JHeimbach LLC

#905

January 21, 2020

Susan J. Carlson, Ph.D., Director
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Carlson:

Pursuant to 21 CFR Part 170, Subpart E, SporeGen Ltd., through me as its agent, hereby provides notice of a claim that the addition of *Bacillus subtilis* strain SG188 to conventional foods is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because SporeGen Ltd. has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, one copy of the GRAS monograph and one signed copy of the conclusion from each member of the Expert Panel are provided. Additionally, I have enclosed a virus-free CD-ROM with the GRAS monograph and the signed statements of the Expert Panel.

If you have any questions regarding this notification, please feel free to contact me at 202-320-3063 or jh@jheimbach.com.

James T. Heimbach, Ph.D., F.A.C.N.
President

Encl.

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Generally Recognized as Safe (GRAS) Determination for the Intended Use of *Bacillus subtilis* Strain SG188

Prepared by:
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SporeGen Ltd,
Bourne Laboratories,
Royal Holloway University of London,
Egham, UK.

November, 2019

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Abbreviations and Glossary of Terms

AMR	Antimicrobial Resistance
BW	Body Weight
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EFSA	European Food Safety Authority
CoA	Certificate of Analysis
CFU	Colony Forming Units
DSM	Difco-Sporulation Medium
GI tract	Gastrointestinal Tract
Katal	SI Enzyme Unit (amount that converts 1 mole of substrate per second)
MIC	Minimal Inhibitory Concentration
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
OECD	Organization for Economic Cooperation and Development
ORF	Open Reading Frame
QPS	Qualified Presumption of Safety
RAST	Rapid Annotation using Subsystem Technology
U	Enzyme Unit (amount that catalyzes the conversion of 1 micro-mole of substrate per minute)

Part 1: Signed Statements and Certification

1.1. GRAS Notice Submission

SporeGen Ltd., of Egham, United Kingdom, submits this GRAS notification through its agent James T. Heimbach, president of JHeimbach, LLC, in accordance with the requirements of 21 CFR Part 170, Subpart E.

1.2. Name and Address of Notifier

SporeGen Ltd.
Bourne Laboratories
Royal Holloway University of London
Egham, Surrey, TW20 0EX
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Professor Simon M. Cutting
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1.3. Name of Notified Organism

The subject of this Generally Recognized as Safe (GRAS) notification is the bacterium *Bacillus subtilis*, strain designated as SG188.

1.4. Intended Conditions of Use

B. subtilis SG188 is intended to be added to conventional foods. Target foods include but are not limited to:

- Beverages such as milk drinks, protein high energy sports drinks, hot beverages, and juices
- Dry and shelf-stable products such as cereals, cookies, gums, and confectionary

The intended addition level of *B. subtilis* is a maximum of 10^9 spores/dose or per serving throughout the shelf-life of the product. Since spores are stable, no overage is needed to protect against loss of viability, and it is anticipated that this concentration will exist throughout the shelf life.

1.5. Statutory Basis for GRAS Status

SporeGen's GRAS determination for the intended use of *B. subtilis* SG188 is based on scientific procedures in accordance with 21 CFR § 170.30(b).

1.6. Premarket Exempt Status

The intended use of *B. subtilis* SG188 is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act based on SporeGen's determination that it is GRAS.

1.7. Data Availability

The data and information that serve as the basis for the conclusion that *B. subtilis* SG188 is GRAS for its intended use will be made available to the FDA upon request. At FDA's option, a complete copy of the information will be sent to FDA in either paper or electronic format, or the information will be available for review at the home office of JHeimbach LLC, located at 923 Water Street, Port Royal VA 22535, during normal business hours.

1.8. Freedom of Information Act Statement

None of the information in the GRAS notice is exempt from disclosure under the Freedom of Information Act, 5 USC 552.

1.9. Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the intended use of *B. subtilis* SG188.

1.10. FSIS Statement

Not applicable

1.11. Name, Position and Signature of Notifier

James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
Agent to SporeGen Ltd.

Part 2: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1. Name of the GRAS Organism

The notified organism is a strain of the bacterium *Bacillus subtilis* designated SG188.

2.2. Source of the GRAS Organism

B. subtilis SG188 was isolated from healthy human feces from a study reported previously (Hong et al., 2009). The strain was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) with the deposit number DSM 32444.

2.3. Description of the GRAS Organism

Bhandari et al. (2013) reported that "the genus *Bacillus* is a phylogenetically incoherent taxon with members of the group lacking a common evolutionary history." Based on complete genomic date from over 30 different Bacillus species, these authors identified two clearly differentiated groupings, a "*Bacillus subtilis* clade" and a "*Bacillus cereus* clade." They proposed that "the genus *Bacillus sensu stricto* should comprise only the monophyletic subtilis clade that is demarcated by the identified conserved signature indels, with *B. subtilis* as its type species" (Bhandari et al., 2013). This genus is then distinguished from that including *B. cereus* and similar species.

The genus *Bacillus senso stricto* includes about 90 species with new species appearing frequently (Logan, 2004). A defining feature of these bacteria is their ability to form endospores or spores. They are Gram-positive, catalase positive, generally mesophilic, and motile (by peritrichous flagella).

The ability to form spores means that they can survive extreme environmental conditions, for example, exposure to high temperatures, desiccation, and contact with noxious chemicals such as solvents (Nicholson et al., 2000). It also means that they are typically found in abundance in the environment, for example in soil. This has led to *Bacillus* bacteria being considered soil microorganisms. In fact, while they are found in the soil and environment, this is almost exclusively as spores. Some species of *Bacillus* (e.g., *B. thuringiensis*) are able survive in the vegetative cell form associated with plants in the top soil, where they may benefit the plant host by producing toxins that kill insect predators (Nicholson, 2002).

More recent work has shown that spores may provide a means to survive and persist in the environment until consumed by animals. Ingestion of plants and soil contaminated with spores provides entry to the gastrointestinal (GI) tract. Here the spore form enables safe passage through the stomach to the intestines, where large numbers of spores can germinate and proliferate. As the bacteria are pushed through the GI tract by peristalsis, they re-sporulate and are shed in the feces, thus completing their life cycle (Hong et al., 2009; Tam et al., 2006). It is estimated that counts of *Bacillus* spores in human feces are about 10^4/g (Hong and Duc, 2004). In studies using spores of *Bacillus clausii* administered in a single oral dose to human volunteers, shedding of spores was observed for about 7 days, after which no detectable counts of *B. clausii* were reported (Ghelardi et al., 2015). This suggests that *Bacillus* spores are unable to establish permanent residency in the human GI tract.

2.3.1. Phenotypic identification

2.3.1.1. Morphology

B. subtilis SG188 exists in two forms, a vegetative cell and a spore. The spore is produced as a response to environmental stress or nutrient deprivation. Figure 2.1 shows a phase contrast image of a culture comprising vegetative cells and spores.

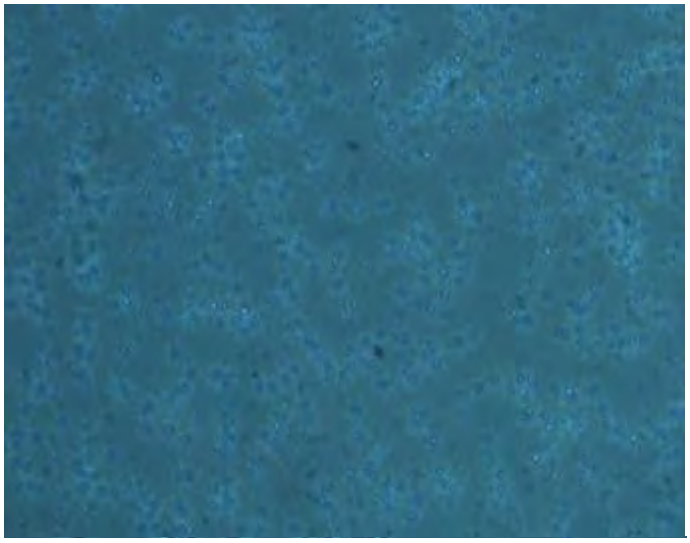

Figure 2.1. *B. subtilis* SG188
Phase contrast image of culture containing sporulating cells. Spores are visible as phase bright ellipsoidal bodies within the cell. In this culture most spores are retained within the cell and have not yet been released.

Vegetative cells of *B. subtilis* SG188 are rod shaped (*bacilli*), the length depending on the stage of growth; e.g., at stationary phase, rods are typically 2 μm in length while in early exponential phase rods can grow to 5 μm in length. Cell diameter is ~1.0-1.2 μm. Cells are motile. Spores are ellipsoidal in shape and ~1.0 μm in length. Spores are placed sub-terminal

or terminal within the sporangial cell and do not cause swelling of the sporangium. Under phase contrast imaging, they appear as refractile, phase-bright bodies (Figure 2.1). After prolonged growth, spores are released from the sporangial cell by lysis.

B. subtilis SG188 is strictly aerobic and was unable to grow anaerobically on media containing nitrite or nitrate as a terminal electron acceptor (Nakano et al., 1997, Nakano and Zuber, 1998). The inability to grow anaerobically makes strain SG188 different from many other strains of *B. subtilis* that can grow anaerobically under certain conditions. The strain is able to digest starch by production of amylase (Cutting and Vander-Horn, 1990).

Table 2.1: Biotype of *B. subtilis* SG188

Attribute	Description
Vegetative cell length	2-5 μm
Vegetative cell diameter	1.0-1.2 μm
Position of spore within cell	Sub-terminal-terminal
Shape of spore	ellipsoidal
Length of spore	~1.0 μm
Motility	+
Storage bodies	-
Digestion of starch	+
Anaerobic growth in presence of nitrite	-
Anaerobic growth in presence of nitrate	-
Catalase production	+
Pigmentation	None
Colony diameter	1-4 mm*

*Colony size depends on medium

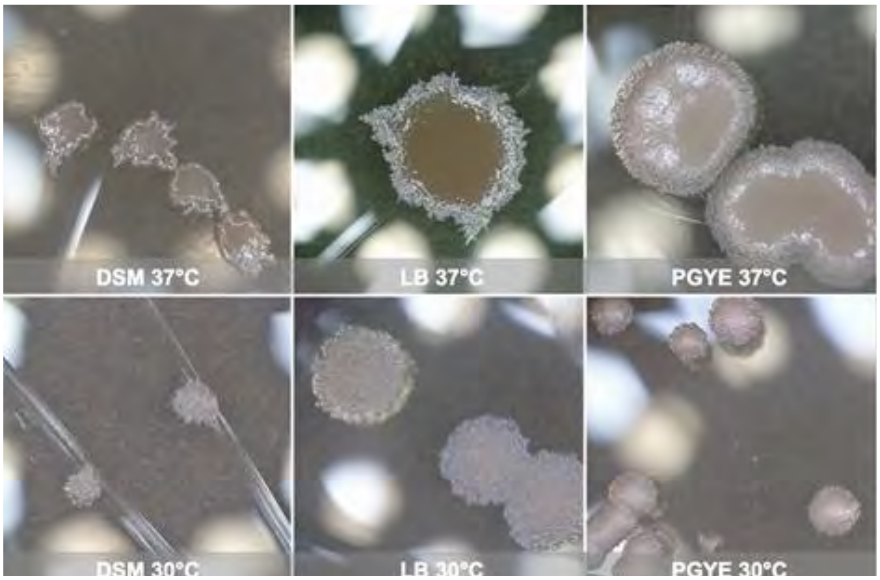

Figure 2.2: Colonies of *B. subtilis* SG188 colonies grown on solid medium

Individual colonies grown on Difco Sporulation Medium, Luria Broth, or Potato Glucose Yeast Extract Medium at 30°C and 37°C. Typical colony size after 37°C incubation was DSM 1-2mm, PGYE 3-4mm, and LB 3-5mm.

2.3.1.2. Heat Resistance

Purified spores are able to survive exposure to 60-70°C for 45 minutes without significant loss of viability. At temperatures above this, some loss of activity occurs, but 60% of spores survived 100°C for 45 minutes (Nicholson and Setlow, 1990). These data are shown in Table 2.2 using *B. subtilis* strain PY79 as a comparator.

Table 2.2: Resistance of *B. subtilis* SG188 pure spores to heat*

Temperature	PY79	SG188
60°C	97	101.3
70°C	90.9	98.1
80°C	83.3	65.0
90°C	84.8	61.3
100°C	69.7	60.1

*Suspensions of pure spores were made by growth of cultures in DSM liquid medium followed by lysozyme treatment to remove residual vegetative cells. Spores were washed with buffer and then water as described (Nicholson and Setlow, 1990). Suspensions of ~2 X 10^9 spores were incubated for 45 minutes in a calibrated oven and the surviving CFU determined by serial dilution and plate counting. Values shown are % of surviving CFU relative to initial untreated CFU.

Sporulation efficiency in growth media at 30°C and 37°C was evaluated after 24 hours by measurement of heat-resistant spores (68°C, 45 minutes) and determination of surviving CFU expressed as a % of untreated CFU/ml (Table 2.3). Sporulation levels were highest using a rice medium (Nicholson and Setlow, 1990).

Table 2.3: Sporulation efficiency

Growth Medium	30°C/24h	37°C/24h
DSM medium	24.2*	42
Brain Heart Infusion Broth	3.3	2.0
BTC Rice medium	93.2	100

*% of heat resistant spore CFU relative to untreated CFU

2.3.2. Genotypic identification

Determination of both 16S rRNA and *gyrA* DNA sequences followed by phylogenetic analysis against available reference type-strains were used to identify species using primer sets described previously (Chun and Bae, 2000, Hoa et al., 2000). Although 16S rRNA analysis is considered a suitable tool for species identification, more recent developments have shown that sequencing of the *gyrA* gene is more informative and enables better discrimination of *Bacillus* strains and species (Chun and Bae, 2000). The sequence of the SG188 16S and *gyrA* sequences are shown in Table 2.4. A phylogenetic analysis based on the *gyrA* sequence is presented in Figure 2.3. This analysis reveals that *B. subtilis* strain SG188 is most closely related to representative strains of *B. subtilis*.

Table 2.4: 16S and *gyrA* nucleotide sequences of *B. subtilis* SG188

Gene target	Sequence (5' – 3')
16S	atttatcggagagtttgatcctggctcaggacgaacgctggcggcgtgcctaatacatgcaagtcgagcggacagatgggagcttgctccctgatgttagcg gcggacgggtgagtaacacgtgggtaacctgcctgtaagactgggataactccgggaaaccggggctaataccggatggttgtttgaaccgcatggttcaa acataaaaggtggcttcggctaccacttacagatggacccgcggcgcattagctagttggtgaggtaacggctcaccaaggcaacgatgcgtagccgacc tgagagggtgatcggccacactgggactgagacacggcccagactcctacgggaggcagcagtagggaatcttccgcaatggacgaaagtctgacgga gcaacgccgcgtgagtgatgaaggttttcggatcgtaaagctctgttgttagggaagaacaagtaccgttcgaatagggcggtaccttgacggtacctaacc agaaagccacggctaactacgtgccagcagccgcggtaatacgtaggtggcaagcgttgtccggaattattgggcgtaaagggctcgcaggcggtttctta agtctgatgtgaaagcccccggctcaaccggggagggtcattggaaactggggaacttgagtgcagaagaggagagtggaattccacgtgtagcggtga aatgcgtagagatgtggaggaacaccagtggcgaaggcgactctctggtctgtaactgacgctgaggagcgaaagcgtggggagcgaacaggattagat accctggtagtccacgccgtaaacgatgagtgctaagtgttagggggtttccgccccttagtgctgcagctaacgcattaagcactccgcctggggagtacg gtcgcaagactgaaactcaaaggaattgacgggggcccgcacaagcggtggagcatgtggtttaattcgaagcaacgcgaagaaccttaccaggtcttga catcctctgacaatcctagagataggacgtccccttcgggggcagagtgacaggtggtgcatggttgtcgtcagctcgtgtcgtgagatgttgggttaagtcc cgcaacgagcgcaacccttgatcttagttgccagcattcagttgggcactctaaggtgactgccggtgacaaaccggaggaaggtggggatgacgtcaaa tcatcatgccccttatgacctgggctacacacgtgctacaatggacagaacaaagggcagcgaaaccgcgaggttaagccaatcccacaaatctgttctc agttcggatcgcagtctgcaactcgactgcgtgaagctggaatcgctagtaatcgcggatcagcatgccgcggtgaatacgttcccgggccttgtacacacc gcccgtcacaccacgagagtttgtaacacccgaagtcggtgaggtaacctttaggagccagccgccgaaggtgggacagatgattggggtgaagtcgta acaaggtagccgtatcggaaggtgcggctggatcacctcctttcta
gyrA	atgagtgaacaaaacacaccacaagttcgtgaaataaatatcagtcaggaaatgcgtacgtccttcttggattatgcaatgagcgttatcgtgtcccgtgctc ttccggatgttcgtgacggtttaaaaccggttcatagacggattttgtatgcaatgaatgatttaggcatgacaagtgacaagccttataaaaaatccgcgcgt atcgttggagaagttatcgggaaataccacccgcacggtgattcagcggtatatgaatccatggtcagaatggctcaggatttcaactaccgttatatgctcg ttgacggtcacggaaacttcggttctgttgacggagactcagcggcggccatgcgttatacagaagcacgaatgtctaaaatctcaatggagattcttcgcg acatcacaaaagacacaatcgattaccaggataactatgacgggtcagaaagagaacctgtcgttatgccttcaaggttcccgaatctgctcgtgaacggt gctgccggcattgcggtaggtatggcaacaaacattcctccgcaccagctgggagaaatcattgacggtgtacttgctgttagtgagaatccggacattaca attccagagcttatggaagtcattccagggcctgatttcccgaccgcgggtcaaatcttgggacgcagcggtatccggaaagcatacgaatcaggccgagg ctctatcacgatccgggcaaaagctgagatcgaacaaacatcttcgggtaaagaaagaattatcgttacagagttaccttaccaagtaaataaggcgaaat taattgagaaaattgctgatctcgtaagggacaaaaagatagagggtatcacagatctgcgtgatgagtcagatcgtacaggtatgagaattgtcattgaaa tcagacgcgatgccaatgcgaatgttatcttaaacaatctgtacaaacaaactgctctacaaacatcttttggcatcaacctgcttgcgcttgttgatggccag ccgaaagttttaactcttaagcaatgcctggagcattaccttgaccatcaaaaagttgtcattagacgccgtactgcttatgaattgcgtaaagcagaagcga gagctcatatcttggaaggattgagagttgcactggatcatctcgatgcagttatctcccttatccgtaattctcaaacggctgaaattgcgagaacaggttta attgaacaattctcactgacagagaagcaagcacaagcgatccttgacatgaggctccagcgtttaacgggactggaacgtgaaaagatcgaagaagaa taccagtctcttgttaaattaattgcagagctaaaagacatcttggcaaatgaatataaagtgcttgagatcattcgtgaagaactcacggaaatcaaagag cgttttaacgatgaaagacgtactgagatcgtcacttctggactggagacaattgaagatgaagatctcatcgagagagaaaatatcgtagttactctgacg cacaacggatacgtcaaacgtcttcctgcatcaacttaccgcagtcaaaaacggggcggaaaaggtgtacaaggtatgggaacaaacgaagatgatttc gttgaacatttgatctctacgtcaactcatgacacgattctcttcttctcgaacaaggggaaagtgtatcgtgcaaaagggtatgaaatccctgaatacggca gaacggcaaaaggaatcccgattattaacctgctggaggtagaaaagggtgagtggatcaacgcgattattccagtcacggaattcaatgcggagctttac ctcttcttcactacaaagcatggggtttcaaaacgaacatcgctatctcaattcgctaatatccgcaacaatggtctaattgctctgagtcttcgtgaagatgat gaactgatgggtgtacgtctgactgacggcacaaaacaaatcatcattggaacgaaaaacggtttactgattcgtttccctgaaacagatgtccgagagatg ggaagaactgcggcgggcgtaaaaggcatcaccctgacggatgacgacgttgttgtcggcatggagattttagaggaagaatcacacgtccttatcgtaac tgaaaaagggtacggaaaacgaactcctgctgaagagtacagaacccaaagccggggcggaaaaggactcaaaacagcgaaaatcaccgagaacaa cggccaactagtagcagtgaaagctactaaaggtgaagaggatctaatgattattacagctagcggcgtactcatcagaatggacatcaatgatatctccat caccggacgtgtcactcaaggtgtgcgtctcatcagaatggcagaagaagagcatgttgctacagtagctttagttgagaaaaacgaagaagatgagaat gaagaagaacaagaagaagtgtga

