly on the target with 1.5 µL matrix solution, and the mixture was dried with the help of hot air. The matrix was 20 mg/mL DHBs (9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) complemented with solubilized in aqueous solution of 50% acetonitrile and 1% trifluoroacetic acid (v/v in H_2O). Generally, if sample spots are readily crystallized mass spectra with sufficient signal-to-noise values were obtained from the edge of the crystals (Stein 2008). Delayed extraction™ (DE) MALDI time-of-flight (TOF) mass spectra were recorded on a Voyager-DE STR instrument (Applied Biosystems Instruments) using a nitrogen laser (λ = 336 nm, repetition rate = 20 Hz) for desorption and ionization with an acquisition mass range from 600 to 15 000 *m/z* and the low mass gate set to 550 *m/z*. The total acceleration voltage was 20 kV with 68.5% grid voltage on the first grid, 0.02% guide wire voltage, 200 ns delay, and a mirror voltage ratio of 1.12. All experiments were carried out with the reflector positive ion mode. Between 500 and 1 000 laser shots were accumulated for each mass spectrum.

Subtilin quantitation

Similar growth profiles were obtained for all *B. subtilis* strains tested after growth in 2 mL Landy medium (37°C, 175 rpm). In standard determinations *B. subtilis* was grown for 16 h, the cultures were centrifuged (4°C, 15 min, 15,000 g), and the subtilin amount was determined in 500 µL aliquots by RP-HPLC using a Beckman Gold HPLC System and an analytical ODS Hypersil column (particle size: 5 µm, width & length: 2 x 250 mm, Maisch, Ammerbuch, Germany) as described previously (Heinzmann et al. 2006).

SDS-PAGE and Western blotting

SDS-PAGE (10% Tris-Glycine gels) and Western blot analyses were performed with an immuno-purified SpaC-directed immunoserum as described previously (Helfrich et al 2007). Molecular standards were purchased from Biorad (München, Germany).

Results And Discussion

Identification of antibiotic producing B. subtilis **and field collected bacterial strains**

Initially we tested twelve*B. subtilis* laboratory strains (*B. subtilis* subsp. *subtilis* and subsp. *spizizenii*, respectively), including well established strains from the German Collection of Microorganisms (DSMZ) on their ability to inhibit the growth of *M. luteus*. Interestingly, a number of *B. subtilis* DSM strains: DSM 618, 1087, 6395, 6405, and 8439 showed promising antimicrobial activities with growth inhibition activities comparable to the well-characterized subtilin-producing strain *B. subtilis* subsp. *spizizenii* ATCC 6633 (representative examples are given in Fig. 2a). The laboratory strains *B. subtilis* DSM 3256 and

DSM 3258 exhibited semi-large inhibition zones. To get more insight into *B. subtilis* strains without laboratory history we took random soil samples from various environmental habitats, nutrient-rich farmland and forest (200 m altitude, Hesse, Germany) and alpine environments (2000 m altitude, Vorarlberg, Austria). In a first pasteurization step, we were able to select the spore-forming microorganism out of the natural probes, which were single colony streaked on agar plates (40 single colonies, TY medium). Antibiotic producing strains were identified using *M. luteus* as a highly sensitive Gram-positive target strain using optimized conditions for the detection of lanthipeptides (Heinzmann et al. 2006) and sactipeptides (Stein et al. 2002). Notably, 20 to 25 percent of the isolated aerobically grown spore-formers inhibited the growth of *M. luteus* (not shown). Twelve probable antibiotic producing strains were selected: Six strains from the farmland (N), one from the forest (IP), and five strains from alpine environments (HS and HI). The strains with large and clear inhibition zones HS and N5, (Fig. 2a), were later identified as producer of subtilin (Sub^+, for a summary see Table 1). In contrast, the strains HI1 and IP exhibited relatively small inhibition zones, both strains were found to be Sub^- in further investigations (for a summary see Tab.1).

