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Bacillus subtilis isolated from the human gastrointestinal tract

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Abstract

As part of an ongoing study to determine the true habitat of *Bacillus* species, we report here the isolation and characterisation of *Bacillus subtilis* from the human gastrointestinal tract (GIT). Strains were obtained from ileum biopsies as well as from faecal samples and their biotypes defined. 16S rRNA analysis revealed that most isolates of *B. subtilis* were highly conserved, in contrast to RAPD-PCR fingerprinting that showed greater diversity with 23 distinct RAPD types. The majority of *B. subtilis* strains examined possessed features that could be advantageous to survival within the GIT. This included the ability to form biofilms, to sporulate anaerobically and secretion of antimicrobials. At least one isolate was shown to form spores that carried an exosporium, a loosely attached outer layer to the mature endospore, this being the first report of *B. subtilis* spores carrying an exosporium. This study reinforces a growing view that *B. subtilis* and probably other species have adapted to life within the GIT and should be considered gut commensals rather than solely soil microorganisms.

Keywords: *Bacillus subtilis*; Probiotics; Spore-formers; Gut microflora

1. Introduction

Although normally considered soil organisms, members of the spore-forming genus *Bacillus* can inhabit the gastrointestinal tract (GIT) of insects and animals [22]. In the case of pathogens such as *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus sphaericus*, entry into the GIT is an essential part of their virulent life cycle [24,33]. It is probable, though, that *Bacillus* spores present in the soil enter the GIT associated with ingested organic matter and this could explain the abundance of spore-formers in soil-dwelling animals (e.g., earthworms [18]). However, a number of studies have also recovered *Bacillus* species in mammals (reviewed in ref. [22]), for example, members of *Bacillus* were readily recovered in the faeces of broiler chickens [4], deer [28,36] as well as from the mouse GIT [42]. A recent study has identified *Bacillus* spore-formers in human faeces [14]. It has been shown recently that spores of a laboratory strain of *Bacillus subtilis*, strain PY79, are able to germinate in the jejunum and ileum of mice dosed orally with spores [6,45]. Surprisingly, germinated spores could outgrow and then, as they progressed into the upper colon, re-sporulate. This phenomenon was also observed with other, natural isolates of *B. subtilis* that had been recovered from human faeces, suggesting that *B. subtilis* could use the GIT for both growth and sporulation. This raises the intriguing question of what is the real habitat for spore-formers? Is it possible that the presence of spores in soil is a byproduct of having been shed into the environment in faeces? If so, then over time the soil would accumulate large quantities of spores that might mistakenly be considered soil organisms due to their 'apparent' presence there in high

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numbers. There are examples of spore-forming bacteria carrying out their entire life cycle within the GIT of animals, and *Metabacterium polyspora* that lives in the intestine of guinea-pigs is one notable case [1]. Here, coprophagia ensures that a proportion of spores shed in the faeces can re-enter the host GIT; indeed, this organism does not appear to be able to survive outside of its host.

Bacillus spores are being used in a surprisingly large number of probiotic preparations (reviewed in ref. [22]). In most cases, the potential benefits, if any, have yet to be proven. However, with some, such as the traditional Japanese staple, Natto, which contains large numbers of viable *B. subtilis* spores, there is a long history of beneficial properties assigned to this product [23]. Another commercial product known as Enterogermina is used prophylactically against diarrhoeal infections and is comprised solely of spores of *Bacillus clausii,* and is one of the few probiotics to be licensed as an OTC (over-the-counter) medicine [22]. To produce a 'beneficial' effect to the host, it seems likely that the spore must germinate and grow within the gut, in agreement with models for how other non-spore-forming probiotic microorganisms function. *B. subtilis* has also been shown to be of importance in stimulating development of the gut-associated lymphoid tissue (GALT) in rabbits and that it was sporulation of *live* bacilli within the GALT that was considered critical to this process [40]. This study in rabbits suggested that spore-formers play an important role in strengthening the immune system at an early stage and supports the role of these bacteria in their vegetative form as probiotics. Accordingly, providing a link between probiosis and intestinal residency is therefore important not only for substantiating the use of *Bacillus* spp. as probiotics, but also for understanding the true habitat of spore-formers.

If indeed *Bacillus* species are intestinal residents or commensals, then not only should it be possible to isolate them readily from the GIT, but it should also be possible to identify strains that carry attributes enabling their survival within the gut. In this paper, we examined *B. subtilis* isolates recovered from the faeces as well as the small intestine of human volunteers. This work identified a remarkable biodiversity between *B. subtilis* isolates and, in some cases, revealed traits that could be beneficial to an intestinal existence.

2. Materials and methods

2.1. Isolation and screening of spore-formers

2.1.1. Faecal samples

Samples of freshly voided faecal material were collected from 29 healthy volunteers at the Ho Chi Minh City University of Medicine & Pharmacy (Vietnam) and stored frozen until processing. No volunteer had taken any form of probiotic within the preceding 12 months. A total of 1 g samples were homogenised in PBS, heated for 1 h at 65 °C, serially diluted and plated for single colonies. After aerobic incubation, selected colonies from each sample were re-streaked on DSM agar to isolate single colonies and checked for formation of phase-bright spores and their catalase reaction. Eighty-eight isolates in total were short-listed for further analysis.