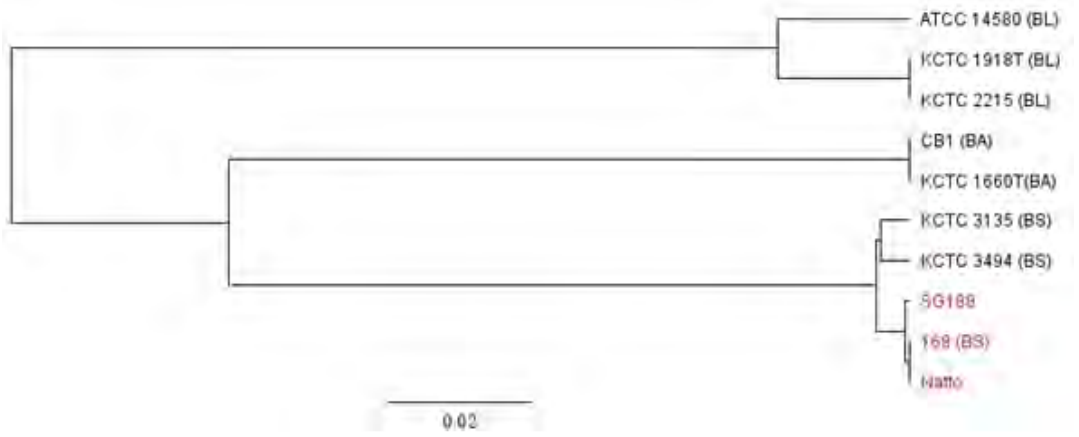

Figure 2.3: Phylogenetic analysis of the SG188 gyrA gene.

The *gyrA* sequence of SG188 was compared with corresponding nucleotide sequences of available reference strains of *B. subtilis* (BS), *B. amyloliquefaciens* (BA) and *B. licheniformis* (BL) from NCBI. The "Genius" program/software was used for construction of trees. Natto is a strain of *B. subtilis* used in fermented foods and 168 a *B. subtilis* type strain.

2.4. Genomic Analysis

2.4.1. Sequencing

A complete genome sequence using Illumina mate-paired analysis was conducted as a definitive demonstration of species[1]. A summary of the genome sequence is presented in Table 2.5 discussed in detail in Part 6.

Table 2.5: Summary of the SG188 Genome Sequence

Feature	SG188
Size (bp)	4,013,943
No. contigs	4
Closest neighbors	*B. subtilis* strain 168
No. coding sequences	4,179
No. RNAs	93
GC content	43.8%

[1] Conducted by Seqomics Biotechnology Ltd. Vallalkozok utja 7, 6782 Morahalom, Hungary. *www.seqomics.hu*

The genome sequence was uploaded on to the RAST database (http://rast.nmpdr.org/). The genome sequence was used for a number of purposes:

1. to demonstrate the integrity of SG188
2. to demonstrate species identity
3. to identify potential genes of benefit to the intended use of the bacterium for human use
4. to conduct a risk assessment for the intended use of this organism in humans

1. Integrity

The genome was complete with no gaps. The genome sequence is held on the RAST database.

2. Species Identity

Using the RAST database, the closest microbial species was *B. subtilis* strain 168. A summary is given in Figure 2.4.

Closest neighbors of Bacillus undefined SG188 (1386.556)

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Genome ID	Score	Genome Name
224308.1	516	Bacillus subtilis subsp. subtilis str. 168
224308.43	513	Bacillus subtilis subsp. subtilis str. 168
1148.72	513	Bacillus subtilis BEST7613
224308.49	512	Bacillus subtilis subsp. subtilis str. 168 [WGS]
565143.3	490	Bacillus subtilis subsp. subtilis str. AUSI98
1089443.3	490	Bacillus subtilis subsp. subtilis str. SC-8
1220533.3	489	Bacillus subtilis QB928
535024.3	470	Bacillus subtilis subsp. subtilis str. SMY
535025.4	469	Bacillus subtilis subsp. subtilis str. JH642
535026.3	447	Bacillus subtilis subsp. subtilis str. NCIB 3610
1192196.3	432	Bacillus subtilis subsp. subtilis str. BSP1
703612.3	366	Bacillus subtilis subsp. spizizenii ATCC 6633
645657.3	347	Bacillus subtilis subsp. natto BEST195
1001582.3	316	Bacillus amyloliquefaciens LL3
936156.3	295	Bacillus subtilis BSn5
1051503.3	285	Bacillus subtilis subsp. spizizenii DV1-B-1
720556.3	272	Bacillus atrophaeus Detrick-1
655816.3	270	Bacillus subtilis subsp. spizizenii str. W23
720557.3	261	Bacillus atrophaeus Detrick-2
720555.4	253	Bacillus atrophaeus 1942
1034836.4	253	Bacillus amyloliquefaciens XH7
720558.3	250	Bacillus atrophaeus Detrick-3
334727.3	246	Bacillus atrophaeus C89
723888.3	239	Bacillus atrophaeus 1013-1
1127744.3	237	Bacillus sp. JS
1236548.3	235	Bacillus subtilis subsp. inaquosorum KCTC 13429
326423.3	229	Bacillus amyloliquefaciens FZB42
723889.3	228	Bacillus atrophaeus 1013-2
1052588.4	222	Bacillus subtilis subsp. subtilis RO-NN-1
903507.3	220	Bacillus subtilis gtP20b

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Figure 2.4: Closest bacterial neighbors to SG188 based on the genome sequence.

As determined using the RAST database with available sequences.

2.4.2. Annotation of the Genome

The genome of SG188 has been subject to a complete annotation.

2.4.2.1. Beneficial Genes

A squalene hopene cyclase (Peg. 2198) is a squalene present only in the spore of SG188 and other *B. subtilis* strains. It is an immune adjuvant (Bosak et al., 2008) and would support the known immunostimulatory properties of this species (Huang et al., 2008).

2.4.2.2. Genome-Based Risk Assessment

No genes were identified that indicate potential risk to humans under the intended conditions of use. This is reported in detail in Part 6 of this monograph, but the key findings are:

1. No evidence of plasmids
2. No evidence of insertion sequences or transposable elements
3. No genes for medically important antibiotic resistance.

2.5. Production Process

B. subtilis SG188 is produced by Atani Holdings[2] in Vietnam under current Good Manufacturing Practice (cGMP). Atani Holdings is a manufacturer of *Bacillus* spores (www.biospring.com.vn/en*)*. Production is conducted at the ELCOM Building, Alley No. 15, Duy Tan St., Dich Vong Hau Ward, Cau Giay Dist. Hanoi, Vietnam. Atani Holdings have the following accreditation:

- ISO 9001:2015
- GMP-WHO: CAC/RCP 1-1969 Rev 4-2003 (GMP) issued by Quacert

2.5.1. Storage of Master Stocks

B. subtilis strain SG188 is deposited at the DSMZ in Germany (https://www.dsmz.de*)* as a safe-deposit (No. 32444). This means that lyophilized stocks are maintained indefinitely and represent security. SporeGen also have stocks as aliquots held in two separate freezers (-80°C). Atani Holdings have received a master plate of SG188 and this is stored as aliquots at -80°C in two separate freezers.

From the master stock, a working cell stock is made comprising 200 tubes of SG188 frozen at -80°C. This is replenished when needed from the master stock. The working cell stock is checked for strain identity by sequencing of the *gyrA* gene, which must be identical to the sequence shown in Table 2.4.

[2] Registered Address: Atani Holdings JSC, Floor 16, ICON Tower, No. 243A, De La Thanh St., Lang Thuong ward, Dong Da Dist, Hanoi, Vietnam

The process is shown in Figure 2.5.

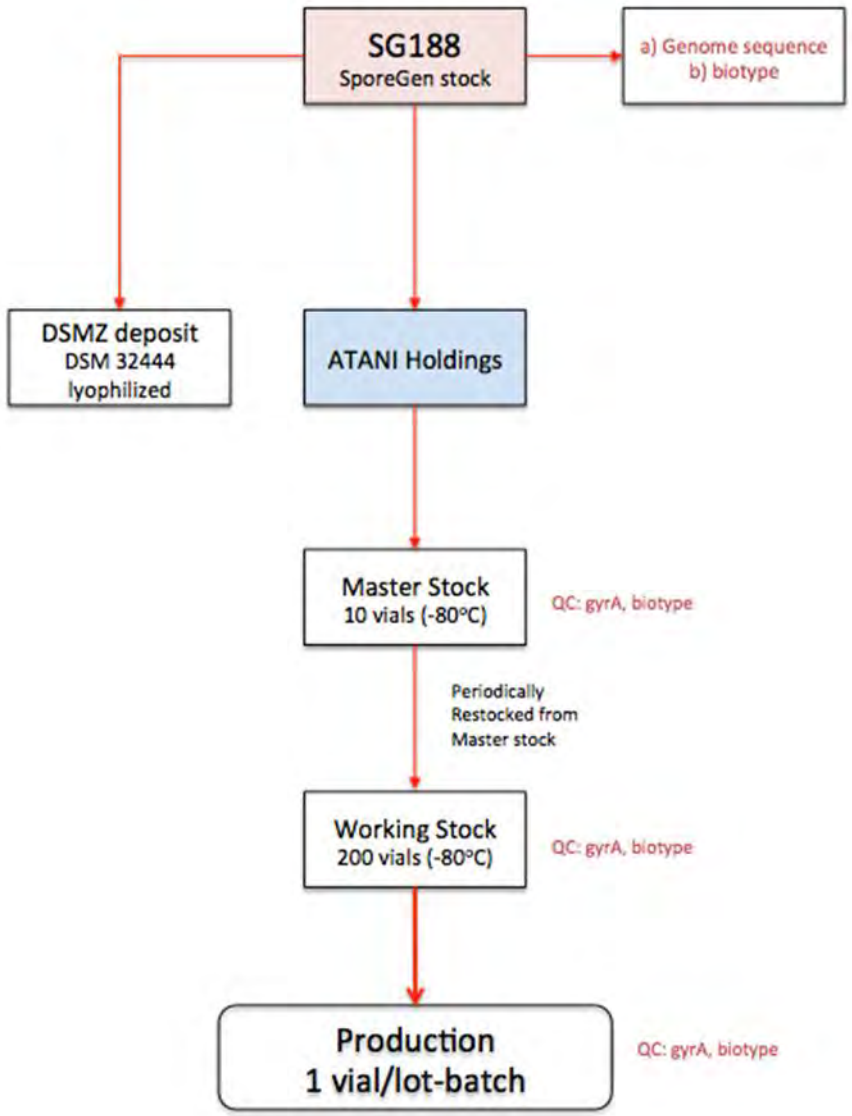

Figure 2.5: SG188 Stocks and QC.

2.5.2. Production

The production of spores is as follows and shown schematically in Figure 2.6.

1. SG188 is stored as aliquots frozen at -80°C in two separate freezers.
2. A fresh agar plate is cultured for single colonies at 30°C.
3. Colony integrity is checked by pigmentation, appearance, and presence of spores.

4. 25 ml of BTC medium, a meat-free growth medium, is inoculated with a single colony and incubated at 37°C in a shaking incubator.
5. At mid-log phase (OD_{600nm} 0.4), 10 ml of culture is used to inoculate 1000 L of BTC medium in a bioreactor.
6. Cells are grown for 2 days at 37°C, with aeration.
7. After 48 hours, the culture comprises ~90% spores and the remaining vegetative cells are mostly dead.
8. The culture is concentrated using a continuous centrifuge and suspended in 1 L of 1M food-grade NaCl and centrifuged using a bench centrifuge.
9. This process is then repeated with 0.5 ml food-grade KCl and then 3 times with sterile water.
10. The final spore suspension is heat-treated at 68°C for 1 hour, brought to room temperature, and then freeze-dried.
11. The freeze-dried powder is milled to reduce aggregates and sealed in bags.

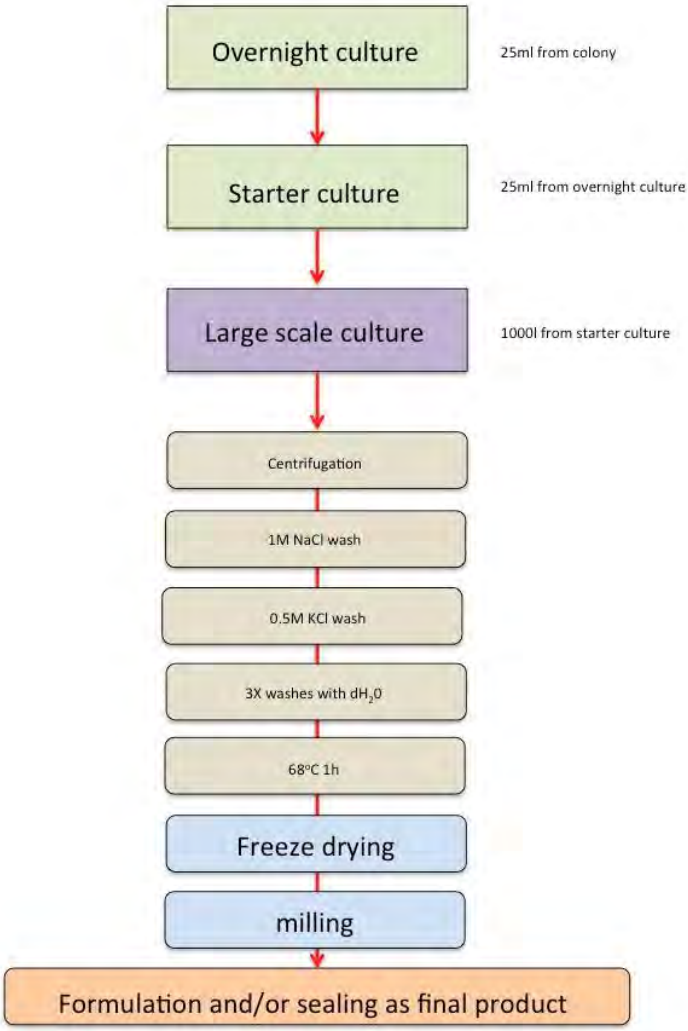

Figure 2.6: The Production Process

2.5.3. Formulation

The freeze-dried powder is blended with permitted food-grade excipients (depending on customer requirements but typically maltodextrin). The total yield from 1000 L of BTC medium is ~$5x10^{14}$ spores.

2.5.4. Quality Control

Each batch of spores is assessed for quality by a QC unit at Atani Holdings.

2.6. Stability

2.6.1. Genetic Stability

Routine passage of *Bacillus* strains is likely to introduce changes in the genome. Evidence for this comes from studies made on the type strain of *B. subtilis* known as strain 168 (Zeigler et al., 2008). Passage of a strain is likely to lead to incremental changes in the genome sequence and it is essentially not possible to identify these using any method other than complete genome sequencing. Additionally, large-scale growth of bacteria in bacterial bioreactors or fermenters is likely to induce mutations in the chromosome resulting from the physiological stress of high-density growth.