Phylogenetic analyses of laboratory and field-collected Bacillus **strains; genomic organization of SSU rRNA encoding** rrn **alleles of** B. spizizenii **and** B. subtilis

The 16S rDNA of all spore-forming microorganisms examined (laboratory and field-collected strains) were PCR amplified, sequenced, and their nucleotide sequences were taxonomically classified. *B. subtilis* strains 168 and ATCC 6051 have a total of ten *rrn* alleles (Tab. S2, Supplement), each 1552-1554 nucleotides in length; MSA analyzes indicated a few (two to three) nucleotide variations within these individual genomes (Tab. S3, Supplement). It is noteworthy, that *B. spizizenii* ATCC 6633 has ten identical *rrn* alleles in both length and nucleotide sequence, whereas for its close relative, the W23 strain, only eight *rrn* alleles (1552-1554 nucleotides) were found. The *B. spizizeni* typing strain TU-B-10 contains ten *rrn* genes, but the genome seems to be organized differently: The organization of the first five *rrn* alleles is similar to the organization of B. subtilis 168 or B. spizizenii ATCC 663, but the positions of alleles 6-10 indicate extensive genetic re-organization (Tab. S2 Supplement).

Notably, the *rrn* sequences of the field-collected *Bacillus* strains HS1 and HS2 (hereinafter referred to as strain HS) and N1, N5, and N6 (referred to as strain N5), as well as the laboratory strains DSM 618 and DSM 6405 were identical to *rrn* sequences of *B. spizizenii* ATCC 6633 and W23 (data not shown), suggesting their classification as *B.spizizenii* (Nakamura et al. 1999; Zeigler et al. 2008; Zeigler 2011). On the other hand, the field-collected strains IP and HI1 (referred to as strain HI) as well as the laboratory strain DSM 3258 are classified as further members of *B. subtilis* (see Fig. 3a for phylogenetic analyses). As indicated in Fig. 3b, MSA analyses revealed that position 181 of the individual *rrn* alleles can be used for clear species differentiation between *B. subtilis* and *B. spizizenii*. Interestingly, position 600 of the *B. spizizenii* typing strain TU-B-10 is identical to the *B. subtilis* sequence, whereas a transition (T/C exchange) was found at this position for the *B. spizizenii* ATCC 6633 and W23 *rrn* alleles.

BLAST searches revealed, that the field collected *Bacillus* strains HI2 and HI3 belong to *B. macroides* and *B. licheniformis*, respectively. Both species are known for members with a high potential to produce antibiotics. The strain N2 was classified as *B. thuringiensis*, a class of *Bacillus* strains some members of which produce the lipopeptide kurstakin (Hathout et al. 2000). No sequence was obtained for strain N3, strain N4 was discarded because no further significant antibiotic activities could be identified under the test conditions used.

SpaS **sequence of different** B. spizizenii **strains, presence of the SpaC protein**

For rapid PCR amplification of the subtilin structural gene *spaS* oligonucleotide primers (SpaS-Seq1 and SpaS_Seq2) complementary to the –35 region of the *spaS* promoter and the *spaS-spaI* intergenic region were used. The presence of the *spaS* gene was verified in the case of *B. subtilis* strains DSM 618, 1087, 6395, 6405 and 8439, as well as the natural isolates HS and N5 (Fig. 2b; for a summary see Tab.1). Sequences of both the *spaS* genes and their flanking regions (ribosomal binding site, -10-region) were identical to their counterpart in the ATCC 6633 strain (the GeneBank records for *B. subtilis* DSM 618, 6405, HS1, and N5 are DQ452514-17, respectively; ATCC 6633: NZ_CP034943.1, and TU-B-10: NC_016047.1). Since the *spaS* gene was detected within the DNA of all *B. spizizenii*, we expected also the subtilin maturing proteins in the cultures of these strains. This hypothesis was confirmed by detection of the subtilin cylase SpaC (Fig. 2c) and subtilin dehydratase (SpaB, not shown) within PAGE-separated cell extracts of the W23 strains using SpaC or SpaB specific immunosera, respectively. Most likely, all observed W23-type SpaC proteins are closely related, as EriC of *B. subtilis* A1/3 with only 85% sequence identity to the SpaC counterpart from the ATCC 6633 strain (Stein et al. 2002a) gave only weak immunoblotting signals. Consistent with these observations is that for the strains which lack *spaS* (Fig. 2b, strains 168, 3258, HI, and IP), no SpaC protein could be detected in the associated cell extracts either (Fig. 2b); the same applies for SpaB (not shown).