2.1.2. Ileum samples

Six patients (two men, four women; mean age 45 years) who had been referred to the Gastroenterologic Unit (A. Gemelli Hospital, UCSC, Rome) to undergo an upper gastrointestinal endoscopy for dyspeptic symptoms were enrolled in this study. Demographic details of patients were recorded, including age, sex, habits and concurrent medical conditions. None of the included patients had a previous history of gastrointestinal surgery or antibiotics, laxatives, probiotics or proton pump inhibitory usage in the month before gastroscopy. Signed informed consent was obtained from each patient. A push and pull endoscopy based on an anterograde approach using a double balloon enteroscopy system (Fujinon EN-450 P5/P20, Fujinon Inc., Japan) was performed by the same endoscopist on all patients. Two biopsies of about 3 mm in length were collected from each patient at the proximal part of the small bowel (approximately 40 cm after the pylorus). All samples were stored at −80 °C. To isolate spore-formers, each biopsy was weighed and homogenised in 200 µl sterile H_2O using a syringe and needle. The homogenised tissue was diluted 1:1 in ethanol (final concentration 50%) and incubated for 1 h at room temperature. Subsequent plating of 10-fold serial dilutions was made on LB plates. Plates were incubated aerobically at 37 °C for 2 days. Colonies were re-streaked on DSM (Difco sporulation medium) agar plates to isolate single colonies and checked for formation of phase-bright spores and their catalase reaction.

2.2. Reference strains

Reference *B. subtilis* strains used for biotyping, 16S rRNA and RAPD-PCR analysis were as follows: PY79, a prototrophic derivative of the 168-type strain [48]; type strain 168 obtained from the *Bacillus* Genetic Stock Center [BGSC] as strain 1A1; Natto (*B. subtilis* var *natto*); W23 (BGSC strain 2A1); 3A23 [10]; and ATCC 9799.

2.3. General methods and preparation of spores

Methods for measurement of heat-resistant spores were as described [8,34]. Spores were prepared for *in vitro* experiments by the exhaustion method [34] using DSM medium. Spore suspensions were lysozyme-treated and then heat-treated (68 °C for 1 h) to remove residual vegetative cells, and spore titres were determined by serial dilution and plating out, as well as microscopically, using a cell counting chamber. Aliquots were stored at −20 °C prior to use. For analysis of sporulation efficiencies, the resuspension method was used [34]. Starch hydrolysis was determined as described elsewhere using agar plates containing 1% soluble starch [8]. Haemolysis was evaluated by streaking onto tryptose blood agar containing sheep's blood at 5% and incubation for 24 h at 37 °C.

Motility was tested by the method of Hendrickson [17] using growth in 0.4% agar and incubation for 2 days at 30 °C.

2.4. PCR amplification and DNA sequencing of 16s rRNA

To identify tentative bacterial species for each isolate, the entire 16S rRNA gene (*rrnE*) was amplified as described previously [20]. The 1400 bp amplicon was then sequenced completely on both strands and compared to *rrnE* sequences contained in the Ribosomal Database Project (RDP) II database (http://rdp.cme.msu.edu/). Closest known species were recorded with percentages of identity and confirmed as *B. subtilis* if the 1st, 2nd and 3rd matches to the submitted sequence were the same species. The accession numbers of the *B. subtilis* isolates are given in Supplementary data.

2.5. Statistical parsimony based network analysis

We aligned 1274–1282 bp of 16S rRNA (*rrnE*) sequence data from the 31 *B. subtilis* GIT isolates together with corresponding sequences from three reference strains, 168, PY79 and Natto, and then calculated the absolute number of pairwise differences between haplotypes using PAUP v4.0b10 [44]. We used this distance matrix to infer a haplotype tree using TCS v1.21 [7,46].

2.6. RAPD-PCR

Random amplified polymorphic DNA-PCR (RAPD-PCR) was carried out using three 10-nucleotide single-strand primers OPA3 (5′-AGTCAGCCAC-3′), OPH3 (5′-AGACGTCCAC)-3′ and OPL12 (5′-GGGCGGTACT). These primers were selected from 30 primers of the OPA, OPH, OPL and OPS series that have been shown to yield distinctly different and reproducible polymorphic patterns and described previously [38]. The extracted genomic DNA was amplified in a 25 µl reaction mixture each containing 0.5 µl Taq polymerase (Promega), 5 µl 5X buffer, 2 µl 50 mM $MgCl_2$, 1 µl dNTPs (Bioline), 0.5 µl primer (1 pmol/ml), 15 µl DNase-free water and 1 µl DNA. Amplification consisted of an initial denaturation at 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 36 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Control reaction mixtures lacking template DNA were also included in each experiment. The PCR products were separated on 1.3% agarose gel electrophoresis running at 65 volts for 50 min. A GeneRuler™ 1 kb DNA ladder (Fermentas) was used as a size standard in each run. Gels patterns were visualised by ethidium bromide staining and photographs taken under UV light. After photography, RAPD patterns were scanned and analysed with GelCompar II 5.0 software (Applied Maths, Belgium). Similarities were calculated using the Pearson correlation coefficient and strains were grouped with the UPGMA (unweighted pair group method with arithmetic averages) algorithm. The bands subjected to cluster analysis included PCR products ranging from 0.2 to 1.6 kb in size. RAPD patterns recovered in a cluster were then examined by visual comparison of the banding patterns. Strains having the same band patterns or differing by one or two bands were considered to be closely related types and, those having three or more different bands were allocated to different RAPD types.

2.7. Anaerobic growth

Strains were streaked on solid DSM agar plates and grown at 30 °C. For anaerobic growth, potassium nitrate at a concentration of 5 mM and potassium nitrite at 2.5 mM were added to the medium as electron acceptors [31,47]. Streaked plates with control strains were incubated in an anaerobic chamber (Don Whitley). Growth was test and monitored after 72 h. In addition to this, for those that grew anaerobically, we determined the presence or absence of phase-bright spores by microscopy. *Clostridium perfringens fD00385* (obtained from Prof. N. Fairweather, Imperial College) was included as a positive 'anaerobic' control and *Bacillus pumilus* SC2200 as a negative control. For quantification of anaerobic sporulation, plates were incubated for 72 h at 30 °C anaerobically or aerobically, after which the entire bacterial lawn was recovered from each plate. The suspension was then serially diluted and plated out for CFU or heat-treated (68 °C, 45 min) before serial dilution to determine spore counts.