Most changes are phenotypically silent but, as noted for *B. subtilis* strain 168, over time considerable changes occur. Importantly, methods such as RAPD-PCR that are commonly used to verify strains do not identify silent mutations. Therefore, the only way to control strain integrity is by:

a) storage of the master strain in a depository,
b) storage of a master stock comprising freeze-dried ampoules, and
c) construction of a working cell stock.

As was discussed above, all of these methods are employed in assuring the genetic stability of *B. subtilis* SG188.

2.6.2. Stability of Lyophilized SG188 Powder

Freeze-dried spores of SG188 were stored at ~18-20°C for 12 months with sampling every 3 months. As shown in Table 2.7, there was essentially no decline in spore CFU, demonstrating that spores are stable in the freeze-dried form. However, it is worth noting that if spores are plated directly for CFU determination, the resulting CFU is lower than when spores are pre-treated with a short exposure to 75°C. The reason for this is super-dormancy (Wei et al., 2010) and occurs when spores age, and, following freeze-drying, this is accentuated and hinders spore germination on plates. Thus, the true number of spores is better reflected in the CFU determined from pre-treatment with heat.

Table 2.7: Stability of Freeze-dried SG188 powder*

Storage time	CFU/g with no pre-treatment with heat	CFU/g after heat treatment
0	1×10^{11}	1×10^{11}
3	6.2×10^{10}	7×10^{10}
6	6×10^{10}	6.8×10^{10}
9	5.2×10^{10}	6×10^{10}
12	5×10^{10}	6×10^{10}

*Powder is suspended in PBS and serially diluted before plate counting or suspended in PBS and heated at 75°C for 15 minutes before dilution and plate counting.

2.6.3. Stability in Food Matrices

Bacillus spores are notably resistant to heat. This is shown from data for SG188 presented above. A recent example is demonstration that spores of *B. subtilis* HU58 can survive exposure to 235°C for 8 minutes when incorporated in biscuit mix that was then baked. Only a 1-log reduction in CFU was reported (Permpoonpattana et al., 2012). This suggests that SG188 may well have similar utility in food matrices.

Stability tests for different foods will be conducted according to the customer's requirements.

2.7. Specifications

SporeGen has established the specifications shown in Table 2.8 for food-grade *B. subtilis* SG188. Also shown in the table are the results of testing of 5 lots of product. All lots are within specification.

Table 2.8: Specifications and Results of Testing of Five Lots of *B. subtilis* SG188.

Parameter	Specification	Method	Tested Lots (Date of Manufacture)				
			20170403	20170406	20170508	20170525	20170605
Appearance	Opal powder	Inspection	Conforms	Conforms	Conforms	Conforms	Conforms
Taste	Sweet	Inspection	Conforms	Conforms	Conforms	Conforms	Conforms
Odor	None	Inspection	Conforms	Conforms	Conforms	Conforms	Conforms
Water content	≤10%	ISO 712.2009	8.12%	8.15%	8.10%	8.20%	8.50%
Ash content	≤2.0%	ISO 5984.2002	1.60%	1.57%	1.61%	1.65%	1.70%
Spore density	$\geq 10^{11}$ spores/g	SG-SOP5	1.07×10^{11}	1.10×10^{11}	1.03×10^{11}	1.01×10^{11}	1.02×10^{11}
Microbiological purity							
E. coli (in 1 g)	Not detected	ISO 07251.2005	ND*	ND	ND	ND	ND
Salmonella spp. (in 25 g)	Not detected	ISO 06579.2002	ND	ND	ND	ND	ND
Coliforms (in 1 g)	≤10	ISO 40831.2006	ND	ND	ND	ND	ND
S. aureus (in 1 g)	≤5	ISO 06888.1999	ND	ND	ND	ND	ND
C. perfringens (in 1 g)	≤10	ISO 07937.2004	ND	ND	ND	ND	ND
B. cereus (in 1 g)	≤10	ISO 7932.2004	ND	ND	ND	ND	ND
Fungal spores (in 1 g)	≤10	ISO 21527.2008	ND	ND	ND	ND	ND
Heavy metals							
Lead	≤0.5 mg/kg	AOAC 972.25	ND (0.02)	ND (0.02)	ND (0.02)	ND (0.02)	ND (0.02)
Mercury	≤0.1 mg/kg	AOAC 971.21	ND (0.05)	ND (0.05)	ND (0.05)	ND (0.05)	ND (0.05)
Cadmium	≤1 mg/kg	AOAC 973.34	ND (0.02)	ND (0.02)	ND (0.02)	ND (0.02)	ND (0.02)
Unwanted Chemicals							
Aflatoxin B_1	≤5 µg/kg	LC-MS/MS	ND (0.1)	ND (0.1)	ND (0.1)	ND (0.1)	ND (0.1)
Aflatoxin $B_1B_2G_1G_2$	≤15 µg/kg	LC-MS/MS	ND (0.1)	ND (0.1)	ND (0.1)	ND (0.1)	ND (0.1)
Patulin	≤50 µg/kg	HPLC	ND (2.0)	ND (2.0)	ND (2.0)	ND (2.0)	ND (2.0)
*ND = not detected (limit of detection)							

Part 3: Dietary Exposure

B. subtilis SG188 is intended to be added to conventional foods at concentrations considered beneficial to human health, 10^9 spores/serving. Target foods include but are not limited to:

- Beverages such as milk drinks, protein high energy sports drinks, hot beverages, and juices
- Dry and shelf-stable products such as cereals, cookies, gums, and confectionary

Since the spores are stable, no overage is needed to provide for viability throughout the shelf life. In an extreme case, an individual might consume as many as 5 servings of foods containing the bacterium in a day, thus ingesting up to $5x10^9$ viable spores.

Part 4: Self-limiting Levels of Use

There is no technological or organoleptic limitation to the concentration of *B. subtilis* SG188 in foods.

Part 5: Experience Based on Common Use in Food

The conclusion that the intended use of *B. subtilis* SG188 is GRAS is based on scientific procedures rather than experience based on common use in food prior to 1958.

Due to the ubiquity of *B. subtilis* and other *Bacillus* species in the environment and the spores' robustness and ability to persist indefinitely (Nicholson, 2002), humans (as well as other animals) are exposed on a daily basis to spores through inhalation of dust and ingestion of plants and other food matter.

There is a worldwide record of safe use of *Bacillus* species, including *B. subtilis*, with humans, land animals, and farmed fish and shrimp. In humans, the principal *Bacillus* species currently being used are *B. clausii, B. coagulans*, *B. licheniformis*, and *B. subtilis*. In animals, mostly *B. subtilis* and *B. licheniformis* are employed.

5.1. Use of *Bacillus* Species in Foods and Dietary Supplements

Bacillus species are used in humans worldwide. Normally, they are consumed in foods or taken orally in tablets or capsules or in powders and liquid suspensions. These bacteria are used in humans and in animals (as alternatives to antibiotics as growth promoters). The use of *Bacillus* species has been reviewed extensively; for example:

- Activity of *Bacillus subtilis* spores in the management of intestinal disorders (e.g., diarrhea) associated with alterations in the qualitative and quantitative composition of the normal human intestinal flora (Mazza, 1994)
- Review of preparations from spore-forming bacteria used in Russia (Osipova et al., 2003)
- Brief reports of human studies with *B. coagulans* and *B. subtilis* (Sanders et al., 2003)
- Additional reports of human studies with *B. clausii* and *B. coagulans*, and animal studies with *B. subtilis* and *B. licheniformis* (Hong et al., 2005)
- Review summarizing the current use of *Bacillus* species, their safety, and mode of action (Cutting, 2011)

The important advantage of *Bacillus* is that it forms spores as a part of its life cycle. Spores are heat stable and can routinely withstand extremes of temperature, typically 80-85°C (indefinitely) and even higher temperatures for shorter periods of time. Spores can also withstand exposure to noxious chemicals (e.g., solvents) and to acid. The latter attribute is

important for use of *Bacillus*, since spores can be ingested and survive transit through the stomach. Most types of bacteria (for example, *Bifidobacteria* and *Lactobacillus*) are heat labile and sensitive to gastric fluids. Thus, *Lactobacillus* products stored at room temperature have a limited lifespan before their viability is reduced. Consumption reduces CFU (by as much as 10%) so the combined negative effects of heat and acid reduce the active dose of bacteria consumed (since by definition, probiosis is linked to viable, live, bacteria). The use of spores addresses the issue of providing a product that carries a specific concentration; spores can be produced in a dried form (lyophilized or spray dried) and maintain viability and thus level of intake.

5.2. *Bacillus subtilis*

Using molecular techniques, *B. subtilis* has been reported to be able to grow and proliferate in the murine gut (Hoa et al., 2001; Casula and Cutting, 2002; Tam et al., 2006). Substantial evidence suggests that this species and other *Bacilli* are minor gut commensals that have adapted to a life within and outside the mammalian GI tract (Hong et al., 2009). *B. subtilis* is readily found in humans, pigs, and other animals (Barbosa et al., 2005, Fakhry et al., 2008, Hong et al., 2009). Intestinal residency is an important aspect of the use of this bacterial genus since it reveals that *Bacilli* have a natural relationship with their intended host.

B. subtilis spores are an important component of the popular Japanese staple Natto (Hosoi and Kiuchi, 2004). *B. subtilis* is used to seed the fermentation of soybeans leading to a product that carries greater than 10^8 live spores of *B. subtilis* per gram of product. Natto has a long history of safe use in Japan, where the typical daily consumption is between 100-400 g Natto/day, suggesting intake of over 10^{10} spores per day. This history of safe use supports the use of *B. subtilis* spores in food products.

Part 6: Narrative

6.1. Safety of *Bacillus* Species

This section briefly summarizes what is known about the safety of *Bacillus* species. The safety of *Bacillus* as a genus has been reviewed many times; the review by Logan (2004) is the most comprehensive. In addition, EFSA has provided reports of safety concerns arising from the use of *Bacillus* species in foods (EFSA, 2007; EFSA, 2008). The European Scientific Committee on Animal Nutrition (SCAN) has provided a number of reports relating to the use of *Bacilli* in animal feed products that are directly relevant (SCAN, 2000; SCAN, 2001).

Two *Bacillus* species, *Bacillus anthracis* and *Bacillus cereus*, are known human pathogens No other single species can be considered a *bona fide* human pathogen (*Bacillus thuringiensis* is an insect pathogen), although in many cases *Bacilli* other than *B. anthracis* and *B. cereus* have been linked to illness or infection. Commenting on this fact, Logan (2004) reported:

> "This must be put in perspective. Most such cases occurred in persons debilitated by neoplastic disease, immunosuppression, alcoholism and other drug abuse, or some other underlying condition, and reports of infections with *B. clausii, B. coagulans, B. pumilus, Brevibacillus laterosporus* and *Paenibacillus polymyxa* are exceedingly rare. Infections with *B. subtilis* are also rare, but with some of the earlier reports we may entertain some doubts as to the accuracies of the identifications. These occasional cases do not seem to be sufficient grounds upon which to recommend the withdrawal of products containing these species" (Logan, 2004).

6.1.1. *B. anthracis* and *B. cereus*

B. anthracis is well known as a human and animal pathogen where disease (anthrax, often fatal) is due to phagocytic uptake of *B. anthracis* cells and secretion of a potent toxin (Mock and Fouet, 2001). Symptoms of anthrax are well defined and the identification of this pathogen is relatively straightforward. *B. cereus* is a common form of food poisoning (Stenfors Arnesen et al., 2008). Two specific types of illness arise, a diarrheal-type and an emetic-type. The diarrheal-type is caused by the action of one or more enterotoxins while the emetic-type is caused by the action of one, the emetic toxin cereulide.

6.1.2. Other *Bacilli*

Bacillus species other than *B. cereus* and *B. anthracis* have been linked to illness and infection. A summary is shown in Table 6.1. To explain these data, a number of issues must be considered/addressed.

Table 6.1: Illnesses associated with *Bacillus* species other than *B. cereus* and *B. anthracis*

Species[b]	Illness	Comments
B. subtilis	Various cases of bacteremia, septicemia,	Isolated from tissue
	Endocarditis	In drug user
	Bovine abortis	
	Food poisoning	Linked to bacterial load?
B. licheniformis	Found in blood in L-form	L form found in association with erythrocytes of arthritic persons
	Food-borne illness	Has been linked to infant fatality. A toxin has been found in one strain.
	Bovine abortion	Linked to contamination of silage
	Various cases of bacteremia, septicemia, orbital injury	Isolated from tissue
B. clausii	Fatal septicemia	In immunocompromised drug user
B. circulans	meningitis	Isolated from tissue
	endocarditis	Isolated from tissue
B. coagulans	Corneal infection	Isolated from tissue
	Bovine abortion	Isolated from tissue
B. sphaericus	Lung pseudo-tumor	Isolated from tissue
B. pumilus	Bovine mastitis	Isolated from tissue
	Rectal fistula infection	Isolated from tissue

[a] Information taken from Logan, 2004.

[b] In each case illnesses and reports refer to individual strains, not the species.

Was the species isolated from the clinical tissue and the specimen correctly identified?

In many cases, bacterial typing was done in a hospital and reports date from decades ago when rapid taxonomic tools (e.g., the API kit) and molecular tools (e.g., 16S rRNA sequencing) were unavailable. It is possible that robust heat-resistant spores of a *Bacillus* species were present in a clinical sample but possibly as a contaminant and their original identification (often as the presence of Gram-positive bacteria or phase bright spores) was misleading and incorrect. However, there are cases where a *Bacillus* species was correctly identified.

Was the species the only bacterium present?

It is probable, especially in wound infections, that bacterial spores could have been present as a contaminant and thus led to misidentification.

Does the isolate cause disease in a reinfection model, i.e., does it fulfill Koch's postulates? This type of experiment is absolute proof that a bacterium is pathogenic, but it has never been reported as such with *Bacillus* isolates. However, numerous studies have shown that *Bacillus* species, and notably *B. subtilis* (Hong et al., 2008, Sorokulova et al., 2008, Tompkins et al., 2008), can be administered to animals without producing adverse effects.

Although a number of species of *Bacillus* have been implicated, rightly or wrongly, in bacterial infections, there is, to our knowledge, no case on record involving *B. subtilis*.

6.1.3. Bacterial Load and the Risk of Consuming Large Quantities of *Bacilli*

As discussed above and shown in Table 6.1, *Bacillus* species other than *B. anthracis* and *B. cereus* have on occasion been implicated in cases of illness, and *Bacillus* species have been reported in infections. It is highly probable that the reported presence of *Bacilli* arose from misdiagnosis or contamination. In other cases, it cannot be ruled out that isolates of *Bacillus* species carried the potential for virulence, enabling them to proliferate and cause illness in the human host. In some cases it is possible that some *Bacillus* species actually encode toxins similar to those expressed in *B. cereus*, and to date these toxins have not been identified. For this reason genome assessments are vital to assure safety.

The concept of the action of bacterial load is emerging and needs to be assessed when considering the use of a *Bacillus* isolate. If ingestion of *Bacillus* spp. can, in some isolates, lead to illness, then what could be the cause? One possibility is that the *Bacillus* isolate carries one or more enterotoxin genes that encode an active product, or alternatively, an emetic-type toxin is produced and when ingested produces illness. Evidence exists that some *Bacilli* other than *B. cereus* carry enterotoxin genes (Rowan et al., 2001, Phelps and McKillip, 2002, From et al., 2005). Evaluation of the presence or absence of potential toxin genes is a prerequisite for establishing the potential safety of a new *Bacillus* isolate for food use. (Note that this has been done for *B. subtilis* SG188.)

EFSA concluded that it is highly unlikely that *B. cereus*-like enterotoxins found in *B. subtilis* and other *Bacillus* species are either produced or active. As stated by EFSA, "*Any toxicogenic potential in such species appears far more likely to arise from the production of surfactins*" (

EFSA, 2014a). Therefore, analysis of toxin genes alone is not sufficient and it must be assumed that other factors may contribute to illness and that the potential of these must also be assessed. Examples of potential virulence factors are discussed below and include surfactins and other lipopeptide antimicrobials (e.g., bacteriocins), phospholipases, and hemolysins that could have cytotoxic potential.

It is important to note that the genomic analysis undertaken as part of the safety assessment of the intended use of *B. subtilis* strain SG188 included investigation of genes potentially encoding these factors.

6.2. Safety of *B. subtilis*

B. subtilis was assessed for safety along with other *Bacillus* species in a safety review by Logan (2004). No safety issues were identified. The microorganism is Class 1, having the lowest level of risk as defined by the following authorities:

- UK: Advisory Committee on Dangerous Pathogens. 2013. "The Approved List of biological agents" 3rd Edition. Health and Safety Executive
- Europe: Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work
- USA: NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules.
- Canada: Risk Groups, Containment Levels, and Risk Assessments (2013); In Canadian Biosafety Standards (1st ed.). Government of Canada

This species is not linked to any human health issues. Known bacteriocins produced by *B. subtilis* are shown in Table 6.2. The bacteriocins identified to date in *B. subtilis* are megacins and these are plasmid encoded (Von Tersch and Carlton, 1983a, 1983b; Kiss et al., 2008). They are therefore not present in *B. subtilis* strain SG188 which, as discussed below, has no plasmids.

Table 6.2: *B. subtilis* Bacteriocins (Urdaci and Pinchuk, 2004; Abriouel et al., 2011).

Bacteriocin	Mwt	Type
Subtilin A	3.34	lantibiotic
Subtilin B	3.42	lantibiotic
Subtilosin A	3.39	lantibiotic
Subtilosin B	3.41	lantibiotic
Sublancin 168	3.88	lantibiotic
Mersacidin	1.82	lantibiotic
Betacin	-	lantibiotic
MJP1	4.5	lantibiotic
Ericin S	3.44	lantibiotic
Ericin A	2.98	lantibiotic

6.3. Safety of *B. subtilis* Strain SG188

6.3.1. Genome Analysis

The complete genome of *B. subtilis* strain SG188 was examined for coding sequences (ORFs) that could encode gene products that may be of potential concern regarding safety for use in humans or animals.