MALDI-TOFMS profiling of B. subtilis **culture supernatants**

MALDI-TOFMS analyses of butanolic extracts of *B. subtilis* culture supernatants resulted in well resolved peak clusters (Fig. 4). Prominent cluster between m/z 3280 and 3520 representH^+-, Na^+- and K^+-adducts of the lanthipeptide subtilin and its succinylated species (Chan et al. 1993; Heinzmann et al. 2006) and the sactipeptide subtilosin, respectively (see Tab. 1 for summary). Furthermore, the nonribosomal generated lipoheptapeptide surfactin was identified by MALDI-MS experiments due to characteristic *m/z* values of its different isoforms. Remarkably, all investigated *B. subtilis* strains produced surfactin (see Tab. 1 for a summary), notably, with the exception of the laboratory-adapted *B. subtilis* strain 168, which is known as a surfactin non-producer due to a mutation within the 4'-phosphopantetheine transferase *sfp* gene which posttranslationally modifies the required surfactin synthetase enzymes (Lambalot et al. 1996). Further MS/MS-fragmentation experiments according to Leenders et al. (1999) unambiguously assign the observed *m/z* values to the lipoheptapeptide sequence Glu-Leu-Leu-Asp-Val-Leu-Leu which can be clearly assigned to the peptide moiety of surfactin (data not shown). However, the detection of surfactin within the culture supernatants of all investigated *B. subtilis* strains, and the widespread

frequent appearance of the potential to produce surfactin among strains of the genus *Bacillus* (Peypoux et al. 1999; Kalinovskaya et al. 2002; Torres et al. 2016) restricts the usage of the phenotype "*surfactin production*" as biomarker for subspecies classification/differentiation (e.g. between *B. subtilis* and *B. spizizenii*).

Quantitative determination of subtilin

Subtilin was quantitatively determined in culture supernatants of stationary grown *Bacillus* cells in Landy medium (Fig. 5a/b). Whereas the production yields of *B. subtilis* ATCC 6633 (4.9 mg/mL) and the field-collected strainN5 (4.2 mg/mL) were comparable, the strain DSM 618 produces three-fold higher amounts (14.9 mg/mL). The largest subtilin yield was obtained from the laboratory strain *B. subtilis* DSM 6405 (33 mg/mL) and the field-collected *B. subtilis* HS (30 mg/mL). Representative chromatograms for these strains are shown in Fig. 5a. A further observation was that the proportion of N-terminally succinylated subtilin also increased with increasing yields of subtilin, especially for the high producer strains 618 and 6405. The production yields of the DSM strains 1087, 6395 and 8439 were similar to the ATCC 6633 strain (data not shown).

GC-Content of the subtilin gene cluster spa

Our results show that for all *B. spizizenii* strains characterized so far, the Sub^+ phenotype is a characteristic feature. The analysis of the base compositions of a given genome is a common strategy to investigate gene history (Garcia-Vallvé et al. 2000; Popa et al. 2001). Remarkably, in all analyzed

B. spizizenii genomes the average GC-content of the subtilin gene cluster with 36 % is significantly lower (about 8 %) than the GG content of about 44 % of respective GC-content average of the respective *B. spizizenii* host genome (Fig. 6 and Tab S3, supporting information). This observation is a strong hint that *B. spizizenii* acquired the subtilin gene cluster most likely from another organism by a recent horizontal gene transfer event as is hypothesized for a number of lanthipeptide producers (Zhang et al. 2012).