2.8. Biofilm formation

For analysis of dendritic growth, biofilms and surface colonisation phenotype strains were grown on a casein digest-mannitol (CM) medium solidified with 0.3% (wt/vol.) agarose (Fisher Scientific, low EEO grade) as described elsewhere using 12 mm well microtitre plates [15]. Briefly, CM medium contained (per litre) 10 g casein digest (N-Z Amine A, Sigma Aldrich Co.), 10 g of D-mannitol and a trace metal mixture (H medium metals). Use of unbuffered CM agarose medium gave more discrete dendritic growth, while CMK medium containing 7 mM K_2HPO_4 (added separately from a 100× stock; final pH 7.3) as a potassium source produced extensive surface colonisation [15,26]. For biofilm formation, CM medium was solidified with 1% (wt/vol.) agar, and for restricted growth (to obtain single colonies), 2% (wt/vol.) agar was used [2]. To observe biofilm formation and adherence to a surface, the general method described by O'Toole et al. [35] was used. Bacterial strains were grown overnight in LB broth and 10 µl was used to inoculate 0.5 ml CM medium in polypropylene tubes; these cultures were incubated overnight at 37 °C with mild shaking. After discarding the medium, the tubes were rinsed once with 1% (wt/vol.) crystal violet and then with water. Finally, the adherent biofilm was lysed by adding 90% ethanol and optical density was measured at 590 nm.

2.9. Adhesion studies

Bacterial strains were grown in brain heart infusion (BHI) broth (Oxoid) at 30 °C for 15–18 h (note that bacteria grown in BHI do not form spores) and washed two times with PBS.

Bacteria were then suspended to approximately 10^7–10^8 CFU/ml in complete cell culture medium supplemented with HEPES. Adherence to HT29-16E (a mucus-secreting cell line; [2,29]) was determined using the methods described by Rowan et al. [41].

2.10. Antimicrobial activity

Antimicrobial activity was assessed with a colony overlay assay to screen *B. subtilis* isolates; the assay was adapted from the method described by Pugsley [39]. Overnight cultures of *Bacillus* strains grown in LB medium were inoculated as a 5 µl spot on DSM agar plates and incubated at 30 °C for 24 h prior to killing cells by exposure to chloroform vapour for 30 min. Covers were replaced and plates allowed to aerate for 20 min before being overlaid with 1.5% tryptose or MH agar (according to the requirements of the indicator strains) that had been inoculated with an overnight culture of the indicator strain. These indicator strains included three Gram-positive and two Gram-negative strains, *Staphylococci aureus* (CIP 20256), *Listeria innocua* (CIP 8011), *B. subtilis* 168, *Salmonella indiana* (CA11) and *Escherichia coli* (CIP 54.8), respectively. Zones of inhibition around the spots after 48 h incubation at 37 °C (30 °C for *L. innocua* and *B. subtilis* 168) were scored as positive.

2.11. Surfactin

The ability to measure surfactin produced by the bacteria has been described by Youssef et al. [49].

2.12. Transmission electron microscopy (TEM)

Spores were processed for ultramicrotomy according to standard procedures. Spores were fixed for 2 h in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.4) and post-fixed for 1 h at 4 °C with 1% osmium tetroxide in the same buffer. Sample pellets were dehydrated with ethanol and embedded in Epon-Araldite. Thin sections were stained successively with 5% uranyl acetate and 1% lead citrate. TEM observation was performed with a FEI CM120 operated at 120 kV.

3. Results

3.1. Isolation of spore-forming bacteria from the GIT

Spore-forming bacteria were recovered from two locations in the human GIT, from ileum biopsies and from faecal samples. Eighty-eight heat-resistant faecal isolates, prefixed HU1–88, obtained from a previously described study in Vietnam [45] were examined in detail in this work. Ileum biopsies were taken from six volunteers at the Gemelli Hospital (Italy), and low numbers of ethanol-resistant colony-forming units were detected (Table 1), among which 14, prefixed GB1–GB14, were taken forward for further analysis. All isolates tested catalase-positive, distinguishing them from the anaerobic spore-forming *Clostridium* spp.

Table 1
Spore-formers recovered from the ileum.

Volunteer	Biopsy weight (mg)	Total recovered spores/biopsy
1	5.5	11
2	6.6	2
	7.3	5
3	5.1	7
	3.0	4
4	3.3	18
	4.5	4
5	2.0	3
	4.3	4
6	2.4	9
	4.0	8

For all HU and GB isolates, the entire 1400 bp 16S rRNA gene, *rrnE*, was PCR-amplified from chromosomal DNA and sequenced in its entirety. Sequences were compared first to those deposited in the GENBANK database and based on this analysis, 22 of the HU strains and nine GB isolates were short-listed as most probably *B. subtilis* (>98% homology). Next, for these short-listed candidates, we examined the relatedness of the *rrnE* sequences to those contained in the Ribosomal Database Project (RDP) II database. This analysis revealed that with the exception of three isolates, all were most similar to *B. subtilis* entries and carried *B. subtilis* sequences as first-, second- and third-closest matches. The three exceptions, HU2, HU79 and HU83, were found to most closely match *Bacillus amyloliquefaciens* Accn. No. AY651023, but the second and third most related species were *B. subtilis*. It is possible, then, that the AY651023 entry was misclassified and is, in fact, *B. subtilis*.