The purpose of this study was to objectively examine the genome of *B. subtilis* strain SG188 for coding sequences that could encode gene products that may be of potential concern regarding safety for use in humans or animals. An Excel file showing all open reading frames (ORFs) identified from the SG188 genome was scanned item-by-item to identify all putative genes of interest falling under two classes, virulence-associated and phage-associated. Next, the genome uploaded on the RAST server was examined. The RAST server highlights potential genes of interest and these are categorised under several categories, listed below:

1. Transposons & plasmids
2. Antibiotic resistance
3. Genes potentially involved in virulence
    - Resistance to heavy metals
    - Other genes of potential interest
4. Phage associated genes
5. Other genes identified on the RAST server and requiring explanation

6.3.1.1. Transposons & Plasmids

B. subtilis SG188 carries no transposon sequences or plasmids. Plasmids would have been identified from the sequencing contractor and in any event have been confirmed experimentally by failed attempts to identify plasmid DNA from cultures of SG188.

6.3.1.2. Antibiotic Resistance

Consideration is made of issues defined by the European Food Safety Authority (EFSA) and the Scientific Committee on Animal Nutrition (SCAN) as being of importance for the use of live bacteria as food or feed supplements. EFSA enunciated the following principles (EFSA, 2012):

- Where all strains within a given taxonomic group show a common resistance to an antimicrobial, the resistance could be intrinsic to the taxonomic group. Provided that the gene (or genes) conferring resistance is (are) not associated with plasmids or transposable elements, the risk of transfer to other organisms can be considered as minimal.
- Where resistance has been acquired by a strain belonging to a taxonomic group naturally susceptible to an antibiotic, the degree of risk of transfer is generally considered to be substantially greater than that associated with intrinsic resistance, unless it can be shown that the genetic basis of the acquired resistance is due to chromosomal mutation.
- Resistance by mutation of chromosomal genes presents a low risk of horizontal dissemination.

The risk in focus when evaluating safety of ingested microorganisms is the possible transfer of antibiotic resistance rather than its presence in itself.

Intrinsic resistance exists for a variety of reasons, e.g., lack of drug target or inability of drug to reach its target, and is based on the basic biology of the microorganism. Acquired resistance occurs following the acquisition of antibiotic resistance genes from another organism, usually by phage transduction, DNA-mediated transformation, and/or conjugation. It is normally considered that antibiotic resistance acquired on plasmid vectors is the most problematic since plasmids can be easily mobilized and are capable of inter-species gene transfer. Following this, the presence of transposable elements or IS elements on the genome can in principle provide a mechanism for gene transfer either for gene duplication or for transfer from chromosome to plasmid. Acquired resistance also encompasses the mutation of host genes and their functional products. EFSA considers that resistance which arises by mutation does not pose any particular risk (EFSA, 2012), stating that, “Any bacterial strain carrying an

acquired resistance to antimicrobials that is shown to be due to chromosomal mutation presents a low potential for horizontal spread and generally may be used as a feed additive."

EFSA has identified nine antibiotics of human and veterinary importance and recommended that bacteria used for food or feed supplements should ideally carry minimum inhibitory concentrations (MIC) at or below a defined 'breakpoint' as listed in Table 6.3 (based on EFSA, 2012).

Table 6.3: Antibiotic Resistances for SG188 and EFSA Breakpoint Values

Antibiotic type	Antibiotic	*Bacillus subtilis* SG188 MIC (µg/ml)	EFSA Breakpoint for *Bacillus* spp. (µg/ml)	Putative Mechanism for Resistance Present in Genome?	Conclusion
Aminoglycoside	Gentamicin	<0.0625	4	yes	Not a risk
	Kanamycin	0.125	8	yes	Not a risk
	Streptomycin	8	8	yes	Not a risk
	Neomycin	nt*	n.r.*	yes	Not a risk
Tetracycline	Tetracycline	2	8	no	Not a risk
Macrolide	Erythromycin	2	4	yes	Not a risk
Lincosamide	Clindamycin	1	4	yes	Not a risk
Chloramphenicol	Chloramphenicol	8	8	yes	Not a risk
β-lactam	Ampicillin	nt	n.r.	yes	Not a risk
Glycopeptide	Vancomycin	0.125	4	no	Not a risk
Streptogramine	Virginiamycin	nt	n.r	no	Not a risk
Oxazolidinone	Linezolid	nt	n.r	no	Not a risk
Diaminopyrimidine	Trimethoprim	nt	n.r	no	Not a risk
Fluoroquinolone	Ciprofloxacin	nt	n.r.	no	Not a risk
Rifamycin	Rifampicin	nt	n.r.	no	Not a risk

*nt = not tested; n.r. = not required by EFSA

B. subtilis strain SG188 carries no MIC value above breakpoint values so this strain satisfies QPS requirements.

However, 2 groups of coding ORFS are present on the genome that could be involved in antibiotic resistance:

GROUP 1: genes directly involved in antibiotic resistance or share homology with genes known to be involved in antibiotic resistance.

GROUP 2: genes that indirectly have the potential to be involved in antibiotic resistance, e.g., by providing efflux of antibiotics from the cell.

GROUP 1: ORFs Potentially Encoding Antibiotic Resistance

Chloramphenicol

Chloramphenicol acetyltransferase encoded by the *cat* gene is well known as the enzyme produced by bacteria that confers resistance to chloramphenicol. Whether the gene is repressed or not fully functional is not fully known.

SG188 carries a *cat* gene which is well characterised for producing resistance to chloramphenicol (Schwarz et al., 2004) and other studies have demonstrated clearly the presence of low levels of resistance in *B. licheniformis* (Adimpong et al., 2012). The coding ORF is most likely responsible for the MIC cut off value that is at the threshold dictated by EFSA. The same gene is found in other *Bacilli* and *Clostridia* and examination of the RAST server revealed its presence in *B. anthracis* and *B. clausii*. In the case of *B. clausii,* resistance is conferred by the chromosomal borne cat_{Bcl} gene and is postulated to have been acquired at some point by *B. clausii* (Galopin et al., 2009). The same gene was found in at least 20 *Bacillus* species by BLASTP searching. The presence of
this gene in so many *Bacillus* species strongly suggests that resistance is intrinsic.

Peg.3964 is labelled as encoding a chloramphenicol resistance protein but BLASTP searches reveal it to be a MFS transporter in *B. subtilis* and *B. licheniformis* (and ubiquitous in *Bacillus*), so this is an incorrect annotation on RAST, although as a transporter it could be involved in efflux of chloramphenicol.

Streptohricin

satA encoding Streptohricin acetyltransferase is present in 18 strains of *Bacillus* whose genomes have been sequenced. This includes *B. subtilis* strain 168. This suggests the resistance gene may be intrinsic to the *Bacillus* taxonomic group. Streptothricins (STs) are broad-spectrum antibiotics that inhibit protein biosynthesis in bacteria and also that of eucaryotes (yeast, fungi, insects and plants). STs are not used in humans as antibiotics due to their nephrotoxicity (Hoffmann et al., 1986a; Hoffmann et al., 1986b). Accordingly, the identification of a resistance gene in SG188 is not of immediate concern.

Ciproflaxin

Fluoroquinolones are synthetic broad-spectrum antibiotics, examples of which are ciproflaxin, leveoflaxin and moxifloxacin. There are numerous reports of quinolone toxicity and serious adverse reactions, and in the USA the FDA has concluded that fluoroquinolones may cause long-term damage in rare cases. The quinolones prevent the unwinding of bacterial DNA and thus inhibit cell replication. The fluoroquinolones are still used, for example, in the treatment of pneumonia and genito-urinary infections but resistance is rising (Acar and Goldstein, 1997). Due to the possibility of adverse effects fluoroquinolones are not used as first-line antibiotics but rather only after other antibiotics have failed. Quinolones have been used extensively in animals.

Examination of the SG188 sequence reveals 4 genes whose products (topoisomerase IV subunit A, topoisomerase IV subunit B, DNA gyrase subunit A and DNA gyrase subunit B) are the targets of the fluoroquinolones. These could serve as sites for developing resistance by mutation (Drlica and Malik, 2003). Resistance acquired through mutation is not a cause for concern by EFSA (EFSA Journal 2012;10(6):2740). No flanking sequences carried IS or Tn sequences that could infer mobilization. These sequences carry similar genes in a multitude of other *Bacillus* genomes.

Stepwise accumulation of resistance to ciproflaxin has been shown for *B. anthracis* and results from increased expression of multidrug transporters (Price et al., 2003). In principle, resistance could develop although there is no evidence for resistance with SG188.

Vancomycin

Vancomycin is a glycopeptide antibiotic used in the prophylaxis and treatment of infections caused by Gram-positive bacteria. It is a naturally occurring antibiotic made by the soil bacterium *Actinobacteria* species *Amycolatopsis orientalis* (formerly designated *Nocardia orientalis*). Vancomycin acts by inhibiting cell wall synthesis in Gram-positive bacteria. Due to the different mechanism by which Gram-negative bacteria produce their cell walls and the various factors related to entering the outer membrane of Gram-negative organisms, vancomycin is not active against Gram-negative bacteria (except some non-gonococcal species of *Neisseria*).

Vancomycin resistance (*van*^{R}) gene clusters (*vanR, S, H, A, X, Z, W*) are found in human pathogens such as *Enterococcus faecalis, Enterococcus faecium* and *Staphylococcus aureus*, glycopeptide-producing actinomycetes such as *Amycolotopsis orientalis,*

Actinoplanes teichomyceticus and *Streptomyces toyocaensis* and the non-glycopeptide producing actinomycete *Streptomyces coelicolor*. Expression of the *van* genes is activated by the VanS/VanR two-component system in response to extracellular glycopeptide antibiotic. Two major types of inducible vancomycin resistance are found in pathogenic bacteria; VanA strains are resistant to vancomycin itself and also to the lapidated glycopeptide teicoplanin, while VanB strains are resistant to vancomycin but sensitive to teicoplanin.

The *vanB*-type resistance is due to synthesis of peptidoglycan precursors ending in the depsipeptide D-Ala-D-Lac instead of the dipeptide D-Ala-D-Ala (Arthur et al., 1996). The organization and functionality of the *vanB* cluster is similar to that of *vanA* but differs in its regulation, because vancomycin, but not teicoplanin, is an inducer of the *vanB* cluster. *vanW* is a subtype of *vanB*. Interestingly in *B. subtilis* 168, the function of *vanW* is unknown. SG188 has only the *vanW* gene on the chromosome. Since *vanS* and *vanR* genes are not present in the SG188 chromosome and these genes control the expression of the *van* genes, the *vanW* gene might not be expressed. However, it is possible that the gene is regulated by other 2-component regulators which are not yet known or identified in the genome sequence. This could be an epistatic or indirect effect and low levels of vancomycin resistance may be observed.

Peg.2227 was found in >20 other *Bacilli* strains but in most cases the homologous genes were not identified as *vanW*. Vancomycin, though, targets the cell wall to exert its bactericidal effect and resistance to vancomycin would be achieved by altering the drug target, i.e., the cell wall. Tentatively, Peg.2227 could be involved in vancomycin resistance, but other genes appear to be lacking. A simpler explanation, based on the absence of other gene members is that the annotation on the RAST database is incorrect and *vanW* is not related to a role in vancomycin resistance and rather is involved in cell wall maintenance.

Bicylcomycin

Bicyclomycin is used to treat diarrhea. The Bcr/CflA protein is a drug transporter and has 12 membrane spanning domains in *E. coli* (Kohn and Widger, 2005). In *E. coli* it produces resistance to Bicyclomycin but in *Salmonella* and *Pseudomonas* it can produce resistance to chloramphenicol. The gene is present in many other *Bacilli* and in *B. thuringiensis* the protein is well described. It is not clear how this resistance determinant has arisen; most studies have been made with Gram-negatives, and these genes may contribute to resistance to Bicyclomycin and other antibiotics.

Tetracycline

Two proteins (Peg.3763 and Peg.3776) are annotated on the RAST database as tetracycline resistance proteins. BLASTP searches reveal that their closest relatives are MFS transporters from *B. licheniformis*. One homologous sequence from *B. subtilis* strain RO-NN- 1 has been labelled as encoding a tetracycline resistance protein (NCBI Reference Sequence Accession:NC_017195). This is a provisional assignment and may be an incorrect annotation. On the other hand, an MFS transporter could remove unwanted substances such as tetracycline by active efflux.

BLASTP analysis revealed >20 homologous proteins in other *Bacilli,* and in *B. subtilis* the closest homologues are MFS transporters where they have been linked to tetracycline efflux (Saier et al., 2002). Since the gene is ubiquitous and chromosomal borne it most probably accounts for a low level of intrinsic resistance reflecting modification or use of an existing efflux system.

Fosfomycin

Fosfomycin is a broad spectrum antibiotic produced by some species of *Streptomyces*. It is used for oral treatment of urinary tract infections, primarily those caused by *E. coli* (Falagas et al., 2010). It is becoming more frequently used due to the emergence of antimicrobial resistance to the Enterobacteriaceae. The European Committee on Antimicrobial Susceptibility Testings has suggested a breakpoint of susceptibility of Enterobacteriaceae to fosfomycin of 32 mg/l or less (www.srga.org/eucastwt/MICTAB/index.html). Fosfomycin inhibits cell wall synthesis and development of resistance is common in patients under therapy. Fosfomycin specifically targets and irreversibly inactivates MurA (UDP-Nacetylglucos-amine enolpyruvyltransferase) which catalyses the first step of peptidoglycan biosynthesis in bacteria. Resistance to fosfomycin in strains of Enterobacteriaceae may be high (Ellington et al., 2006). No breakpoints for fosfomycin have been provided by EFSA as of this date.

Fosfomycin resistance could arise in SG188 due to the presence of the FosB protein. FosB is found in *B. subtilis* strain 168 and other *Bacilli* and in all cases the gene is chromosomal and is a general housekeeping system involved in detoxification considered intrinsic to *Bacillus* (Cao et al., 2001b; Castañeda-García et al., 2013).

β -lactams

SG188 carries 10 ORFs that could encode proteins involved with resistance to β-lactams. β-lactamases are enzymes that can break the -lactam ring of β-lactam antibiotics (e.g., ampicillin, penicillin). β-lactams are broad-spectrum antibiotics and work against Gram-negatives and Gram-positives. They are secreted efficiently with over 90% of the protein exported. Ampicillin resistance in strains of *B. subtilis* varies considerably and MIC breakpoints for ampicillin have never been set or required by EFSA (EFSA, 2008b; EFSA, 2012). Bla is the class A β-lactamase and is found in numerous *Bacilli* (>22 genome sequences and most with 95-100% homology). *bla* is the key gene that encodes β-lactamase resistance in *Bacilli.*

Fosfidomycin

Fosfidomycin is an antibiotic produced by *Streptomyces* and is used in the treatment for malaria. It is not recognised by EFSA as an antibiotic of concern and is found in other *Bacilli*.

Aminoglycosides (Gentamicin, Kanamycin and Streptomycin)

Aminoglycoside resistance is conferred most commonly through enzymatic modification of the drug, enzymatic modification of the target rRNA through methylation or through the overexpression/modification of efflux pumps. Enzymatic modification can be achieved by the Aminoglycoside N6'–acetyltransferases (a member of the aminoglycoside adenylyltransferases) and in *B. subtilis* the *aadK* gene and its gene product (aminoglycoside adenylyltransferases) has been identified as being responsible for streptomycin resistance. Two putative coding ORFs are present that could contribute to resistance to aminoglycosides (Peg.1226 and Peg.1571). BlastP shows that these genes are found in a large number of *Bacillus* species so they are most likely intrinsic.

Therefore, it would be expected that SG188 carries some resistance to one or all of the above-mentioned aminoglycosides. Resistance would be further enhanced by the presence of efflux pumps (see section below on efflux pumps). Typically, aminoglycosidic modifying enzymes have been acquired at some point (Alekshun and Levy, 2007). Since the gene is isolated on the chromosome and is also found in a large number of *Bacilli,* it is probably intrinsic to the genus.

Erythromycin & Clindamycin

Erythromycvin, a macrolide, is normally modulated by efflux pumps in *Bacillus* but specific resistance mechanisms do occur. The phosphotransferase (Peg.4022) and esterase (Peg.4046) are probably involved in imparting resistance.

Clindamycin is a lincosamide class antibiotic and binds to the 23S portion of the ribosome and is essentially similar to the action of erythromycin. As noted above, the genome carries one ORF (Peg.4022) that would encode for a macrolide phosphotransferase. It is highly probable that this same enzyme also provides resistance to clindamycin. This assumption is based on the fact that in *Streptococcus*, strains that are resistant to macrolides (using a macrolide phosphotransferase) are usually also resistant to the lincosamides (Asmah et al., 2009; Prabhu et al., 2011). Resistance could also be achieved by one or more of the potential efflux pumps present on the chromosome.

GROUP 2: ORFs That May Indirectly Affect Antibiotic Resistance

Multidrug Resistance Proteins

The potential for resistance to multiple antibiotics is well-described and specific proteins (often membrane bound) that act as efflux pumps can play a role in resistance to multiple drugs but also to toxic compounds present in the environment (Neyfakh et al., 1991; Piddock, 2006). Using RAST, these ORFs are classified as resistance proteins and as efflux pumps but they should be considered as a single group of proteins that could potentially play a role in drug resistance and can serve as a mechanism for increasing resistance to toxic compounds or antibiotics. It should be noted that these proteins are thought to provide an intrinsic mechanism for resistance of the host and they are ubiquitous in almost all bacteria studied to date.