Nakamura et al. (1999) proposed the classification of *Bacillus subtilis* strains into two classes: The 168-type strains into (1) *B subtilis* subsp. *subtilis*, and W23-type strains into (2) *B. subtilis* subsp. *spizizenii*. Classical chemotaxonomy differentiates between both classes by the composition of their cell wall teichoic acids: Whereas 168-type strains are endowed with the essential major teichoic acids poly(glycerol phosphate) and the non-essential minor teichoic acids poly(glucopyranosyl N-acetylgalactosamine 1-phosphate), the W-23-type mainly consists of poly(ribitol phosphate) (Lazarevic et al. 2002). Very recently, *B. subtilis* subsp. *spizizenii* was promoted to species status on the basis of comparative genomics and secondary metabolite (mycosubtilin and bacillaene) production (Dunlap et al. 2020). The experiments presented in this study have revealed that all *B. spizizenii* strains from both laboratory (ATCC 6633, DSM 618, 1087, 6395, 6405, and 8439) as well as natural origin (HS and N5) produced identical mixtures of antibiotics including the lanthipeptide subtilin, the sactipeptide subtilosin, as well as nonribosomally biosynthesized lipopeptides from the surfactin class (Tab. 1). However, the lanthipeptide subtilin cannot be used as a characteristic for closely related species differentiation since

the production of subtilin and subtilin-related structures has also been found in *B. subtilis* A1/3 (Stein et al. 2002a), and other *Bacillus* strains (*Bacillus* sp., Velho et al. 2013, (*B. mojavensis*, Reva et al. 2020; *B. vallismortis*, Kim et al. 2018; *B. intestinalis*, Xu et al. 2015).

Our finding that different strains produce different amounts of subtilin - the yield of the HS strain was 7-fold superior to the original subtilin producer *B. subtilis* ATCC 6633 (Heinzmann et al. 2006), imply differential efficiencies in subtilin production. The examined *B. spizizenii* strains may have developed different genetic elements for the regulation of the extremely complex system of subtilin biosynthesis, such as repressor (AbrB) or activator (Sigma factor H) elements or variations in the promoter regions (-35 region) (Stein et al. 2002b and 2003; Kleerebezem 2004; Kleerebezem et al. 2004; Spieß et al. 2015). Furthermore, also the subsequent steps in subtilin biosynthesis for example post-translational dehydration of serine (threonine), addition of neighboring cysteines (Kiesau et al. 1997; Helfrich et al. 2007), and final processing (Stein and Entian 2002; Corvey et al. 2003) might exhibit differential efficiency in the investigated strains.

Recently, it was shown that succinylation of subtilin is a consequence of high glucose concentrations in the culture medium, for example the Landy medium (Bochmann et al. 2015). We found especially for the strains with very high production rates of subtilin - HS and DSM 6405, that the proportion of succinylated subtilin species were significantly increased compared to the unsuccinylated species (data not shown). Since N-terminal succinylated subtilin shows less antimicrobial activity, this could also be a necessary self-protection mechanism of *B. subtilis* cells against the antibacterial effect of their own product (Heinzmann et al. 2006; Geiger et al. 2019). The *B. spizizenii* strain TU-B-10 turned out to be an extraordinary exception, here the production of a subtilin isoform EtnS (entianin) was identified, which differs from subtilin in three conservative amino acid exchanges: L6V, A15L, and L24I (Fuchs et al. 2011).

Conclusion

In an age in which bacterial resistance to antibiotics is becoming increasingly important, systematic screening for new antibiotic agents with new mechanisms of action is still an important strategy. Microorganisms from the genus *Bacillus* are able to produce a large number of different antimicrobial substances (Stein 2005; Zhao et al. 2018; Tran et al 2022). It is to be expected that a large number of new active antimicrobial agents and isoforms of known antibiotics with minor chemical modifications will be found in the future through systematic screening, a strategy which is strongly supported by subsequent genome sequencing and novel genome mining bioinformatic tools (Walker et al. 2020). The findings from this work show that many *B. spizizenii* strains always produce a cocktail of antibiotic agents (Stein 2004 and 2020; Mülner et al. 2020), in particular always several lipophilic membrane-active agents like subtilin, subtilosin, surfactin, and fengycin. As a result, future studies should increasingly consider the synergistic effects of these interesting drug classes which have in common an amphiphilic structure with effects either in the cytoplasmic membrane itself (surfactin, fengycin, or the sactibiotic subtilosin) or in membrane-associated processes like cell wall biosynthesis (lanthipeptide subtilin). In particular, production strains of such antibiotic cocktails or strains that are suitable for use as probiotics should be

examined under more realistic growth conditions, such as co-cultures of different microorganisms in the same habitat (microbiome), in more natural behavior and production conditions, e.g. in biofilms (Kolter 2010). These aspects should be considered when further developing *B. subtilis* or other relates microorganisms as a biosynthetic platform for lanthipeptides (Schmitt et al. 2019), a very promising strategy to genetically engineer lanthipeptide structures to obtain drugs with novel properties.