Other species identified in the faecal samples but not described here were *Bacillus pumilus* (four isolates), *Bacillus licheniformis* (four isolates), *B. amyloliquefaciens* (six isolates), *Bacillus cereus* (20 isolates), *Bacillus megaterium* (nine isolates), *Bacillus flexus* (three isolates), *Brevibacterium halotolerans* (two isolates) as well as nine pigmented *Bacillus*. Of these pigmented isolates, three produced pink colonies and were identified as *Bacillus firmus*, and the other six were yellow-coloured and were closely most related to *Bacillus indicus*. These yellow-pigmented isolates have been reported in a recent study and the pigmentation was shown to result from the production of more than one carotenoid [11]. The remaining nine faecal isolates were found to produce spores, but could not be classified and were not taken forward for further analysis, likewise, five biopsy isolates that were not *B. subtilis* were not further studied.

We decided to focus only on isolates of *B. subtilis* that comprised one quarter of the faecal isolates (22/88) and two-thirds (6/9) of the biopsy samples. We aligned 1274–1282 base pairs (bp) of sequences from the 16S ribosomal RNA of the 31 isolates and three reference strains, 168, PY79 and Natto. As the sequences showed a very limited amount of divergence, we inferred haplotype trees (Fig. 1) using the statistical parsimony approach [46] implemented in the program TCS [7]. We collapsed 15 identical sequences into a single haplotype (including the type strain *B. subtilis* 168,

Table 2
Identification and characteristics of *B. subtilis* GIT isolates.

Isolate[a]	Ori[b]	RAPD type[c]	Adhesion[d]	Anaerobic growth[e]				Biofilms[f]			Surface growth[g]		Surf.[h]	Starch hyd.	Haemolysis[i]	Lec.[j]	Sporulation[k]
				NO_2		NO_3		Liquid Ad.	CM	CMK	Dendritic	Profuse					
				VC	Sp	VC	Sp										
PY79	n/a	−	0.03 ± 0.05	+	−	+	−	0	−	−	−	−	−	+	γ	−	16
B. cereus	n/a	−	0.397 ± 0.09	+	−	+	−	0	+	−	Type D	+	−	+	α	+	NT
Natto	n/a	−	0.01 ± 0.01	−	−	±	+	0.56	−	++	Type B	+	+	−	γ	+	7
HU75	F22	15	0.036 ± 0.11	+	−	+	+	0	+	+	−	+++	−	+	α	−	16
HU15	F2	3	0.005 ± 0.01	−	−	+	−	0	−	−	−	−	−	−	α	−	16
HU31	F6	4	0.007 ± 0.01	±	−	+	−	0	−	−	−	−	−	−	α	−	16
HU68	F19	13	0.01 ± 0.04	−	−	+	−	0	−	−	−	−	−	−	α	−	16
HU87	F24	19	0.016 ± 0.04	+	++	+	++	0.14	+	++	Type B	++	−	+	β	−	7
HU71	F20	14	0.036 ± 0.03	+	−	+	++	1.52	+	++	−	+	+	+	β	−	8
HU78	F23	16	0.017 ± 0.04	±	+	+	+	1.98	+	++	Type B	+++	+++	+	β	−	7
HU39	F9	5	0.05 ± 0.00	±	−	±	+	1.58	+	+++	Type A	++	+++	+	β	−	10
HU48	F11	6	0.09 ± 0.02	+	++	±	−	0.63	+	++	Type A	+++	+	+	β	+	10
HU50	F12	7	0.031 ± 0.03	+	+	+	+	1.67	+	+++	Type B	+++	+++	+	β	−	6
HU58	F16	8	0.020 ± 0.08	+	+	+	+	1.51	+	+++	Type A	+++	+++	+	β	−	6
HU60	F16	9	0.041 ± 0.04	−	−	±	−	0.02	+	+++	Type B	++	++	+	β	+	8
HU1	M1	1	0.03 ± 0.1	−	−	+	++	0.77	+	+++	Type B	+++	++	+	β	+	6
HU2	M1	1	0.05 ± 0.07	±	−	+	++	0.56	+	+++	Type B	+++	++++	+	β	+	7
HU5	M2	2	0.08 ± 0.00	+	−	±	++	2.15	+	+++	Type A	+++	+	+	β	−	10
HU24	F5	2	0.04 ± 0.05	+	++	+	++	1.71	+	+++	Type A	+++	+++	+	β	−	16
HU61	F17	10	0.04 ± 0.020	±	++	+	++	0.125	+	+++	Type B	++	−	+	β	+	6
HU63	F17	11	0.05 ± 0.01	−	−	±	+	0.18	+	+++	Type A	++	+	+	β	−	7
HU67	F19	12	0.03 ± 0.04	−	−	±	−	1.27	+	+++	Type B	++	+	+	β	+	8
HU70	F20	13	0.01 ± 0.02	±	−	+	++	0.44	+	+	Type A	+++	++++	+	β	−	8
HU79	F23	17	0.004 ± 0.09	−	−	+	++	1.8	+	+++	Type A	+++	++	+	β	+	10
HU83	M5	18	0.006 ± 0.07	+	+	+	+	0.57	+	+++	Type B	+++	++++	+	β	+	7
GB2	V1	20	0.09 ± 0.03	+	+	+	+	1.28	+	+++	Type A	+++	+++	+	β	−	8
GB5	V2	20	0.06 ± 0.04	±	−	±	−	1.11	+	+++	Type C	+++	++	−	β	−	10
GB4	V2	20	0.04 ± 0.04	±	−	+	−	0	−	−	−	−	−	+	α	−	16
GB6	V5	20	0.03 ± 0.02	−	−	+	−	0	−	−	−	−	−	+	α	−	16
GB10	V4	21	0.004 ± 0.20	−	−	+	−	0	−	−	−	−	−	−	α	−	16
GB11	V4	22	0.002 ± 0.01	−	−	+	−	0	−	−	−	−	−	−	α	−	16
GB12	V6	23	0.12 ± 2.06	±	−	−	−	0	±	−	Type D	±	−	+	β	+	8
GB13	V6	23	0.013 ± 0.05	+	−	−	−	0	−	−	−	±	−	+	β	+	8
GB14	V3	23	0.11 ± 0.03	+	−	−	−	0	+	±	−	+	−	+	β	−	8