There are a considerable number of copies of a gene encoding the multidrug resistance protein B. In Gram-negative bacteria this protein enables the efflux of antibiotics from cells and could account for drug resistance to unspecified antibiotics (Alekshun and Levy, 2007). Since evidence for multi-drug resistance in *B. subtilis* has been reported (Neyfakh et al., 1991), it is possible that SG188 has the potential to develop resistance to unspecified drugs using a similar system of drug efflux.

In addition to multidrug resistance protein B, there are ORFs encoding proteins with homology to MarC, EbrA and EbrB . These proteins have been described elsewhere, but in *E. coli* can provide resistance not only to antibiotics (chloramphenicol and tetracycline) but also toxic compounds. They can also develop mutations conferring increased levels of resistance (Miller and Sulavik, 1996). A similar phenomenon could arise in SG188.

These proteins might contribute to the low levels of resistance to vancomycin, macrolides and tetracycline found in SG188.

Putative Efflux Pumps

One mechanism for bacterial resistance to antibiotics is active export of antibiotics using efflux pumps (Alekshun and Levy, 2007; Piddock, 2006). These proteins can be specific for a particular class of antibiotics. Five families of efflux pump have been described in bacteria: RND (resistance-nodulation-cell division), MF/MFS (major facilitator), SMR (staphylococcal/small multidrug resistance), ABC (ATP-binding cassette), and MATE (multidrug and toxic compound extrusion).

Energy for efflux can come from different sources, for example, the MFS, RND and SMR families use a transmembrane electrochemical gradient of protons while the MATE family uses sodium ions and the ABC family uses ATP-hydrolysis (Alekshun and Levy, 2007; Piddock, 2006).

Peg.3959 and Peg.4005 encode proteins with putative homology to the EmrB/QacA family of drug transporters. In *E. coli*, these proteins are involved in multi-drug resistance (Lomovskaya and Lewis, 1992).

It is probably not fruitful to speculate as to the precise functions of these coding ORFs since their specific action may vary between bacterial species. For example, the ErmB/QacA family of proteins may be involved in efflux of different antibiotics in different species. Moreover, these efflux pumps could also play a role in efflux of toxic compounds such as arsenic, bile etc. These genes are found in many *Bacillus* genomes (Saier et al., 2002).

These genes identified in the SG188 genome may or may not be involved in the efflux of antibiotics but where antibiotic resistance is found these genes and their encoded proteins are targets for the acquisition of spontaneous mutations. If mutated one or more of these genes may provide a low level of resistance. Since most of these genes are found in other *Bacilli*, this is of minor concern.

6.3.1.3. Other Genes Potentially Relevant to Virulence

Bacteriocins

Bacitracin

(1421.7 Da) is a non-ribosomally produced polypeptide antibiotic with activity against Gram-positive bacteria, and inhibits peptidoglycan biosynthesis (Johnson et al., 1945). Most of the bacitracin genes are also found in *B. subtilis* strain 168. Bacitracin is rarely used systemically or orally in humans since it is nephrotoxic but it is still used topically as an ointment (Phillips, 1999). Typically, bacitracin is produced from *B. licheniformis*. Studies in *B. subtilis* show that bacitracin production is inhibited during sporulation (Vitkovic and Sadoff, 1977). The presence of only 3 genes suggests that this antimicrobial cannot be synthesized.

Bacilysin

(271 Da) is one of the simplest peptide antibiotics and is a dipeptide comprised of L-alanine and L-anticapsine (Ozcengiz et al., 1990; Walker and Abraham, 1970).

Subtilosin

Subtilosin A is a 35 aa cyclic peptide with activity against *L. monocytogenes* and *B. cereus* (Babasaki et al., 1985; Zheng et al., 1999).

Biofilms

TasA is a 31 kDa sporulation-specific protein with broad spectrum bactericidal properties and is secreted into sporulation medium where it adsorbs to the spore surface (Stover and Driks, 1999). SG188 carries 5 genes involved in TasA biosynthesis indicating that TasA is synthesized. TasA is involved in biofilm production which, in turn, indicates that SG188 should be able to form biofilms (Wood et al., 1990).

Resistance to Bile Salts

The symporter participates by transporting bile salts to the cell cytoplasm. Resistance to bile suggests that this bacterium has developed a method to exist in the GI-tract.

Lipases and Amylases

There is historical evidence that lipases can result in Type-1 hypersensitivity. This was an issue for those involved in the production of these enzymes from *Bacilli* and laundry workers who are exposed to laundry detergent carrying these enzymes.

The lipase and lipase/acylhydrolases are both similar enzymes involved in degrading lipids. Lipases are found commonly in *B. subtilis* and are of commercial interest (Ma et al., 2006;

Nthangeni et al., 2001).

A detailed risk assessment by the EU sponsored program HERA (Human & Environmental Risk Assessment on ingredients of household cleaning products; November 2005; accessed at http://www.heraproject.com/ RiskAssessment.cfm?SUBID=38) assesses the risk of microbial derived lipases and amylases in laundry products. Following extensive toxicity testing (including oral and skin sensitization) the report concludes "*In conclusion it can be said, that use of amylases, cellulases and lipases in laundry and cleaning products represents no safety concerns for consumers."* This report allays recent concerns over the potential safety issues following exposure to *B. subtilis* (and other *Bacilli*) that produce lipases and amylases.

Adhesion Factors

The SG188 genome carries one gene that could encode a protein involved in binding to the mucosal cell matrix, i.e., fibronectin. This is a cell wall anchor protein. Since this organism is not a pathogen, the interpretation of this must be cautionary. If spores should germinate and liberate live vegetative cells in the GI-tract, then these would have superior adhesion properties than *Bacillus* species that lack either the collagen or fibronectin-binding proteins. In fact, adhesion of live cells to the gut mucosa is considered a beneficial trait for a beneficial bacterium.

6.3.1.4. Phage-Associated Genes

Phage genes in SG188 are found in two clusters. Cluster I probably represents an intact prophage. *Siphoviruses* (Lorenz et al., 2013) are common to *Bacillus* and this may correspond to a member of the Siphoviridae. Cluster II carries a lower number of phage like genes and is most probably PBSX. PBSX is common to most strains of *B. subtilis* and is a defective phage unable to transduce (Wood et al., 1990).

6.3.1.5. Biogenic Amines

Biogenic amines are biomolecules with one or more amine groups, and are nitrogenous organic bases of low molecular weight. They are produced by the microbial decarboxylation of precursor amino-acids by substrate-specific enzymes (Calzada et al., 2013). Histamine (regulated by histidine decarboxylase) and tyramine (regulated by tyrosine decarboxylase) are the most studied biogenic amines (Hagel and Facchini, 2005; Masini et al., 2005).

Some strains of *B. subtilis* are used in the production of fermented foods such as Natto where *B. subtilis* is used to impart texture, smell and taste to soybeans. The levels of biogenic amines in fermented soybeans have been reported (Eom et al., 2015). The presence of biogenic amines has been reported in some *Bacillus* strains and enzymes capable of degrading biogenic amines have been reported in other *Bacillus* species (Kim and Kim, 2006; Mah and Hwang, 2009). It has been proposed that amine degradation activity is common to the *Bacillus* genus (Zaman et al., 2010).

The mechanisms by which *Bacillus* strains control levels of biogenic amines are interesting and only now being researched, but it appears that, although biogenic amines can be produced, the bacterium also plays a regulatory role in reducing these compounds (Eom et al., 2015; Zaman et al., 2010) and is a consequence of the putrifying properties of these bacteria.

The SG188 genome carries a number of amine oxidase enzymes that might potentially be able to degrade biogenic amines. It could be expected that the action of one or more of SG188's decarboxylases could produce biogenic amines if suitable substrates were available. However, there seems to be mounting evidence that *Bacillus* as a genus has the ability to degrade these same amines. Thus, the risk of production of biogenic amines is minimal or highly unlikely based on existing evidence.

6.3.1.6. Summary

B. subtilis SG188 carries no plasmids, transposable elements, or prophages so the possibility of gene transfer is low other than by DNA-mediated transformation. It carries few genes mediating antibiotic resistance, mostly genes that could be involved in the efflux of antibiotics, and none of these appears to be transferable. Examination of the complete genome suggests that this strain should be considered benign.

6.3.2. *In Vitro* Tests

6.3.2.1. Hemolysis

SG188 showed no hemolysis and was γ hemolytic on sheep blood agar plates. Hemolysis, whether complete (β) or partial (α), suggests virulence. For *Bacillus* species, EFSA no longer requires hemolysis testing for strain approvals since this can be misleading due to the large numbers of surfactin-related antimicrobials/bacteriocins that are secreted by *Bacillus* species (Abriouel et al., 2011; EFSA, 2014a).

6.3.2.2. *In Vitro* Cell Cytotoxicity

A cytotoxicity test is suggested by EFSA as an important indicator of potential toxicity in members of the *Bacillus* genus outside of the *B. cereus* group (EFSA, 2014a). EFSA-compliant methods were used by SporeGen Ltd. to assess cytotoxicity to HT29 and Vero cells with comparators *B. subtilis* strain PY79 and two strains of *B. cereus*; data are shown in Table 6.4.

Table 6.4: *In Vitro* Cytotoxicity of *B. subtilis* strain SG188 (%)[a]

Expt. 1 (Dec. 14, 2016)	Toxicity on HT29 (%)	Toxicity on Vero (%)
B. subtilis SG188	0	0
B. subtilis PY79 (reference strain)	0	0
B. cereus SC2329 (positive control)	92	34
B. cereus SC2331 (positive control)	100	100
Triton-X-100 positive control	100	100
LB medium negative control	0	0

Expt. 2 (March 17, 2017)	Toxicity on HT29 (%)	Toxicity on Vero (%)
B. subtilis SG188	0	0
B. subtilis PY79 (reference strain)	0	0
B. cereus SC2329 (positive control)	99	97
B. cereus SC2331 (positive control)	100	100
Triton-X-100 positive control	100	100
LB medium negative control	0	0

[a] Experiment has been repeated and each run in triplicate with similar findings.

The results show that *B. subtilis* SG188 exhibits no toxic activity on the growth of HT29 and Vero cells. The genome analysis of SG188 revealed a number of surfactant-like molecules (Bacitracin, Bacilysin, Subtilosin) that could be synthesized by SG188 but these did not contribute to any cytoxicity. Therefore, SG188 satisfies the EFSA *in vitro* cytotoxicity test, which is normally only considered necessary if strains are found to be hemolytic (which SG188 is not).

EFSA has stated that cytotoxicity should use the Vero cell assay of Lindback and Granum (Lindback and Granum, 2005). Vero cells are derived from the kidneys of monkeys. Although the data show little difference between HT29 and Vero cells, HT29 is more representative since HT29 are of intestinal origin.

6.3.2.3. Adhesion

In the GI tract, *B. subtilis* SG188, as vegetative cells, might have the ability to adhere to the mucus lining. If this occurs, it could enable persistence within the GI tract. Adhesion is usually conducted with Caco-2 or HT29 cells since these are of human intestinal origin. However, for adhesion studies, intestinal cells (HT29-MTX) that produce mucus should be used since mucus forms a thick layer that coats the GI tract and is the most likely point of first contact. Adherence of *B. subtilis* strain SG188, 4 additional strains of *B. subtilis* (PY79, SG336, SC4025, and SG183), and one strain (SG42) of *B. pumilus* on HT29-MTX mucus-producing intestinal cells was assessed. As shown in Figure 6.1, strain SG188 showed low levels of adhesion to mucus in comparison to some of the other strains, notably *S. subtilis* strains SC4025 and SG183 and *S. pumilus* strain SG42. The data suggest that the adhesion ability of the vegetative cell form of SG188 is limited.

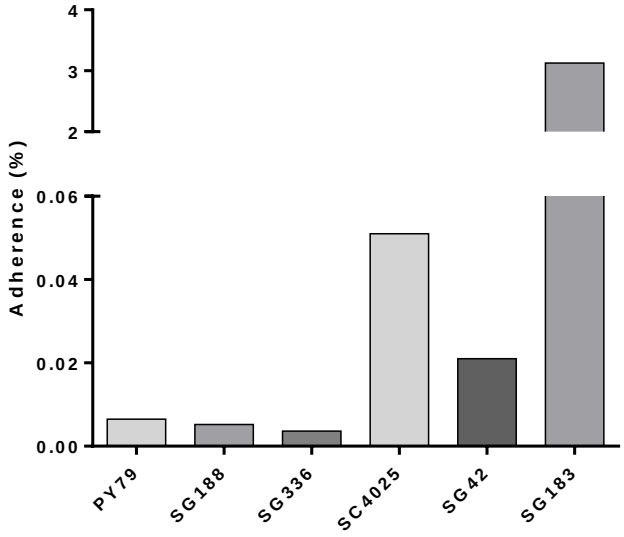

Figure 6.1: Adhesion of SG188 to mucus

Cells of *B. subtilis* SG188, *B. pumilus* SG42 and four strains of *B. subtilis*, SG183, SG336, SG2405 and PY79 were incubated on mucus producing cell lines for 2 hours, after which non-adhering cells were washed off. Remaining CFU was determined and expressed as a % of the initial inoculum.

6.3.2.4. Gastric Stability

The ability to survive in the GI tract could enable limited growth and proliferation of *B. subtilis* SG188. This would also support the use of this bacterium if survival can be demonstrated. In an unpublished study by SporeGen Ltd., a single colony from a fresh DSM agar plate was suspended in 2 ml of sterile LB broth in a yellow-capped test tube and incubated for 6 hours at 37° C in rotor. Next, 200 µl of culture was transferred into a new test tube containing 2 ml of DSM broth (for sporulation). Cultures were incubated overnight at 37° C in a roller drum. The next day cultures were examined under the microscope for the presence of spores. Then cultures were pre-heated for 45 minutes at 65° C to kill all vegetative cells. Pre-heated cultures were serially diluted and plated out onto DSM agar plates in duplicate to access the initial CFU number. They were then treated in one of the following conditions for 1 hour:

- 0.2% saline (2 mg NaCl/1 ml dH_2O)
- 0.2% bile salts (1 mg sodium cholate + 1 mg sodium deoxycholate/1 ml 0.2% saline)
- Simulated Intestine Fluid (SIF; 1 mg pancreatin/1 ml 0.2% saline)
- Simulated Gastric Fluid (SGF; 3.5 mg pepsin/1 ml of 0.2% saline); pH adjusted to 4, 3 or 2 using concentrated hydrochloric acid

Table 6.5: Gastric and Bile Resistance

Treatment	Initial CFU (Spores)	Final CFU (Spores)	% of Initial
0.2% saline	1.5 X 10^6	1.4 X 10^6	93%
SIF	1.5 X 10^6	8.0 X 10^4	5%
SGF pH 2	1.5 X 10^6	1.0 X 10^6	67%
SGF pH 3	1.5 X 10^6	1.6 X 10^6	110%
SGF pH 4	1.5 X 10^6	1.0 X 10^6	67%

B. subtilis SG188 spores showed resistance to gastric fluids, including pH 2, which reflects the robustness of spores. This demonstrates that SG188 could be used for oral administration since the stomach is rarely at pH 2 and a pH range of 3-4 is more typical.

6.3.3. Animal Studies

6.3.3.1. Persistence in the GI Tract

Groups of 4 BALB/c inbred mice (female, aged 7 weeks) were given 1 dose of $2x10^9$ pure spores of *B. subtilis* strain SG188 by oral gavage (0.2 ml). *B. subtilis* strains PY79, SG336, and SC4025 served as comparators. Feces were collected at daily intervals and homogenized in PBS buffer. Homogenates were heated at 68°C for 45 minutes to kill all vegetative microbiota and the suspension was serially diluted and plated on DSM agar plates. *S. subtilis* strain SG188 had a relatively fast transit time through the GI-tract and was not detectable after 3 days. Strain SG188 did not persist as long as strain SC4025 (the Natto strain of *B. subtilis*). This agrees with the *in vitro* adhesion studies discussed above that showed that strain SG188 had lower adhesion to gut enterocytes than the Natto strain SC4025. It is concluded that SG188 has the potential to persist briefly within the GI tract but is relatively quickly shed.

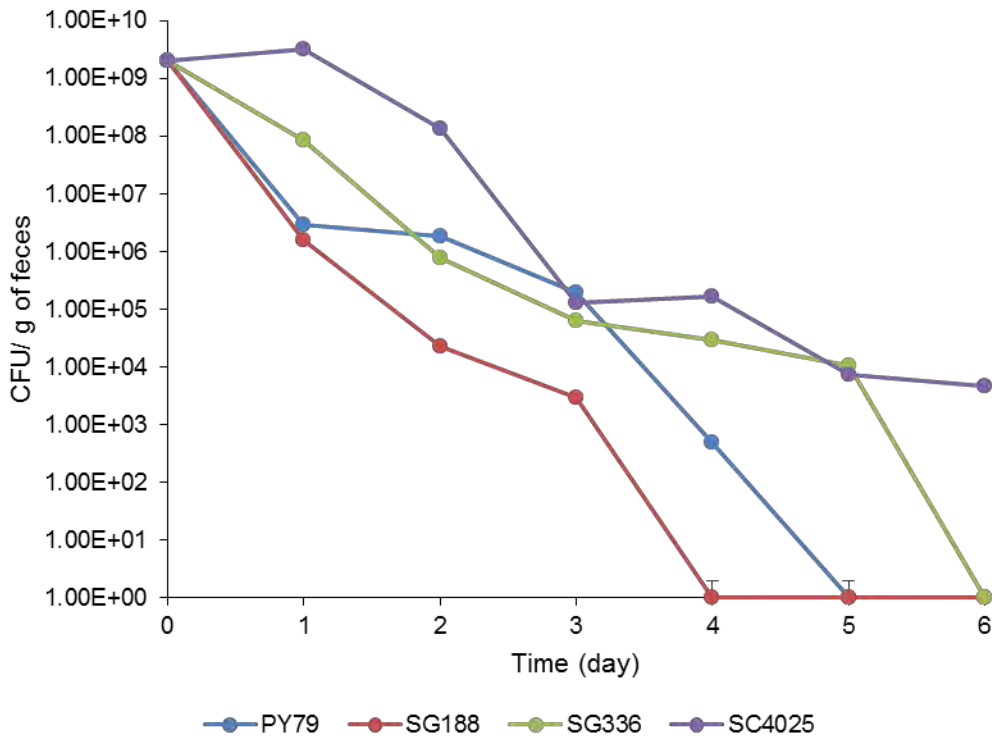

Figure 6.2: Shedding of *B. subtilis* from the Murine GI Tract

Groups of mice were dosed once with spores of SG188 and 3 other strains of *B. subtilis* following a single (i.g.) dose of pure spores (2 X 10^9 spore CFU) in mice. CFU of heat-resistant spores in the feces determined thereafter.

