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The Intestinal Life Cycle of *Bacillus subtilis* and Close Relatives†

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Bacillus subtilis **is considered a soil organism for which endospore formation provides a means to ensure long-term survival in the environment. We have addressed here the question of what happens to a spore when ingested. Spores displaying on their surface a heterologous antigen, tetanus toxin fragment C (TTFC), were shown to generate anti-TTFC responses not to the antigen contained in the primary oral inoculum but to those displayed on spores that had germinated and then resporulated. We then used reverse transcription-PCR to determine expression of vegetative genes and sporulation-specific genes in the mouse gut following oral dosing with spores. Significant levels of germination and sporulation were documented. Using natural isolates of *B. subtilis* that could form biofilms, we showed that these strains could persist in the mouse gut for significantly longer than the laboratory strain. Moreover, these isolates could grow and sporulate anaerobically and exhibited a novel phenomenon of being able to form spores in almost half the time required for the laboratory isolate. This suggests that spores are not transient passengers of the gastrointestinal tract but have adapted to carry out their entire life cycle within this environment. This is the first report showing an intestinal life cycle of *B. subtilis* and suggests that other *Bacillus* species could also be members of the gut microflora.**

Spores of *Bacillus* species are found in soil, dust, and water as well as in the air (27). Their primary reservoir though, has long been considered soil, and indeed, they can be found there in abundance. By associating with plant matter, it would be expected that *Bacillus* spores could enter the gastrointestinal tract (GIT) of animals by ingestion. Some regions of the GIT in particular (e.g., the stomach) would provide a formidable barrier to the vegetative bacterium, yet the spore would transit unimpeded. The question then, is what happens next, does the spore simply pass through the GIT or can it germinate and then proliferate? The GIT, especially the small intestine, is rich in nutrients, and although the pH is low, it can reach neutrality at the lower end of the colon. The primary signals that induce spore germination are nutritional (24), and in some *Bacillus* species, low pH has also been shown to help activate the germination process (12). For these reasons, it would be argued that spore germination should occur readily in the GIT, and using a molecular approach, this has recently been confirmed (4). For a soil organism, though, germination and outgrowth in the GIT must cope with a particularly toxic environment in which to survive, including anoxia, low pH, bile salts, and an extremely high concentration of commensal bacteria (reaching 10^{12}/g of fecal contents in the colon). Several lines of evidence suggest, though, that *Bacillus* spore formers have adapted to live and survive within the GIT.

First, an ever-increasing number of studies have shown the presence of *Bacillus* spores in the guts of animals. Most of these studies are detailed elsewhere (20), but notable examples include insect symbionts (*Bacillus sphaericus*, *Bacillus cereus*, and *Bacillus pumilus*) (15, 16), insect pathogens, (*Bacillus thuringiensis* and *Paenibacillus popilliae*), and spores recovered from the gut of earthworms (22). A recent exhaustive study has shown species of *Bacillus* to be readily found in the GIT of broiler chickens (2). In addition, two species, *Bacillus anthracis* and *Bacillus cereus* are known to be important gastrointestinal pathogens (22).

Second, an intriguing study shows that *Bacillus subtilis* plays a primary role in development of the gut-associated lymphoid tissue (GALT) and the preimmune antibody repertoire in rabbits (29). This study also showed that sporulation, and not vegetative cell growth, is essential for GALT development.

Finally, studies in mice have shown that animals given a fixed, oral dose, of spores excreted more spores in their feces than were administered (19). The only explanation for these results is that spores germinated and then resporulated in the GIT.

In this work, we have used a molecular approach to prove that orally administered *B. subtilis* spores germinate, proliferate, and then resporulate within the gut of a mouse model. We interpret this as evidence that *B. subtilis* has adapted to the intestinal environment as part of its natural life cycle.

MATERIALS AND METHODS

Bacterial strains. PY79 is a laboratory, or domesticated, strain and is Spo$^+$, prototrophic, and derived from the type strain 168 (31). RH103 (*amyE*::*cotB-tetC*) has been described previously (21) and carries a chimeric gene, *cotB-tetC* that encodes an 82-kDa chimeric CotB-TTFC (tetanus toxin fragment C) polypeptide that is assembled into the spore coat inserted at the *amyE* locus of PY79. TTFC is derived from *Clostridium tetani* tetanus toxin (encoded by *tetX*), as described previously (21). *tetC* refers to the 3-sequence of the *C. tetani tetX* gene (codons 123 to 573) that encodes the C-terminal fragment C (13). UL12 (*amyE*::*cotB-tetC gerD-cwlB*Δ::*neo*) was created by transforming competent cells