[a] Isolate number taken from faeces (HUn) or gut biopsies (GBn). n/a, lab strain.
[b] Volunteer number male (M) or female (F), or for gut biopsies, volunteer (V) number.
[c] Assignment of strain to group based upon RAPD PCR analysis (see Supp. Data).
[d] Percentage of vegetative cells that bound to cultured HT29-16E cells. The experiment was repeated three times.
[e] Anaerobic growth in the presence of nitrite or nitrate. Evaluation of vegetative cell growth (VC):+ after 72 h, ± limited growth in the form of a tiny colony. For evaluation of sporulation (Sp): − no visible sporulation, +, less than 25% sporulation, ++, more than 50% sporulation.
[f] Biofilms were evaluated using a liquid adherence assay (see Section 2) where OD was measured at 590 nm. The assay was performed three times and representative values from one experiment are given. Biofilms on solid media used CM or CMK agarose.
[g] Dendritic growth was determined on CM agarose and isolates assigned to one of four types, A–D; −, no dendritic growth. Rapid surface growth was determined on CMK agarose.
[h] Surfactin was measured using an oil displacement method; − is not measurable activity, +, represents a diameter of oil displacement of <10 mm, ++, 10–15 mm; +++, 16–30 mm; +++, >30 mm.
[i] hem = haemolysis, haemolysis on sheep blood agar; α, complete haemolysis with a clear zone around colonies; β, partial haemolysis; γ, no changes.
[j] Lecithinase production; + blue precipitation of hydrolysed lecithin around peacock blue colonies (indicative of *B. cereus*); −, no changes; ±, weak staining.
[k] Time, in hours, at which maximal spore counts were determined using sporulation in DSM medium. NT, not tested.

PY79 and Natto). Although frequency data was not included in the analysis, this haplotype was assigned the largest outgroup weight. The ''starlike'' shape of the phylogeny, clustered around this modal haplotype, is suggestive of a rapid increase in population size [3].

3.2. *RAPD PCR genotyping of* B. subtilis *isolates*

To evaluate the genetic heterogeneity of the *B. subtilis* isolates RAPD-PCR analysis was performed using OPA3, OPH3 and OPL12 primers used previously for *B. subtilis* typing [38]. RAPD-PCR patterns comprised five or more bands largely in the 0.2–1.6 kb range for most of the strains tested; however, some strains produced fewer bands. The relationships, expressed as percentage similarity between the pattern profiles, were displayed as dendrograms derived from UPGMA (unweighted pair group method with arithmetic averages) clustering of similarities created using the Pearson correlation coefficient. Dendrograms were then used for visual determination of the RAPD types, demonstrating that 90% of

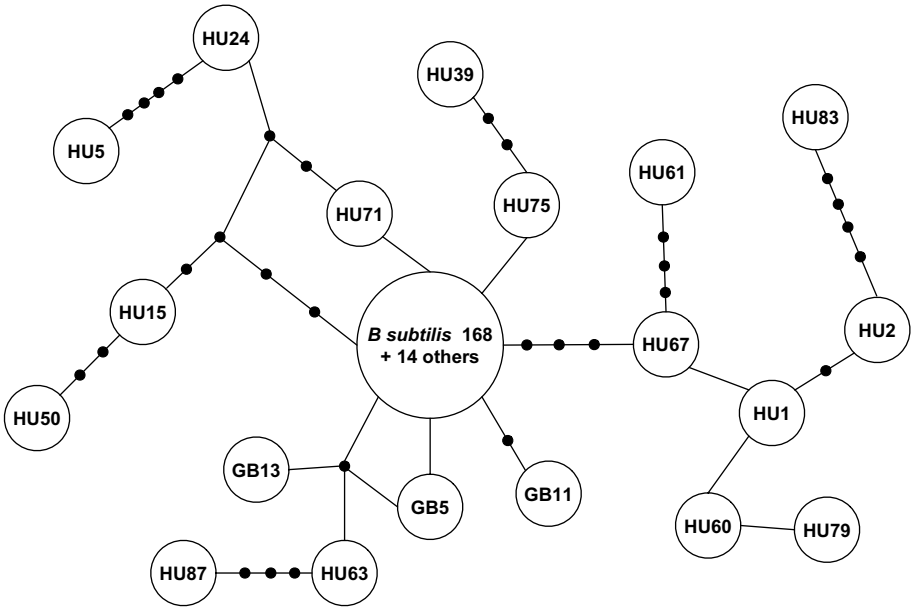

Fig. 1. Haplotype tree of GIT isolates. Statistical parsimony-based network showing relationships between isolates discussed in the text. The core haplotype consists of the type strain 168, PY79, Natto and 12 GB and HU isolates. Each branch represents a 1 bp change and dots represent a hypothetical haplotype.

the strains showed >80% similarity with each other (Table 2 and Supp. Data). The primer OPA3 generated 10 different profiles for the 31 strains tested, OPH3 12 different profiles and OPL12 15 distinct profiles. After visual comparison of the patterns obtained with these three primers, the 31 strains of *B. subtilis* were allocated to 23 distinct RAPD biotypes (Table 2 and Supp. Data).