6.3.3.2. Toxicity Studies

Toxicity testing has been performed both for living *B. subtilis* and for enzymes and other substances expressed by the bacteria. These studies are briefly summarized below:

- Biziulevichius and Arestov (1997) reported on acute and subchronic tests of oral toxicity of lysosubilin (a preparation of lytic enzymes from *B. subtilis*) carried out in mice, rabbits, and calves. In mice, oral gavage administration of doses of lysosubilin up to $2x10^6$ U/kg bw ($3.3x10^4$ mkatal/kg bw) produced no reported toxic effects, as did doses of $2x10^4$, $8x10^4$, and $2x10^5$ U/kg bw/day given by gavage for 30 days. Rabbits receiving the same doses for the same period of time also exhibited no reported evidence of toxicity. In calves, no adverse effects were reported from doses of lysosubilin up to $4x10^4$ U/kg bw/day for 30 days. All doses were choses as multiples of normal dosing levels in veterinary clinical practice.

- Harbak and Thygesen (2002) reported that *in vitro* genotox testing of xylanase expressed by *B. subtilis*—a bacterial reverse mutation assay and a chromosomal aberration test--found no evidence of mutagenic potential or chromosomal aberrations.
- Lampe and English (2016) reported on a toxicological assessment of nattokinase, a fibrinolytic enzyme produced by *B. subtilis*. *In vitro* testing included a bacterial reverse mutation assay and chromosomal aberration test, while *in vivo* testing comprised
  - acute oral toxicity in Sprague-Dawley rats (gavage doses of 0, 1000, and 2000 mg/kg bw; 6 rats/sex/dose),
  - 28-day oral toxicity in Sprague Dawley rats (gavage doses of 0 and 167 mg/kg bw/day; 6 rats/sex/dose), and
  - 13-week oral toxicity in Sprague-Dawley rats (gavage doses of 0, 100, 300, and 1000 mg/kg bw/day; 12 rats/sex/dose).

  The authors reported that no adverse effects were observed and the NOAEL in the 90-day study was1000 mg/kg bw/day, the highest dose tested. Lampe and English (2016) also reported that nattokinase at 10 mg/kg bw/day for 4 weeks was well tolerated by 5 healthy males and 6 healthy females.
- As part of an assessment of the safety of *B. subtilis*, Hong et al. (2008) reported an acute oral toxicity study in which guinea pigs (5 animals/ sex/dose) received gavage doses of 0 or $1x10^{12}$ spores of *B. subtilis*, and a repeated-dose oral toxicity study in which male New Zealand white rabbits (6 rabbits/dose) received daily gavage of 0 or $1x10^{9}$ spores for 30 days. The authors concluded that no evidence of toxicity or virulence was observed.
- MacKenzie et al. (1989) assessed the safety of α -amylase expressed by *B. subtilis* in repeated-dose studies in Fischer 344 rats and beagle dogs. In the rat study, the F0 generation (26 rats/sex/dose) received 0, 36, or 72 U/g feed for 4 weeks before breeding and through weaning of the F1 pups (20 F1 rats/sex/dose), which received the same diets for 13 weeks from weaning. Four beagles/sex/dose received similar diets for 13 weeks. The authors reported that there were no intervention-related adverse effects on clinical signs, reproductive performance or outcome, ophthalmology, hematology, macroscopic findings at necropsy, or microscopic/histopathologic findings. Male and female dogs and rats receiving 72 U α -amylase/g food showed significantly reduced weight gain and male dogs had some statistically significant deviations in

clinical chemistries. The authors concluded that "the no-observable-effect level for alpha-amylase fed to dogs or rats is 36 units/g food."

- Nakamura et al. (1999), in a subchronic oral toxicity study with Fischer 344 rats (10 rats sex/dose), assessed the potential toxicity of *B. subtilis* gum at dietary concentrations of 0, 0.18, 0.55, 1.66, and 5%. No adverse effects were reported on mortality, feed intake or body weight, biochemistry, urinalysis, or histopathology, and the authors concluded that "*B. subtilis* gum in the diet for 90 days does not exert toxicity in rats even at the highest dose."
- Sorokulova et al. (2008) assessed the safety of strain *B. subtilis* VKPM B2335 in a study of acute oral toxicity in male BALB/c mice and repeated-dose studies in mice, rabbits, and pigs (sex and strain not reported). In the acute study, 40 male mice were assigned to 4 groups (10 mice/group) to receive single gavage doses of 0, $5x10^7$, $5x10^8$, or $2x10^{11}$ cfu/ mouse. Mice were observed for 7 days and organs were subjected to histological analysis following necropsy. In the first repeated-dose study with an in-life duration of 10 days, 10 male mice received gavage doses of 0 or 10^6 cfu/day while 10 male rabbits and 10 male piglets received gavage doses of 0 or 10^9 cfu/day; tissues were subjected to histological analysis following necropsy on day 11. In a second repeated-dose study, 10 male rabbits received gavage doses of 0 or 10^9 cfu/day for 30 days; the animals were necropsied and blood subjected to hematological analysis and selected tissues were examined for histopathological effects. The authors reported that no adverse effects were observed in any of the studies, and concluded that "*B. subtilis* strain may be considered as non-pathogenic and safe for human consumption."

6.3.3.3. Feeding Studies

Because numerous strains of *B. subtilis* are widely used in animal husbandry, feeding studies comparing feed with or without added *B. subtilis* have been conducted in a number of species of animals, including fish, chickens and turkeys, swine, and cattle. A small sampling of these studies is summarized below.

- Geng et al. (2011) fed cobia (*Rachycentron canadum*) diets containing 0, $2x10^{10}$, or $4x10^{10}$ cfu *B. subtilis*/kg for 8 weeks and reported enhance growth and survival with no adverse effects.
- Xing et al. (2015) fed 180 one-day-old female Linwu ducks diets containing 0, $5x10^8$, or $5x10^{10}$ cfu *B. subtilis*/kg for 9 weeks and reported that "dietary supplementation with lysine-yielding *B. subtilis* improved gut morphology, increased the population of

beneficial gut microflora, and stimulated increased intestinal immune response of Linwu ducks."

- Wolfenden et al. (2011) fed 7-day-old turkey poults meal containing 0 or 10^6 spores of *B. subtilis* strain PHL-NP122/g feed for 16 days, reporting that inclusion of the bacterium in the feed had no adverse effects while reducing *Salmonella* spp. in the ceca and improving growth.
- Wu et al. (2011) tested the effect of feeding meal containing *B. subtilis* KD1 on the intestinal microbiota of broiler chickens, feeding the chickens meal with 0, 10^9, $5x10^9$, or 10^{10} spores/kg feed. *Lactobacillus* spp. significantly increased while *E. coli* decreased, and the authors concluded that "*B. subtilis* KD1 is a promising probiotic organism in broilers."
- Knap et al. (2011) tested the ability of *B. subtilis* strain DSM17299, to reduce *Salmonella* in broilers. In addition to reducing *Salmonella* loads, inclusion of *B. subtilis* in the feed for 6 weeks produced non-significant improvements in feed conversion and bodyweight gain.
- Forte et al. (2016) fed 600 16-week-old Hy-Line layer hybrids meal with 0 or 500 mg/kg *B. subtilis* for 14 weeks. No adverse effects were reported on clinical signs or clinical chemistries of the laying hens or their eggs, and the authors concluded that the bacterium "had beneficial effects on hen metabolism and welfare."
- Guo et al. (2006) fed *B. subtilis* strain MA139 to 72 35-30-day-old piglets (18 piglets/dose) at concentrations of 0, $2.2x10^5$, $2.2x10^6$, or $2.2x10^7$ cfu/g feed for 28 days. No adverse effects were reported, while loads of *E. coli* decreased, and the authors concluded that "*B. subtilis* MA139 is a promising alternative to antibiotics for use as a feed additive in piglet diets."
- Peng et al. (2012) assessed the effect of *B. subtilis* natto fermentation products on blood metabolites, rumen fermentation, and milk production and composition of dairy cows. Thirty-six Holstein cows were randomly assigned to receive diets supplemented with 0, 6, and 12 g *B. subtilis* natto fermentation product/day (12 cows/group) for 9 weeks. The authors reported that, "The findings show that *B. subtilis* natto fermentation product was effective in increasing lactation performance of early lactation dairy cows possibly by altering the rumen fermentation pattern without any negative effects on blood metabolites."

- Sun et al. (2013) assessed the effect of *B. subtilis* natto on rumen fermentation, ruminal microbiome and milk production and composition of dairy cows. Thirty-six Holstein cows were randomly assigned to receive diets supplemented with 0, $5x10^{10}$, or 10^{11} cfu *B. subtilis* natto for 70 days. No adverse effects were reported, and the authors concluded that "*B. subtilis* natto improves milk production and milk components yield, decreases [milk somatic cell counts] and promotes the growth of total ruminal bacteria, proteolytic and amylolytic bacteria, which indicate that *B. subtilis* natto has potential to be applied as a probiotic for dairy cows."
- Zhang et al. (2016) studied the effect of dietary supplementation with *B. subtilis* strain B27 on growth performance, nutrient digestibility, and stress-related indicators in dairy calves. Sixteen neonatal Holstein calves received diets with 0 or $1.7x10^8$ cfu/calf/day for 58 days. No significant effects, either adverse or beneficial, were reported.

6.3.4. Human Studies

Most human studies of *B. subtilis* strains have had efficacy in treating disease or disorder as the primary objective, but a number of published studies included safety assessments.

- In a study unusual in that it focused on safety and tolerance rather than efficacy, Hanifi et al. (2015) established oral-dose tolerance and gastrointestinal viability of *B. subtilis* R0179 in a randomized, prospective, double-blind, placebo-controlled trial in 81 healthy adults who received the strain at doses of 0, 0.1, 1.0, or $10x10^9$ cfu/day for 28 days. GI transit viability of *B. subtilis* was assessed and general wellness during the trial was evaluated with a daily questionnaire addressing GI, cephalic, ear-nose-throat, behavioral, emetic, and epidermal symptoms. The authors reported that, "General wellness and GI function were not affected by oral consumption of *B. subtilis* R0179 at any dose." They concluded that, "*B. subtilis* R0179 survives passage through the human GI tract and is well tolerated by healthy adults at intakes from 0.1 to $10x10^9$ cfu/day."
- Vukovic (2001) assessed the efficacy and safety of *B. subtilis* strain IP5832 in the management of patients with acute non-typhoid *Salmonella* gastroenteritis in a multicenter, randomized, prospective, double-blind, placebo-controlled clinical trial with 63 patients of both sexes aged 20-52 years of age. Patients received 6 capsules per day, each capsule containing 0 (32 patients) or 10^9 spores of *B. subtilis* (31 patients) for 7 days. No strain-related adverse effects were reported while the intervention significantly reduced the *Salmonella* load.

- Zhao et al. (2004) gave capsules containing *B. subtilis* and *Enterococcus faecium* to 25 patients with liver cirrhosis for 14 days and measured blood ammonia, fecal pH, fecal ammonia, and plasma endotoxin. All tested markers moved significantly in beneficial direction with no reported adverse reactions to the bacteria.
- Pushkarev et al. (2007) evaluated the use of *B. subtilis* strain 3H in the management of patients with nosocomial urinary tract infections. Seventy-one patients with infravesical obstruction received capsules containing 0 (35 patients) or $5x10^9$ spores (36 patients). No adverse effects of the intervention were reported, and it significantly reduced the pathogen load.
- Lee et al. (2010) tested the use of a preparation containing *B. subtilis* and *Streptococcus faecium* for pre-colonoscopy cleansing in both constipated and healthy patients. Patients received either the experimental preparation (51 constipated and 53 healthy patients) or placebo (53 constipated and 54 healthy patients) for 2 weeks. No adverse reactions were reported and the experimental preparation improved cleansing and reduced postendoscopic gastrointestinal symptoms in constipated patients while having no significant effect in healthy patients.
- Li et al. (2012) evaluated the safety and efficacy of management with *B. subtilis* and *E. faecium* of functional constipation in a multicenter, prospective, randomized, double-blind, placebo-controlled trial. A total of 216 patients received lactulose plus either 0 (n = 112) or $3x10^9$ cfu (n = 104) for 28 days. No adverse effects were reported and the authors concluded that "the regimen of live combined *B. subtilis* and *E. faecium* capsules plus lactulose offers better efficacies in the treatment of functional constipation."
- Lefevre et al. (2015) studied the ability of *B. subtilis* strain CU1 to reduce infectious disease incidence in healthy elderly. In a randomized, prospective, double-blind, placebo-controlled trial, 100 apparently healthy men and women aged 60-74 years consumed capsules providing 0 or $2x10^9$ *B. subtilis* spores each day during four 10-day periods, with 18 days separating each test period. Any benefits of the intervention were statistically non-significant, but the authors reported that, "There were no abnormal values of biological parameters at the end of the study, and no clinically significant variation was observed during the study, on renal and hepatic functions." They concluded that the use of *B. subtilis* in elderly humans is safe. In a follow-up publication (Lefevre et al., 2017), the authors presented a more detailed assessment of safety, concluding that "*B. subtilis* CU1 was safe and well-tolerated in the clinical

subjects without undesirable physiological effects on markers of liver and kidney function, complete blood counts, hemodynamic parameters, and vital signs."

- McFarlin et al. (2017) screened 75 apparently healthy men and women for post-prandial dietary endotoxemia, and the 28 responders were randomized in a prospective, double-blind, placebo-controlled trial to receive capsules providing 0 (n = 13) or $4x10^9$ spores of *B. subtilis* HU58, *B. indicus* HU36, *B. coagulans, B. licheniformis*, and *B. clausii* (n = 15) for 30 days. The authors reported that the supplementation reduced symptoms indicative of "leaky gut" syndrome with no reported adverse effects.

In a review article, Tompkins et al. (2010) discussed the findings of 23 published studies involving over 1,800 adults of a preparation comprising 10^9 spores of *B. subtilis* R0179 and *E. faecium* R0026. The preparation was used in patients with acute and chronic diarrhea, irritable bowel syndrome, ulcerative colitis, and *Helicobacter pylori* infection. Tompkins et al. (2010) summarized reports of adverse events as follows:

> Several of the clinical trials reported adverse event details. Most studies reported no adverse events even when liver and kidney biochemical function parameters and blood tests were evaluated. [One trial] reported one case of nausea in the [probiotic] + sulfasalazine group and one case of nausea with vomiting and one case of dizziness in the sulfasalazine-only arm. No patients were withdrawn from the trial and the symptoms persisted after the conclusion of the intervention phase but disappeared shortly thereafter. In [one study] with patients with diarrhoea, a number of events were noted in the probiotic groups but nothing was mentioned for the control groups. In the probiotic treated groups they observed one case of nausea, one headache, another with dizziness and one report of being flustered. There were also two cases of urinary infection that cleared up after treatment and were not attributed to the probiotic bacteria. Similarly, one case of relapse in a patient with chronic prostatitis was also not caused by the probiotic microbes. Finally there was one case with left ventricle blockage as shown by electrocardiogram which was detected prior to the intervention. Again, there was no mention of withdrawals and all patients completed the treatment phase. In summary, all of the studies conducted thus far conclude that no adverse reactions, nosocomial or otherwise, were directly linked to the use of [the probiotic]" (Tompkins et al., 2010).

6.4. Decision-Tree Analysis

A published decision tree (Pariza et al., 2015) was utilized to assess the evidence regarding the safety of the intended use of *B. subtilis* SG188. Significant questions follow:

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? YES
2. Has the strain genome been sequenced? YES
3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? YES
4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? YES
5. Does the strain produce antimicrobial substances? NO
6. Has the strain been genetically modified using rDNA techniques? NO
7. Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')? NO (THE STRAIN WAS ISOLATED FROM THE FECES OF A HEALTHY HUMAN)
8. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? WHILE STRAIN SG188 HAS NOT BEEN TESTED, NUMEROUS OTHER *BACILLUS SUBTILIS* STRAINS WITH CLOSE HOMOLOGY TO STRAIN SG188 HAVE BEEN TESTED AND FOUND TO BE NON-PATHOGENIC AND NON-TOXIGENIC.

The outcome of this decision-tree analysis is that “the strain is deemed to be safe for use in the manufacture of food … for human consumption” (Pariza et al., 2015).

6.5. Reviews by Authoritative Bodies

Noting that a wide variety of microbial species are used in food, some with a long history of apparent safe use, and facing the need to set priorities for risk assessment, the European Food Safety Authority (EFSA) proposed a system referred to as “Qualified Presumption of Safety” (QPS; EFSA, 2007a). This system proposed basing the safety assessment of a defined taxonomic group (e.g., a genus or a species) on 4 pillars: established identity, body of knowledge, possible pathogenicity, and end use. If the taxonomic group did not raise safety concerns or, if safety concerns existed, but could be defined and excluded, the grouping could be granted QPS status. Thereafter, “any strain of microorganism the identity of which could be unambiguously established and assigned to a QPS group would be freed from the need for further safety assessment other than satisfying any qualifications specified” (EFSA, 2007a, p1).