Declarations

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Contributions

M.H. performed research, interpreted the data and wrote the paper, K.-D. E. conceived of or designed study, and reviewed the manuscript, and T. S. conceived of or designed study, performed research interpreted the data and wrote the paper. All authors read, reviewed, and approved the final version of the manuscript.

Corresponding author

Correspondence to Torsten Stein

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Consent for publication

The authors declare that they agree to publishing.

Competing Interest

The authors have no relevant financial or non-financial interests to disclose.

Data availability statement

All data generated or analysed during this study are included in this published article and the supplementary information files.

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Figures

Figure 1

A) The 32 amino-acid lanthipeptide subtilin.

The amino acid residues (one-letter-code) are circled and all posttranslational modified amino acid residues are colored. DA (blue), 2,3-didehydroalanine (dehydrated serine) at positions 5 and 32; DB (blue), 2,3-didehydrobutyrine (dehydrated threonine) at position 18. The five ring structures represent intramolecular thioether bridges, namely the amino acids meso-lanthionine (A-*s*-A, linking amino acids 3 and 7) or methyllanthionine (Ab-*s*-A linking amino acids 8 and 11, 13 and 19, 23 and 26, as well as 25 and 28). The posttranslational modified cysteine residue is shown in yellow.

B) The cyclic 35 amino-acid sactipeptide subtilosin.

The amino acid residues (one-letter-code) are circled and the posttranslational modified amino acid residues are colored, namely three cross-links between the sulfurs of Cys4, 7, and 13 (yellow) which are linked to the alpha-position of Phe22, Thr28, and Phe31 (pink), respectively (Kawulka et al. 2003). The N- and C-termini (green, position 1 and 35, respectively) are linked via amide bond forming subtilosins macrolactam.

C) The lipoheptapeptide surfactin is a heptapeptide lactone, which consist of a ß-hydroxy fatty acid (purple) whose carboxyl group is linked to a heptapeptide moiety via amide bond. Lactonization is accomplished by esterification of the carboxy group of the C-terminal leucine (green) with the ß-hydroxy fatty acid. Further non-proteinogenic components are the D-configured amino acids in positions 3 and 6 (orange). Variations with both, the chain length (13-15 carbon atoms) and the branching pattern (n, iso, anteiso) of the fatty acid moiety, as well as the identity of the amino acid in position 7 (exchange from L-Leu to L-Ile or L-Val) lead to a microheterogeneity of natural surfactin produced by *B. subtilis* (Kowall et al. 1998).

Figure 2

Identification of subtilin producing *Bacillus* strains. Representative examples of wild-type and laboratory *Bacillus* strains *spizizenii* DSM 618, DSM 3258, and DSM 6405; the natural isolates are designated HS, N5, HI and IP. *B. subtilis* 168 (Sub^-) and *B. spizizenii* ATCC 6633 (Sub^+), which were particularly well characterized as model organisms, served as references for non-producers and producers of the lanthipeptide subtilin, respectively.

A) Antimicrobial growth inhibition: Analyses of *Bacillus* strains in agar diffusion tests using *M. luteus* as the test organism. The production of antimicrobial agents is indicated by halos caused by growth inhibition of the test organism.

B) Amplification of the subtilis structural gene: The *spaS* gene was PCR amplified using the primers SpaS_Seq1 and 2, as well as DNA from different *Bacillus* cells as template. The right lane contains a nucleotide size standard. The calculated size of the amplified DNA segment containing *spaS* is 480 bp.

c. Western blot: *Bacillus* strains were harvested in the stationary growth phase. After SDS-PAGE separation of cell lysates the proteins were transferred nitrocellulose membranes and the presence of the SpaC protein was analysed with SpaC-directed immunoserum. The calculated molar mass of SpaC is 42,5 kDa.