3.3. Phenotypic characteristics of the B. subtilis isolates

A more detailed characterisation was made of all GIT *B. subtilis* isolates including appropriate reference strains (Table 2). Six isolates were amylase-negative and failed to hydrolyse starch, a feature normally indicative of *B. pumilus* or emetic *B. cereus* [43]. All strains were motile but three: HU15, HU31 and HU68 exhibited weak motility. Interestingly, these same isolates also failed to hydrolyse starch. Growth was measured at 10 and 50 °C and all were able to grow at these extremes. The ability of *B. subtilis* to grow anaerobically has been examined in several reports [4,31,32] where anoxic growth is enhanced in the presence of nitrite or nitrate in the culture medium. We examined the ability of strains to grow on solid medium under anaerobic conditions (Table 2), and for those that grew anaerobically, determined whether sporulation had occurred. We found that the majority of gut isolates, but not all, were able to form varying levels of spores under anoxic conditions. Sporulation efficiency under aerobic conditions was also measured by the resuspension method (Table 2). For the reference strain PY79, maximum numbers of spores were present 16 h after initiation of sporulation. Natto, a second reference strain, sporulated much faster, with maximum levels reached in 7 h. For the 31 GIT isolates, they showed a diverse array of sporulation speeds with some, such as strains HU1, HU58 and HU61, reaching maximum spore counts in just 6 h.

The ability to form biofilms was also examined in liquid and on semi-solid medium (Table 2). The majority of GIT isolates were able to form biofilms, as judged by their appearance on CM and CMK agar, with the exception of HU15, HU31, HU68, GB4, GB6, GB10 and GB11. HU75 was found to be able to form biofilms only on semisolid medium. For those strains that produced biofilms, they fell into two groups as described previously [5], being either a colony-type or a pellicle-like biofilm. Growth on CMK plates containing 2% agar restricted surface growth somewhat and raised the colony profile. As noted before, the laboratory strain PY79 was unable to form biofilms [5]. For almost all of the strains that produced biofilms on CM or CMK agar, these biofilms were also shown to be adherent to plastic using a simple liquid assay. Dendritic growth and surface-film growth was also measured using a semi-solid CM and CMK agarose-containing medium. With a few exceptions, most of the biofilm-producing strains formed dendritic growth on CM agarose. We identified four types of dendritic growth, as shown in Fig. 2. Group A consisted of the most extensive dendritic growth, producing a recognisable 'spoked-wheel' pattern on plates (Fig. 2A), while group B exhibited little to no dendritic growth, but more profuse colony growth (Fig. 2B), group C showed a creamy-coloured rhizoid pattern on agarose (Fig. 2C), and group D had a unique sunflower pattern produced by GB12 (Fig. 2D). *B. cereus* showed very limited dendritic growth, while 12 strains, including domesticated strain PY79, showed no dendritic growth (Fig. 2E). On CMK agarose, colonies assumed a more wrinkled appearance and were more profuse, being able to spread rapidly across the entire agar plate.

Potential virulence characteristics were also examined (Table 2). Although neither of the reference strains was haemolytic, all of the isolates exhibited either complete or

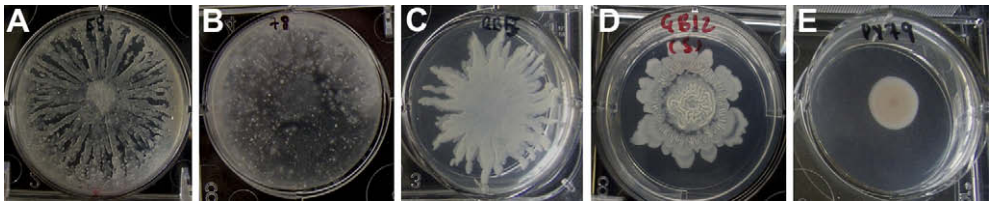

Fig. 2. Dendritic growth. Representative plate cultures showing the appearance of *B. subtilis* isolates growing on CM agarose. Plates were inoculated with a single drop of culture and grown overnight at 37 °C. Panel A, 'spoked wheel' appearance of type A growth; panel B, type B; panel C, rhizoid pattern typical of type C; panel D, 'sunflower' pattern of type D; and panel E, no dendritic growth.

incomplete haemolysis. Interestingly, lecithinase activity was also produced in some isolates, including Natto. PCR was used to evaluate the presence of known *B. cereus* enterotoxin genes in the chromosomes of all strains (*hblC-A*, *nheA-C* and *cytK*). This method was used previously to profile putative food-poisoning *Bacillus* strains [10,12,16,37]. Our analysis showed that no isolate was found to carry any enterotoxin gene (data not shown).

3.4. Antimicrobial activity

Isolates were tested for their antimicrobial activity and ability to suppress the growth of a selection of Gram-positives and Gram-negatives (Table 3). Almost half of the HU isolates had activity against one or more of the indicator bacteria and the majority of these had activity against more than one strain. Activity from the GB isolates was only seen in two strains, GB4 and GB5. We also examined the production of surfactin activity (Table 2), an amphipathic lipopeptide with antimicrobial activity that is produced in some strains of *B. subtilis*, and the Natto strain in particular [30]. Our assay was crude in nature and a negative result did not necessarily prove that surfactin was not produced. On the other hand, the test did show that there were quantitative differences between isolates, with some, such as HU78, able to secrete large quantities into the medium.