EFSA's Scientific Committee was asked to recommend organisms regarded as suitable for QPS status. The list of such organisms proposed by the Committee included a number of *Bacillus* species, including *B. subtilis*. In listing *B. subtilis* and other species of *Bacillus* as suitable for QPS status, the Committee stated, "Where QPS status is proposed, the Scientific Committee is satisfied that the body of knowledge available is sufficient to provide adequate assurance that any potential to produce adverse effects in humans, livestock or the wider environment is understood and capable of exclusion" (EFSA, 2007a, p8) and that the recommendations are "based on a thorough review of the available scientific literature and the knowledge and experience of the scientists involved" (EFSA,2007a, p8).

With regard to *B. subtilis* (along with *B. pumilus, B. clausii, B. licheniformis, B. vallismortis, B. mojavensis, B. lentus, B. coagulans, B. fusiformis, B. atrophaeus,* and *B. amyloliquefaciens*), the Committee noted that these species "can be reliably identified using a 16S rRNA gene sequence" (EFSA, 2007b). It further reported that *B. subtilis* and several other *Bacillus* strains "have been used as probiotics, animal feed supplements, or in aquaculture . . . Furthermore, several *Bacillus* species are involved in the preparation of traditional fermented dishes in Africa and Asia."

At the same time, the Committee did not propose for QPS *Bacillus* spp. belonging to the *Bacillus cereus sensu lato* group (*B. cereus sensu stricto, B. mycoides, B. pseudomycoides, B. thuringiensis*, and *B. weihenstephanensis*) because many strains within this group are toxin producers. The Committee further stated that the bacteria on the QPS list were granted QPS "due to the substantial body of knowledge available about these bacteria," while requiring that, "Since all bacteria within the listed species potentially possess toxigenic traits, absence of toxigenic activity needs to be verified for qualification" (EFSA, 2007b).

Finally, the Committee reported that (as of 2007), "Annotated genome data are currently available for several strains within the species *B. clausii, B. cereus, B. licheniformis, B. subtilis, B. thuringiensis*, and *Geobacillus kaustophilus*, thereby contributing significantly to the body of knowledge and decreasing the probability that unforeseen hazards could be associated with these bacilli" (EFSA, 2007b). Since that time, annotated genome data (based on more complete genome sequences and on more extensive gene-function libraries) have become available on a greater number of *Bacillus* strains, including *B. subtilis* SG188.

In December 2008, EFSA's Panel on Biological Hazards released an opinion reassessing the QPS status of *B. subtilis* and other *Bacillus* strains (EFSA, 2008). The Panel determined that no changes were needed; no new evidence calling into question the QPS status of *B. subtilis* was introduced. EFSA has repeated these same opinions in more recent updates through March 2019 (EFSA, 2019).

In Japan, *B. subtilis* is permitted for use in FOSHU—foods for specified health use (Shimizu, 2003).

In Canada, 8 enzymes derived from *B. subtilis* are permitted in a variety of foods (Health Canada, 2014b) and the bacteria themselves are permitted in 2 natural health products licensed for use in Canada (Health Canada, 2014a).

In the United States, the Environmental Protection Agency reviewed the safety of *B. subtilis* and concluded that it is "a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low" (EPA 1997).

FDA affirmed as GRAS carbohydrase and protease enzyme preparations derived from nonpathogenic and nontoxigenic strains of *B. subtilis* (21 CFR § 184.1148 and § 184.1150, respectively). Since introduction of the GRAS notice program, FDA has accepted without question 9 GRAS notices: GRN20, GRN114, GRN205, GRN274, GRN406, GRN476, GRN579, GRN 592, and GRN 649.

6.6. Safety Assessment and GRAS Determination

6.6.1. Evidence of Safety

A summary of the basis for establishing the safety and for determining the GRAS status of the intended use of *B. subtilis* SG188 at a recommended dose not exceeding 10^9 CFU per serving as a food ingredient is presented below.

B. subtilis SG188 is a Class 1 microorganism with no observed potential for virulence to humans. *B. subtilis* SG188 is designated as QPS and safe for human consumption where

absence of toxicity is confirmed and where no obvious indicators of antibiotic resistance are present.

Both FDA and EPA have indicated a lack of concern with *B. subtilis* or its products.

Evidence that SG188 lacks pathogenicity and toxicity includes the following:

- *B. subtilis* SG188 was not cytotoxic in EFSA-recommended *in vitro* tests;
- Analysis of the complete genome of *B. subtilis* SG188 did not indicate any apparent agents of virulence or the presence of any enterotoxin genes;
- *B. subtilis* SG188 carries antibiotic MICs below threshold levels for antibiotics considered of medical relevance/importance;
- *B. subtilis* SG188 carries no prophages, plasmids, or transposable elements, so the possibility of gene transfer and importantly transfer of antimicrobial resistance is low.
- The genome of *B. subtilis* SG188 shows high homology with those of other strains of the species that have been subjected to toxicity testing, animal feedings studies, and human clinical trials with no reported adverse effects. Additionally, *B. subtilis* strains have a long history of safe use as supplements in animal feeds and in fermented foods widely consumed by humans.

6.6.2. Conclusion of the GRAS Panel

The intended use of *B. subtilis* SG188 in a variety of conventional foods was determined to be safe and suitable and GRAS through scientific procedures set forth under 21 CFR § 170.30(b). This conclusion of the safety and GRAS status of the proposed use of *B. subtilis* SG188 followed the independent and collective critical evaluation of the available information and data on *B. subtilis* SG188 summarized in this dossier and other information deemed appropriate by the GRAS Panel.

Determination of the safety and GRAS status of the addition of *B. subtilis* SG188 to conventional foods has been made through the deliberations of a GRAS Panel consisting of Joseph F. Borzelleca, Ph.D., Michael W. Pariza, Ph.D, and James T. Heimbach, Ph.D. (as editor of the monograph and advisor to the panel), who reviewed a monograph prepared by Dr. Simon Cutting and other information deemed appropriate. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients, including bacteria. They independently critically evaluated the publicly available information and the potential human exposure to *B. subtilis* SG188 anticipated to result from its intended uses,

and individually and collectively determined that no evidence exists in the available information on *B. subtilis* SG188 that demonstrates, or suggests reasonable grounds to suspect, a hazard to either adults or children under the intended conditions of use of *B. subtilis* SG188.

The GRAS Panel prepared the attached statement setting forth their conclusion.

Part 7: List of Supporting Data and Information

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CONCLUSION OF THE GRAS PANEL: DETERMINATION OF THE GRAS STATUS OF THE USE OF *BACILLUS SUBTILIS* STRAIN SG188

Prepared for:
SporeGen Ltd.

November 2019

CONCLUSION OF THE GRAS PANEL:

We, the members of the GRAS Panel, have individually and collectively critically evaluated the publicly available information on *Bacillus subtilis* strain SG188 summarized in a monograph, *Generally Recognized As Safe (GRAS) Determination for the Intended Use of* Bacillus subtilis *Strain SG188* (November 2019), prepared by Prof. Simon M. Cutting, and other material deemed appropriate or necessary. Our evaluation included critical evaluation of the identity, genotypic and phenotypic characteristics of the strain, production methods, potential exposure resulting from the intended use of the strain, and published research bearing on the safety of *B. subtilis* strain SG188. Our summary and conclusion resulting from this critical evaluation are presented below.

Summary

- *Bacillus subtilis* strain SG188 is intended to be incorporated in food matrices at concentrations not exceeding 10^9 spores per serving.
- *B. subtilis* strain SG188 was isolated from healthy human feces and deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen with deposit number DSM 32444. *B. subtilis* is a member of the genus *Bacillus sensu stricto*, which is distinguished from a clade that includes *B. cereus* and similar pathogenic species. All members of the genus are spore-forming, Gram-positive, catalase-positive, mesophilic, and motile.
- A complete genome sequence of the strain revealed a size of 4,013,943 base pairs forming 4,179 coding sequences. The strain has no evidence of plasmids, insertion sequences, or transposons. Annotation identified 2 groups of genes potentially encoding antibiotic resistance (all intrinsic and most apparently non-expressed), and one gene providing resistance to bile salts. No apparent agents of virulence or enterotoxin genes were identified.
- *B. subtilis* strain SG188 is produced under cGMP and each batch is assessed for compliance with food-grade specifications, including spore density, microbiological purity, and levels of heavy metals and mycotoxins. Because the strain is released in the form of freeze-dried spores, it demonstrates a high level of both temporal and thermal stability during storage and in food matrices.
- *Bacillus* species are used worldwide for use in humans. Species that have been employed in human studies include *B. subtilis, B. megaterium, B. coagulans, B. clausii*, and *B. licheniformis. B. subtilis* spores have been a component of the Japanese staple Natto for centuries at a concentration greater than 10^8 live spores/g.
- In *in vitro* testing, *B. subtilis* strain SG188 showed no hemolysis, cytotoxicity, or acquired antibiotic resistance. Study of acute and repeated-dose oral toxicity in mice, rats, guinea pigs, rabbits, dogs, pigs, and calves of *B. subtilis* strains and substances expressed by the bacteria showed no adverse effects. Feeding studies in fish, chickens and turkeys, swine, and cattle demonstrated that addition of *B. subtilis* to animal feeds is safe. The safety of *B. subtilis* species in humans, both healthy and compromised (e.g., with urinary tract infections, constipation, liver cirrhosis, or acute gastroenteritis) was reported in numerous studies.

- A decision-tree analysis (Pariza et al. 2015) determined that the strain is deemed to be safe for use in the manufacture of food for human consumption based on the following responses:
    - Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? YES
    - Has the strain genome been sequenced? YES
    - Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? YES
    - Is the strain genome free of functional and transferable antibiotic resistance gene DNA? YES
    - Does the strain produce antimicrobial substances? NO
    - Has the strain been genetically modified using rDNA techniques? NO
    - Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component (not simply an 'incidental isolate')? NO (THE STRAIN WAS ISOLATED FROM THE FECES OF A HEALTHY HUMAN)
    - Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? WHILE STRAIN SG188 HAS NOT BEEN TESTED, NUMEROUS OTHER BACILLUS SUBTILIS STRAINS WITH CLOSE HOMOLOGY TO STRAIN SG188 HAVE BEEN TESTED AND FOUND TO BE NON-PATHOGENIC AND NON-TOXIGENIC

Conclusion

We, the undersigned members of the GRAS Panel, are qualified by scientific education and experience to evaluate the safety of microorganisms intended for addition to foods.
We have individually and collectively critically evaluated the publicly available information on *Bacillus subtilis* strain SG188 summarized in a monograph, *Generally Recognized As Safe (GRAS) Determination for the Intended Use of* Bacillus subtilis *Strain SG188* (November 2019), prepared by Prof. Simon M. Cutting, and other material deemed appropriate or necessary.

We have individually and collectively determined that no evidence exists in the available information on *B. subtilis* strain SG188 that demonstrates, or suggests reasonable grounds to suspect, a hazard to either adults or children under the intended conditions of use of *B. subtilis* strain SG188.

We unanimously conclude that the intended use as an ingredient added to conventional foods of *Bacillus subtilis* strain SG188, produced consistent with current good manufacturing practice (cGMP) and meeting the food-grade specifications presented in the monograph, is safe and is GRAS by scientific procedures.

It is our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions.

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

[redacted] Date: 21 November 2019

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

James T. Heimbach, Ph.D. (Monograph Editor and Advisor to the GRAS Panel)
President
JHeimbach LLC
Port Royal, Virginia

Signature: _____ Date: _____

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Professor Emeritus
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Signature: _____ Date: _____

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Signature  Date: November 21, 2019

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Madison, Wisconsin

Signature: _____ Date: _____

James T. Heimbach, Ph.D. (Monograph Editor and Advisor to the GRAS Panel)
President
JHeimbach LLC
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Signature: _____ Date: Nov. 21, 2019

From: jheimbach@va.metrocast.net
To: Hice, Stephanie; jh@jheimbach.com
Subject: RE: GRN 000905 - Questions for Notifier
Date: Tuesday, April 7, 2020 8:19:38 AM
Attachments: image001.png
Hice Stephanie 20200407.pdf

Dear Dr. Hice—

Our response to FDA's questions is attached.

Regards,
Jim

James T. Heimbach, Ph.D., F.A.C.N.
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From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Thursday, April 2, 2020 1:07 PM
To: jh@jheimbach.com
Subject: GRN 000905 - Questions for Notifier

Dear Dr. Heimbach,

During our review of GRAS Notice No. 000905, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD

Staff Fellow (Biologist)
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JHeimbach LLC

April 7, 2020

Stephanie Hice, Ph.D.
Staff Fellow (Biology)
Division of Food Ingredients
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Dear Dr. Hice:

On April 2, 2020, you notified us that, during FDA's review of GRN 000905, the reviewers noted a number of questions. Following are our responses to the FDA questions. We believe that the responses we are providing will address these issues to your satisfaction.

This is not germane to your questions, but just for the record, the address and VAT information for the notifier has changed to the following:

SporeGen Ltd.
The London BioScience Innovation Centre
2 Royal College Street
London
NW1 0NH
United Kingdom
Tel: +44 (0)20 7691 2090
www.sporegen.com
Company No. 08001035
VAT No. 178 3570 75

Sincerely,

James T. Heimbach, Ph.D., F.A.C.N.
President

923 Water Street #66, Port Royal Virginia 22535, USA
tel. (+1) 804-742-5548 cell (+1) 202-320-3063 jh@jheimbach.com

1. The notifier states that the intended use level of Bacillus subtilis *strain DSM 32444 is 10^9 spore/dose or per serving. We note that the appropriate units are colony forming units (CFU)/serving. Further, we note that use of the term "per dose" is inappropriate for ingredients added to conventional foods. Please make a statement that corrects this.*

We have replaced "10^9 spores/dose" with "10^9 cfu/serving. We do note (in response to Q4) that over 99% of the colony-forming units in this preparation are in the form of spores.

2. The notifier states that B. subtilis *strain DSM 32444 is intended to be added to conventional foods, but then states that the target foods include, but are not limited to, beverages and dry and shelf-stable products. The notifier should clarify if the scope of the notice is all conventional foods or if it is a subset of foods.*

The scope of the notice is all conventional foods. The mention of beverages and dry and shelf-stable products was simply intended to indicate some of the primary target food categories.

3. Please state whether any of the raw materials used in the fermentation media are major allergens or derived from major allergens.

None of the raw materials used in the fermentation media are major allergens or derived from major allergens.

4. Please confirm that this ingredient is a spore preparation and provide an approximate ratio of spores to vegetative cells.

The product is >99% spores with less than 1% being killed vegetative cells (since heat treatment of 68^{o}C for 1 hour kills any residual vegetative cells). This is checked for each batch.

5. Please state whether the manufacturing process is monitored for contamination, and how often this is performed.

The manufacturing environment and facilities are checked weekly for airborne contamination. Equipment is monitored weekly. The product production process is checked at all stages (starting culture, fermentation, centrifugation, and spray drying) for contamination.

6. Please include a statement indicating that all analytical methods used to analyze the batches for conformance with the stated specifications have been validated for that particular purpose.

Analytical methods are revised and updated yearly by a VILAS (Vietnam Laboratory Accreditation Scheme) certified centre as described at http://www.boa.gov.vn/en/vilas-introducation. All analytical methods have been validated for the purpose of analysing preparations such as that for *B. subtilis* spores.

7. The notifier states that the method used to detect Salmonella *serovars is ISO 06579.2002 (page 22). We note that this method has been revised and replaced by 6572-1:2017, which corresponds to Microbiology of the Food Chain – Horizontal Method for the Detection, Enumeration and Serotyping of* Salmonella *– Part 1: Detection of* Salmonella *spp. Please make a statement that corrects this reference.*

The cited method has been replaced by ISO 6572-1:2017.

8. The notifier states that the method used to detect coliforms is ISO 40831.2006 (page 22). We note that the appropriate citation is ISO 4831:2006, which corresponds to Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of Coliforms – Most Probable Number Technique. Please make a statement that corrects this reference.

The citation of this method has been replaced by ISO 4831:2006; the original citation was a typographical error.

9. The notifier provided results of batch analyses in Table 2.8 (page 22). For some parameters, the results were listed as ND (not detected at the limit of detection) with a numerical value in parentheses following the ND. Please provide the units for the limit of detection for each parameter.

The units for heavy metals (lead, mercury, and cadmium), including the LODs, are mg/kg (ppm). Those for aflatoxin and patulin are µg/kg (ppb), as are the LODs for these parameters.

10. In Part 3: Dietary Exposure, the notifier states that B. subtilis *strain DSM 32444 "… is intended to be added to conventional foods at concentrations considered beneficial to human health, 109 spores/serving" (page 23). We note that the Agency's evaluation of GRAS notices focuses exclusively on the safety of the ingredient in food and not about purported beneficial effects of the substance. Please provide a corrected statement.*

We have deleted the phrase "considered beneficial to human health." The first sentence in Part 3 now reads, "*B. subtilis* SG188 is intended to be added to conventional foods at concentrations not exceeding 10^9 cfu/serving." We then note that over 99% of these colony-forming units are in the form of spores.

11. Please note that the term "probiotic" is neither a regulatory term, nor a scientific term, and its use in the notice appears to have context as a marketing term denoting or

connotating beneficial effects. We note that the Agency's evaluation of GRAS notices focuses exclusively on the safety of the ingredient in food and not about purported beneficial effects of the substance.