Figure 3

Phylogenetic and multiple sequence alignment of SSU encoding *rrn* alelles of different *Bacillus* strains.

A) Neighbor joining tree of *rrn* alelles: All sequenced *rrn* genes of the subtilin producers exhibited 100% identity to the *B. spizizenii* reference strains ATCC 6633 and W23. The strains DSM 3258, HI and IP can be assigned to *B. subtilis* (blue).

B) Multiple sequence alignment: The *rrn* alleles of a given *B. subtilis* strain (n = 10) are almost identical (99.81%; see also Tab. S4, supporting information), but some differences can be determined, such as position 270 (gray). Red box, position 181 can be used for clear species differentiation between *B. subtilis* and *B. spizizenii,* while position 600 of the *B. spizizenii* typing strain TU-B-10 (blue box) is identical to the

B. subtilis sequence. The 8-10 *rrn* alleles of the respective *B. subtilis* strains are almost identical within a given strain, so that only one representative sequence is shown.

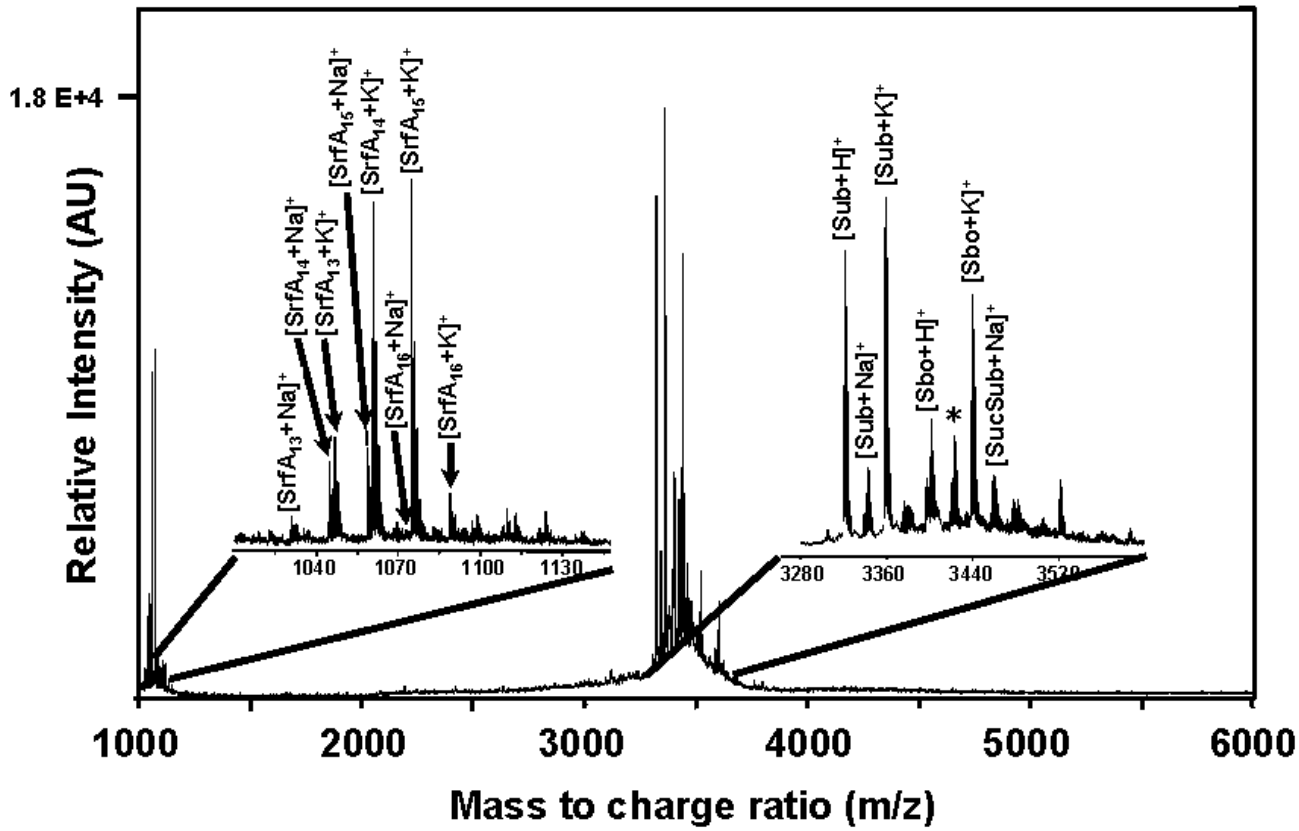

Figure 4

MALDI-TOF MS of butanolic extracts of a representative *B. spizizenii* (DSM 618) culture supernatant: The mass spectrum was recorded in the positive ion modus. Left insert, positively charged sodium (*m/z* 1030.7, 1044.7, 1058.7, and 1072.7) and potassium (1046.7, 1060.7, 1074.7, and 1088.7) adducts of different isoforms of the lipopeptide isoform surfactin A are labeled. The number of carbon atoms within the surfactin acyl chain is denoted in the index of SrfA (Kowall et al. 1998). In the right insert, the proton-adducts of subtilin (Sub, *m/z* 3322.8) and subtilosin (Sbo, 3400.8) are labeled as well as their sodium- and potassium adducts (Sub, 3344.8, 3360.9; Sbo, 3438.9). SucSub represents the potassium-adduct of succinylated subtilin at *m/z* 3460.9; the peak at *m/z* 3422.8 can be interpreted as $[SucSub+H]^+$ or $[Sbo+Na]^+$ (asterix).

Figure 5

Subtilin production by different *B. spizizenii* strains.