3.5. Adhesion

Adhesion of vegetative cells of each strain to HT29-16E cells was evaluated using *in vitro* methods (Table 2). Since spores can spontaneously germinate, it proved impossible to measure adhesion accurately with spores alone. We used the HT29-16E cell line that exhibits differentiation features characteristic of mature intestinal cells, including secretion of mucin, and is therefore more informative than Caco-2 cells [2,9,29]. In each case, a suspension of approximately 10^7–10^8 vegetative cells of each strain were incubated for 2 h with the cultured cell line, after which the number of adhering cells was measured. *B. cereus* was used as a positive control and found to be ten times more competent in binding to HT29-16E cells. Only GB14 showed significantly greater levels of binding compared to other *B. subtilis* isolates, including laboratory strain PY79. GB12 showed high levels of binding, but the ±SD variation was too great to be significant.

3.6. Electron microscopy

Some of the GIT isolates were examined by electron microscopy (Fig. 3). Spores of PY79, Natto and HU58 were found to have a similar and well-defined ultrastructure. Ovoid spores consisted of a core surrounded by a thick layer of peptidoglycan (the cortex) and two layers of spore coat, an

Table 3
Antimicrobial activity of *Bacillus* strains isolated from the human gut using the agar-solid diffusion method.

Strain[a]	*Staphylococci aureus*	*Listeria innocua*	*Salmonella indiana*	*B. subtilis* 168	*E. coli*
SC2329	±	−	−	−	−
PY79	−	±	−	−	−
Natto	−	++	−	+++	+++
HU1	−	+++	−	−	−
HU2	−	+++	−	−	−
HU5	+++	+++	−	+++	++
HU15	−	−	−	−	−
HU31	−	−	−	−	−
HU68	−	−	−	−	−
HU24	+++	±	±	−	−
HU39	−	+	−	−	+++
HU48	++	±	−	++	−
HU50	++	±	−	++	−
HU58	−	−	−	−	−
HU60	−	−	−	−	−
HU61	±	+++	++	−	−
HU63	±	−	−	−	−
HU67	±	++	−	−	+
HU70	−	−	−	−	±
HU71	±	+++	++	−	−
HU78	−	±	−	±	−
HU87	++	±	−	−	−
HU75	−	−	−	−	−
HU79	−	+++	++	−	+
HU83	+	++	−	−	+
GB2	−	−	−	−	−
GB5	−	+	−	−	−
GB4	−	+	−	−	−
GB6	−	−	−	−	−
GB10	−	−	−	−	−
GB11	−	−	−	−	−
GB12	−	−	−	−	−
GB13	−	−	−	−	−
GB14	−	−	−	−	−
Total	11	18	4	5	7

−, no inhibition; ±, weak inhibition; ++, 6–10 mm zone of growth inhibition; +++, >11 mm zone of inhibition.

[a] Isolate number taken from faeces (HUn) or gut biopsies (GBn).

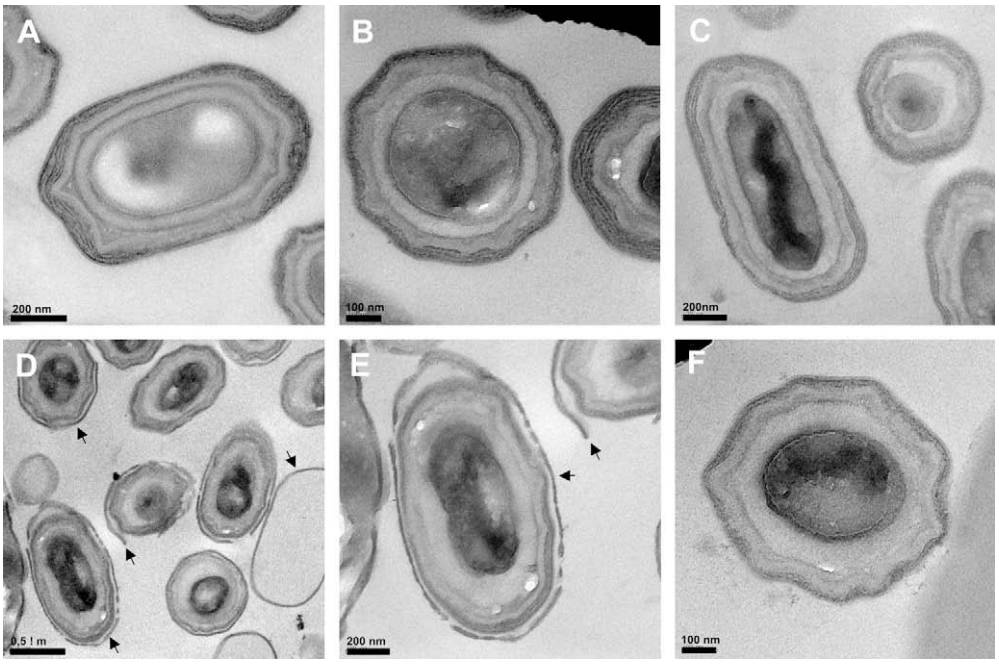

Fig. 3. TEM imaging. Panel A, PY79; panel B, Natto; panel C; HU58; panels D–F, GB14. Many GB14 spores carried a layer of material loosely bound to their outer coat (arrowheads) that was, in some cases, detached from some spores.

inner layer and an outer, electron-dense layer. Although we examined only a selection of isolates we identified one, GB14, that carried an additional layer that appeared loosely attached to the outer coat (arrows in Figs. 3D,E). In some cases, this additional layer was completely detached from the spore (arrowheads in Figs. 3D,F).