We understand that GRAS is concerned with safety and thus considerations of whether ingestion of the microorganism provides a health benefit to the host are irrelevant. We have been careful not to refer to the strain as a probiotic or to state or imply that we are putting it forth as having probiotic benefits. However, others have so referred to it and in quoting their statements regarding safety of *B. subtilis* or its history of use, the word probiotic inevitably appears. We believe these citations are germane to the conclusion that the intended use of *B. subtilis* is safe and cannot be either omitted or misquoted.

Similarly, the word probiotic appears in the titles of a number of journal citations that again are germane to the conclusion of safety.

We again emphasize that we have not used the term probiotic in referring to *B. subtilis*, and the word appears only in direct quotations and titles of journal articles in the list of references.

12. The notifier states that the intended use of B. subtilis *strain DSM 32444 is GRAS based on scientific procedures (21 CFR 170.30(b)), however includes a discussion in Part 5, Experience Based on Common Use in Foods (pages 25-26). Please note that the information provided in Part 5 does not meet the regulatory definition of "Common Use in Foods" as defined by 21 CFR Part 170.245. We note that the provided discussion should be incorporated into Part 6, Narrative, as defined by 21 CFR Part 170.250.*

Part 5 now reads *in toto*: "The conclusion that the intended use of *B. subtilis* SG188 is GRAS is based on scientific procedures rather than experience based on common use in food prior to 1958." The remaining material is moved to Part 6.

13. The notice includes dietary supplements in Part 5: Experience Based on Common Use in Food (pages 25-26). The Agency does not consider GRAS notices for the use of dietary ingredients in dietary supplements. Please provide a corrected statement.

Please note that the cited statement in Part 5 and similar statements in Part 6 simply acknowledge the indisputable fact that *Bacillus* species have been used in dietary supplements in many parts of the world. This in no way asserts or implies an intention on the notifier's part to use *B. subtilis* strain DSM3244 in this way. The intended use is, as stated in Part 3, solely addition to conventional foods.

Nevertheless, we will change the title in the former Section 5.1 (which is now relocated to Part 6) from "Use of *Bacillus* Species in Foods and Dietary Supplements" to "Use of *Bacillus* Species," and will delete the second sentence, which states that, "Normally, they are consumed in foods or taken orally in tablets or capsules or in powders and liquid suspensions."

14. Please provide an updated literature search that discusses the safety of B. subtilis. Please discuss how these studies pertain to the safety of the intended uses of the ingredient. Examples include, but are not limited to, the following:

- La Jeon, Y., Yang, J., Kim, M., Lim, G., Cho, S., Park, T., Suh, J., … Lee, H. (2012). Combined *Bacillus licheniformis* and *Bacillus subtilis* infection in a patient with oesophageal perforation. *J Med Microbiol* 61:1766-1769. doi: 10.1099/jmm.0.042275-0
- Harwood, C. R., Mouillon, J., Pohl, S., and Arnau, J. (2018). Secondary metabolite production and the safety of industrially important members of the *Bacillus subtilis* group. *FEMS Microbiol Rev* 42:721-738. doi: 10.1093/femsre/fuy028

The following discussion is appended to Section 6.1.

This microorganism is Class 1, having the lowest level of risk as defined by the following authorities:

- UK: Advisory Committee on Dangerous Pathogens. 2013. *The approved list of biological agents.* 3rd Edition. Health and Safety Executive
- Europe: Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work
- USA: NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules.
- Canada: Risk groups, containment levels, and risk assessments (2013), In *Canadian Biosafety Standards* (1st ed.). Government of Canada

This species is not linked to any human health issues and its safety was extensively reviewed by Logan (2004) [which was discussed in GRN No. 000905]. No safety issues were identified. Additional reviews are as follows:

***B. subtilis* HU58**

Dound YA, Jadhav SS, Devale M, Bayne T, Krishnan K, Mehta DS. 2017. The effect of probiotic *Bacillus subtilis* HU58 on immune function in healthy human. *Indian Practitioner* 70:15-20.

McFarlin BK, Henning AL, Bowman EM, Gary MA, Carbajal KM. 2017. Oral spore-based probiotic supplementation was associated with reduced incidence of post-prandial dietary endotoxin, triglycerides, and disease risk biomarkers. *World J Gastrointest Pathophysiol* 8:117-126.

***B. subtilis* RO179**

Hanifi A, Culpepper T, Mai V, Anand A, Ford AL, Ukhanova M, Christman M, Tompkins TA, Dahl WJ. 2015. Evaluation of *Bacillus subtilis* R0179 on

gastrointestinal viability and general wellness: a randomised, double-blind, placebo-controlled trial in healthy adults. *Benef Microbes* 6:19-27.

***B. subtilis* DE11**

Kennesaw State University. 2019. The effect of *Bacillus subtilis* DE111® on the daily bowel movement profile for people with occasional gastrointestinal irregularity. *Clinicaltrials.gov* NCT04083521

As noted by Logan (2004) and Harwood et al. (2018), there are occasional reports of *B. subtilis* being implicated in infections in humans, but the evidence for actual involvement is, at most, circumstantial and in some cases the provenance of the strains must be questioned. Nearly always, this arises from either contamination or misdiagnosis. For example, wound infections may be contaminated with soil bacteria from which, even after disinfection, bacterial spores can survive leading to subsequent culture. This aspect was discussed by Logan (2004). Other complicating factors are antimicrobial therapy that does not affect spores, such that subsequent investigation amplifies the numbers of viable *Bacillus*. Similarly, in some countries spore probiotics are used as an adjunct to antimicrobial therapy, complicating diagnosis.

Occasionally there are documented reports of what, *prima facie*, appears as a genuine infection. For example, Jeon et al. (2012) describe a case of bacteremia following an esophageal perforation caused by *B. subtilis* and *B. licheniformis*. Similarly, a recent report (Gu et al., 2019) identified a strain of *B. subtilis* isolated from a deep-sea hydrothermal vent that has virulence potential in animals. In this case the precise mechanism whereby *B. subtilis* can invade vertebrate cells was not identified. As discussed by Harwood et al. (2018), *Bacillus* species can secrete molecules that have cytotoxic potential.

While it is possible that the strains involved may have carried unique features enabling pathogenicity, it does illustrate that even non-pathogenic microorganisms can under some occasions participate in potentially lethal infection requiring clinical treatment. Most importantly, these studies demonstrate the need to conduct safety analysis on a strain-by-strain basis.

Enterotoxins

The presence of one or more *B. cereus* enterotoxin genes has been identified in other *Bacilli*. This includes *B. subtilis, B. licheniformis, B. circulans, B. amyloquefaciens, B. megaterium* and *B. pumilus* [Phelps and McKillip (2002); From et al. (2005); Rowan et al. (2001)]. In many cases the presence of one or more toxin genes was present in strains that also showed potentially virulent characteristics *in vitro*, for example:

1. cell Invasion
2. cytotoxicity
3. hemolysis

While species and strains examined in these studies may, in some cases, be mis-diagnosed, their presence suggests a common ancestry or the possibility of genetic exchange. It is also possible that new toxins (and therefore toxin genes) exist. Similarly, it is possible that other compounds produced by *Bacilli* can cause effects similar to those

of enterotoxins, for example, hemolysins and some antimicrobial compounds (e.g., surfactins) and lecithinase.

Although the presence of individual Hbl and Nhe enterotoxin genes is not necessarily a cause for concern (since each toxin comprises 3 subunits), for the other enterotoxins, for which no meaningful *in vivo* diagnostic test exists, it means that the presence of these genes in all *Bacilli* to be used as food additives must be assessed either by genomic analysis or by PCR methods.

Emetic Toxin

Emetic-like toxins have been found in *Bacilli* other than *B. cereus*, but not in *B. subtilis* (From et al. 2005). In the case of emetic toxin which is non-ribosomally produced, no gene exists, but the *ces* gene is diagnostic. The boar-sperm assay in recent work has shown that a number of gene-markers (*CER, EMI, RE234, CES, Ces3R/CESR2*) can be screened by PCR to determine the likelihood of the emetic toxin being produced (Kim et al. 2010).

Other Potentially Toxic Compounds and Molecules
Lecithinase

Is a phospholipase that can lyse cells. It is found in some *Bacilli* (notably, *B. cereus* and *B. anthracis*) and is considered a potentially virulent marker. It can be diagnosed using a straightforward plate agar test. It has been detected in some isolates of *B. subtilis* although this may well be a miss-diagnosed isolate of *B. subtilis* (Williams 1957).

Hemolysins

These have been noted in some *Bacilli* and are specific enzymes able to lyse erythrocytes (Baida and Kuzmin 1996). In fully sequenced *B. subtilis* strain 168 (Kunst et al. 1997), eight hemolysis-associated genes were identified, *yhdP, yhdT, yugS, yrKA, yqhB, yplQ, yqxC* and *ytjA*.

Antimicrobials

Numerous lipopeptide and potentially cytotoxic antimicrobials (mostly bacteriocins) have been found in isolates of *B. subtilis* (Urdaci and Pinchuk 2004; Hwang et al. 2009) including surfactin, amylosin, and fengycin, and these must, if identified in the genome sequence, be considered potentially virulent and ideally require assessment of cytotoxic potential. However, EFSA has now withdrawn the requirement for *in vitro* cytoxicity testing of these compounds since the tests do not represent the *in vivo* conditions of the GI tract. It should be noted that hemolysis itself is not an indicator of surfactin; other lipopeptide antibiotics could be the cause and genome assessment is necessary.

Of particular note is surfactin, a lipopeptide antibiotic produced in *B. subtilis* for which a locus, *srf*, responsible for its production has been identified (From et al. 2005; Nakano et al. 1988). It is cytotoxic at high concentrations but is believed to have beneficial properties at low physiological concentrations (e.g., anti-proliferation and anti-fibrination) (Hwang et al. 2009). Importantly, subacute toxicity studies in rats have shown clearly that surfactin at 500 mg/kg bw, dosed intragastrically, showed no adverse effects. Higher doses did show symptoms, so the established NOAEL for surfactin was rated at 500 mg/kg bw/day. Considering the doses used in these studies, it seems impossible that *B. subtilis* used as a food additive that produced surfactins could produce

toxicity in healthy individuals. On the other hand, for patients who have internal injuries (e.g., ulcers), the possible action of cytotoxic compounds produced by bacteria should be considered.

Bacteriocins

Known bacteriocins produced by *B. subtilis* are shown in Table 6.2 in GRN No. 000905. The bacteriocins identified to date in *B. subtilis* are megacins and these are plasmid encoded (Kiss et al., 2008; von Tersch et al. 1983). They are therefore not present in *B. subtilis* strain SG188 which, as discussed in GRN No. 000905, has no plasmids.

References

Abriouel H, Franz CM, Ben Omar N, Galvez A. 2011. Diversity and applications of *Bacillus* bacteriocins. *FEMS Microbiol Rev* 35:201-232.

Baida GE, Kuzmin NP. 1996. Mechanism of action of hemolysin III from *Bacillus cereus*. *Biochimica Biophysica Acta* 1284:122-124.

From C, Pukall R, Schumann P, Hormazabal V, Granum PE. 2005. Toxin-producing ability among *Bacillus* spp. outside the *Bacillus cereus* group. *Appl Environ Microbiol* 71:1178-1183.

Gu HJ, Sun QL, Luo JC, Zhang J, Sun L. 2019. A first study of the virulence potential of a *Bacillus subtilis* isolate from deep-sea hydrothermal vent. *Front Cell Infect Microbiol* 9:183.

Harwood CR, Mouillon JM, Pohl S, Arnau J. 2018. Secondary metabolite production and the safety of industrially important members of the *Bacillus subtilis* group. *FEMS Microbiol Rev* 42:721-738.

Hwang YH, Kim MS, Song IB, Park BK, Lim JH, Park SC, Yun HI. 2009. Subacute (28 day) toxicity of surfactin C, a lipopeptide produced by *Bacillus subtilis*, in rats. *J Health Sci* 55:351-355.

Jeon YL, Yang JJ, Kim MJ, Lim G, Cho SY, Park TS, Suh JT, Park YH, Lee MS, Kim SC *et al*. 2012. Combined *Bacillus licheniformis* and *Bacillus subtilis* infection in a patient with oesophageal perforation. *J Med Microbiol* 61(Pt 12):1766-1769.

Kim JB, Kim JM, Park YB, Han JA, Lee SH, Kwak HS, Hwang IG, Yoon MH, Lee JB, Oh DH. 2010. Evaluation of various PCR assays for the detection of emetic toxin producing *Bacillus cereus*. *J Microbiol Biotechnol* 20:1107-1113.

Kiss A, Baliko G, Csorba A, Chuluunbaatar T, Medzihradszky KF, Alfoldi L. 2008. Cloning and characterization of the DNA region responsible for Megacin A-216 production in *Bacillus megaterium* 216. *J Bacteriol* 190:6448-6457.

Kunst F, et al. 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* 390:249-256.

Logan NA. 2004. Safety of aerobic endospore-forming bacteria. In: *Bacterial spore formers: probiotics and emerging applications.* Edited by Ricca E, Henriques AO, Cutting SM. Norfolk, UK: Horizon Bioscience: 93-106.

Nakano MM, Marahiel MA, Zuber P. 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *J Bacteriol* 170:5662-5668.

Phelps RJ, McKillip JL. 2002. Enterotoxin production in natural isolates of *Bacillaceae* outside the *Bacillus cereus* group. *Appl Environ Microbiol* 68:3147-3151.

Rowan NJ, Deans K, Anderson JG, Gemmell CG, Hunter IS, Chaithong T. 2001. Putative virulence factor expression by clinical and food isolates of *Bacillus* spp. after growth in reconstituted infant milk formulae. *Appl Environ Microbiol* 67:3873-3881.

Urdaci MC, Pinchuk I. 2004. Antimicrobial activity of *Bacillus* probiotics. In: *Bacterial spore formers: probiotics and emerging applications.* Edited by Ricca. E, Henriques. AO, Cutting SM; Horizon Bioscience: 171-182Von Tersch MA, Carlton BC. 1983. Megacinogenic plasmids of *Bacillus megaterium*. *J Bacteriol* 155:872-877.

Williams GR. 1957. Haemolytic material from aerobic sporing bacilli. *J Gen Microbiol* 16:16-21.

From: jheimbach@va.metrocast.net
To: Hice, Stephanie; jh@jheimbach.com
Subject: RE: GRN 000905 - Questions for Notifier
Date: Tuesday, April 7, 2020 9:41:56 AM
Attachments: image001.png

Dear Dr. Hice—

Here is the requested correction.

Regards,

Jim

James T. Heimbach, Ph.D., F.A.C.N.
JHeimbach LLC
923 Water Street #66
Port Royal VA 22535
USA
Tel: (+1) 804-742-5543
Cell: (+1) 202-320-3063
Email: jh@jheimbach.com

7. The notifier states that the method used to detect Salmonella *serovars is ISO 06579.2002 (page 22). We note that this method has been revised and replaced by 6579-1:2017, which corresponds to Microbiology of the Food Chain – Horizontal Method for the Detection, Enumeration and Serotyping of* Salmonella *– Part 1: Detection of* Salmonella *spp. Please make a statement that corrects this reference.*

The cited method has been replaced by ISO 6579-1:2017.

From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Tuesday, April 7, 2020 9:05 AM
To: jh@jheimbach.com; jheimbach@va.metrocast.net
Subject: RE: GRN 000905 - Questions for Notifier

Dear Dr. Heimbach,

Thank you for your attention to our comments. Upon review of the provided responses, we noted the following:

Question 7 cites ISO 6572-1:2017 as the revised method that replaced ISO 06579.2002, and corresponds to Microbiology of the Food Chain – Horizontal Method for the Detection, Enumeration and Serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp. We note, that Question 7 includes a typographical error, and the appropriate citation for the revised method is ISO 6579-

1:2017, which corresponds to the same method cited in the question, Microbiology of the Food Chain – Horizontal Method for the Detection, Enumeration and Serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp.

We apologize for this typographical error, and ask for a statement that corrects this reference in your response.

Thank you, and please let me know if you have any questions.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients

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From: jheimbach@va.metrocast.net
To: Hice, Stephanie; jh@jheimbach.com
Subject: RE: GRN 000905 - Questions for Notifier
Date: Wednesday, April 8, 2020 3:33:26 PM
Attachments: image001.png

Dear Dr. Hice:

The units for each microbiological specification (*E. coli*, *Salmonella* spp., coliforms, *S. aureus, C. perfringens, B. cereus*, and fungal spores) are colony-forming units (cfu). We apologize for the omission.

Regards,
Jim

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From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Wednesday, April 8, 2020 3:05 PM
To: jh@jheimbach.com; jheimbach@va.metrocast.net
Subject: RE: GRN 000905 - Questions for Notifier

Dear Dr. Heimbach,

We have one additional question, for clarification purposes. Please find it below:

The notifier provided specifications in Table 2.8 (page 22), however, units were not provided for the microbiological specifications. Please provide the units for each microbiological specification.

Thank you, and please let me know if you have any questions.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
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From: jheimbach@va.metrocast.net
To: Hice, Stephanie; jh@jheimbach.com
Subject: RE: GRN 000905 - Questions for Notifier
Date: Monday, April 13, 2020 11:26:48 AM
Attachments: image001.png

Dear Dr. Hice—

B. subtilis strain DSM 32444 is intended to be used in conventional foods, and the intended use excludes infant formula and all foods regulated by USDA.

Regards,
Jim

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From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Monday, April 13, 2020 11:16 AM
To: jh@jheimbach.com; jheimbach@va.metrocast.net
Subject: RE: GRN 000905 - Questions for Notifier

Dear Dr. Heimbach,

For the administrative record, please clarify whether *Bacillus subtilis* strain DSM 32444 is intended to be used in infant formula and/or foods under the jurisdiction of the U.S. Department of Agriculture (USDA).

Thank you in advance for your attention to our comments.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients

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