A) Identification of subtilin by quantitative RP-HPLC. *B. spizizenii* strains were grown for 16 h in Landy medium to comparable cell densities. 500 µL aliquots of cell free culture supernatants from stationary cells were separated by reverse phase HPLC. Antimicrobial growth assays and MALDI-TOF-MS experiments (not shown) indicated that subtilin elutes as a single peak at 17.5 to 18.5 minutes. The Sub^+ reference strain *B. spizizenii* ATCC 6633 is shown in green, *B. subtilis* 168 was used as a Sub^- control (dotted line in black).

B) Quantitative determination of subtilin produced by different *B. subtilis* strains. The integrals of the peaks eluting at 17.5-18.5 min (Fig 6A) are proportional to the amount of subtilin. The presented values with standard errors of less than +/- 12% are the means of three independent cultures for which the determinations have been performed twice.

Figure 6

GC-content comparison of the subtilin gene cluster and surrounding gene regions to the GC-content of *B. subtilis* and *B. spizizenii* genomes

A) The GC-content of the 12 kb subtilin gene cluster (red balls) of *B. spizizenii* (W23 and ATCC 6633), the upstream localized *opuB* gene cluster (green), and the downstream localized gene region of subtilin non-producers (black) were plotted versus the GC-content of the cognate genomes (black horizontal dotted lines).

B) GC-content of the *spa* gene cluster of *B. spizizenii* ATCC 6633 and its flanking regions (inverse orientation). Insert: The subtilin cluster of *B. spizizenii* ATCC 6633 encoding the subtilin gene cluster *spaBCSFEGRK* (11.8 kbp, nucleotide positions 3,251,415 – 3,263,272, negative strand). Red, *spaS* encoding the subtilin pre-propeptide; blue the posttranslational modification machinery *spaBC* and the

transporter *spaT*; purple, genes *spaIFEG* involved in immunity against matured subtilin; orange, *spaRK* encoding an two component regulatory system (Stein 2005). Black arrows represent different polycistronic transcripts of the *spa*-cluster. Upstream of the *spa* gene cluster the *opuB*-operon (green) is located, which is involved involved in choline transport (Hofmann and Brenner 2017); *catR* downstream of the *spa* cluster belongs to the MarR/DUF24 family of transcription repressors (Chi et al. 2010).

Supplementary Files

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- Helfrichetal.Supportinginformation.pdf