4. Discussion

Although soil is still cited as the primary habitat of the genus *Bacillus*, this may well be misleading, as mounting evidence suggests that spore-forming bacteria may inhabit the intestinal tracts of animals [22]. In this scenario, the robustness of spores enables them to survive transit through the stomach, after which they germinate, proliferate and then re-sporulate before excretion in the faeces [45]. If correct, then many *Bacillus* species would better be considered gut commensals, and this then raises a number of interesting questions regarding the impact of these Gram-positive bacteria on gut microflora, human nutrition and the evolutionary origin of spores themselves. This work is the first comprehensive report of the characterisation of spore-formers from the human GIT. At the outset, it is important to clarify that by the very nature of our experimental approach, the study had a number of obvious limitations. Firstly, for logistical reasons, we examined only a tiny fraction of total spore-formers found in the GIT and therefore we could not determine the total number, identity or distribution of *Bacillus* species within the GIT. Nor did we attempt to identify cells in the vegetative state, and we made the assumption that all spore-formers were culturable and we used only two locations for analysis (ileum and faeces). Despite this, our analysis does show that *Bacillus* spore-formers can be readily obtained from the small intestine and faeces, the latter of which we estimated to carry on average 10^4/g of material [45]. In the absence of an exhaustive screen, it is difficult to determine the total number of *Bacillus* that would reside in the small intestine, but we would estimate numbers greater than 10^9. While we cannot exclude the possibility that this number originates from accidental ingestion with food, we believe it highly unlikely and, as a case study, we focused our attention on *B. subtilis*, since this species is most familiar to the scientific community and most often cited as a soil organism. Interestingly, despite the geographic origins of the samples, almost half of the *B. subtilis* isolates formed a single haplotype, exhibiting 100% identity with the 168 type strain. Most importantly, our study of *B. subtilis* revealed that despite genetic conservation there was considerable diversity in the biotypes.

In addition to identifying *B. subtilis* in the human GIT, our second objective was to determine whether these isolates exhibited any trait that could be relevant for intestinal residency. First and foremost would be the ability to grow and sporulate under anaerobic conditions. All isolates grew anaerobically, and despite reports suggesting that *B. subtilis* cannot sporulate under anoxic [21] conditions, the majority of our isolates were able to form spores. It is probable that the small intestine carries sufficient oxygen to permit growth and sporulation, but the ability to form spores under anaerobic conditions is likely to be beneficial as bacteria enter the large intestine or form biofilms. Our work also revealed that most of the GIT isolates could form biofilms, and these 3D structures might enable *B. subtilis* to survive within the GIT, either attached to the mucosal wall or food particles, or in mixed biofilms. Biofilm formation by undomesticated strains of *B. subtilis* is well recognised and it has been shown that biofilms facilitate the formation of fruiting bodies whose apical

tips support sporulation-specific gene expression and differentiate into spores [5]. In the GIT, the biofilm would also serve to protect the colony from antimicrobials produced by competing bacteria and possibly shield cells from gastric and bile juices present within the gut lumen. One interesting discovery from this work is that the isolates showed remarkable diversity in their sporulation rates. Some strains, including domesticated strain PY79, sporulated relatively slowly, reaching maximal levels after 16 h. In contrast, a number of strains sporulated very quickly, within half of this time. This phenomenon has been observed previously [45] and we could correlate this with biofilm formation. Strains that did not form biofilms sporulated very slowly, whereas those that did form biofilms sporulated in 10 h or less (the one exception to this rule was HU24). We have suggested that rapid sporulation would enable a gut *B. subtilis* strain to adapt to an intestinal environment where the ability to protect oneself is at a premium [45]. It should be noted that we used liquid culture to measure sporulation rates, and under these conditions, biofilms did not form. This therefore shows that the mechanism for inducing rapid sporulation is not dependent upon any signalling mechanism that might exist in the biofilm. Surfactants are thought to be important for biofilm formation, since surfactant mutants (*sfp*) failed to make proper biofilms [5,25]. With a few exceptions (i.e., HU87 and HU61), strains that produced no detectable surfactin failed to produce a biofilm using the assays employed here. We found that, in all cases, *B. subtilis* cells had a strikingly low capacity to bind to mucus-secreting cells. Perhaps, then, biofilms could provide a mechanism enabling cells to adhere to the mucosal epithelium.

We were surprised to find that all isolates were haemolytic and the surface active lipopeptide, surfactin, may account for this. However, our results do not seem to support this view, since some strains (e.g., HU15 and HU31) that produced no surfactin produced complete haemolysis. There was, however, a close correlation between surfactin-producing strains and antimicrobial activity, but again there were exceptions, such as HU61 and HU87, that had significant antimicrobial activity yet no surfactin production. We also observed that the majority of faecal isolates were found to produce antimicrobials (16/22) compared to those obtained from the ileum (2/9). The numbers of isolates were too few for statistical conclusions but might indicate the presence of different *Bacillus* communities within the GIT. Our assay for surfactin was crude, and we cannot rule out a low level of surfactin, yet it seems likely that surfactin alone does not account for the antimicrobial activities recorded alone. Although we did not examine all isolates, we did find one, GB14, that produced spores that carried an additional layer to the spore coat. This layer was loosely attached and we propose that it is an exosporium, a feature described in a number of *Bacillus* species including *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. clausii* and *B. pumilus*, but not as yet in *B. subtilis* [19]. The role of the exosporium is unclear, but it may be involved in adhesion, where it would increase the hydrophobicity of the spore surface [13,27]. More surprising, though, was the fact that GB14 was a member of the 168 haplotype whose type strain, 168, was devoid of this layer.

In conclusion, our study shows that *B. subtilis* is present in the human GIT as a diverse population of strains. These seem to be armed with attributes that could enable survival within the GIT and support the hypothesis that this species, and probably other *Bacillus* species, are gut commensals. The next question to be addressed is their impact on the gut microflora and function.

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Appendix. Supplementary material

Suplementary data associated with this article can be found, in the on line version, at doi:10.10161j.resmic.2008.11.002.

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