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of strain TB1 (*gerD-cwlB*Δ::*neo*) with chromosomal DNA from RH103, followed by selection for chloramphenicol resistance (Cm^r) carried by the *cotB-tetC* cassette. TB1 has the *gerD-cwlB* region of the chromosome replaced with a neomycin resistance gene, and spores of this strain are germination defective (<0.0015%) compared to wild-type spores of strain PY79 (8). DL237 (*amyE*::*rrnO-tetC*) was created by transforming competent cells of PY79 with plasmid pDL229 carrying the *PrrnO-tetC* cassette with selection for Cm^r. These transformants arose from a double-crossover recombination at the *amyE* locus of PY79. DL291 (*amyE*::*cotB-tetC thrC*::*rrnO-tetC*) was created by transforming competent cells of RH103 with plasmid pDL264 carrying the *PrrnO-tetC* cassette with selection for erythromycin resistance (Erm^r). Erm^r transformants arose from a double-crossover recombination at the *thrC* locus. HU58 and HU78 are both Spo^+, undomesticated isolates of *B. subtilis*, isolated from human feces as reported in this work. BT17 (*amyE*::*rrnO-tetC* Cm^r) was constructed by introducing the *PrrnO-tetC* into the genome of HU58 by transformation with pDL229. BT47 (*amyE*::*cotB-tetC*, Cm^r) was created by transforming cells of HU58 with pNS6 that carries *cotB-tetC* at the *amyE* locus (21). DL1033 (SC2329) is a pathogenic strain of *B. cereus* (18).

Plasmid constructions. (i) pDL229. Plasmid pDL229 carries a cassette, *PrrnO-tetC*, that expresses the *Clostridium tetani tetC* gene downstream of the *B. subtilis rrnO* gene promoter, which is vegetatively expressed. Translation signals were provided by the *sspA* gene of *B. subtilis*. This cassette was contained in plasmid pDG364 that, when linearized, allows integration by a double-crossover recombination into the *amyE* locus of the *B. subtilis* chromosome (6, 17). Selection is made for Cm^r. To construct the *PrrnO-tetC* gene fusion, two steps were used: first, construction of a plasmid carrying the promoter of the *rrnO* operon (encoding rRNAs) fused to a ribosome binding site (RBS) and multiple cloning site (MCS); second, insertion of the *tetC* gene (corresponding to codons 123 to 573 of *C. tetani tetX*) (13) adjacent to and downstream of *PrrnO-RBS*. The *rrnO* promoter was first amplified by PCR from the *B. subtilis* chromosome using two oligonucleotides (forward, 5′-gaagatctGCATGACCATTATGACTAG; reverse, 5′-gctctagaACAGGTTAAGTTCACCGCATCC) as primers, resulting in a 244-bp amplicon containing the −35 and −10 regions of the *B. subtilis rrnO* promoter. The forward primer carried a 5′ BglII restriction enzyme site, and the reverse primer carried a 5′ XbaI site (within lowercase type in the sequences). The RBS of the *B. subtilis sspA* gene was artificially created by annealing two oligonucleotides (forward, 5′-ctagaACAAGGAGGTGAGACc; reverse, 5′-catg gGTCTCACCTCCTTGTt). This created 5′ and 3′ sticky ends corresponding to the XbaI and NcoI sites, respectively. The amplified P*rrnO* promoter PCR was cleaved with BglII and XbaI and together with the *sspA* RBS DNA cloned in plasmid pET28b (Novagen) between the BglII and NcoI restriction enzyme sites, resulting in plasmid pDL180. This plasmid carries the *PrrnO-RBS* sequence inserted upstream of the MCS carried in pET28b. The *PrrnO-RBS* sequence and flanking MCS from plasmid pDL180 were then amplified using two primers (forward, 5′-gccagctgCGATGCGTCCGGCGTAGAGGATCG; reverse, 5′-gcca gctgGCAGCCGGATCTCAGTGGTGGTGG). These primers annealed to pET28b sequences in pDL180 upstream and downstream of *PrrnO*-RBS-MCS. The PCR product was cut using PvuII and then cloned in plasmid pDG364 (6, 17) between the EcoRI and BamHI restriction enzyme sites which had been blunt ended using Klenow fragment, yielding plasmid pDL205. pDG364 carries a cassette comprised of the *cat* gene (encoding Cam^R) and a EcoRI-HindIII-BamHI cloning site placed between the left and right segments of the *amyE* (amylase) gene of *B. subtilis*. The *C. tetani tetC* fragment (encoding TTFC) was amplified by PCR from chromosomal DNA of strain RH103 (carrying the *cotB-tetC* gene fusion) (21) using two oligonucleotides (forward, 5′-ctagctagcAAAA ATCTGGATTGTTGGG; reverse, 3′-cccaagcttTTAATCATTTGTCCATCC TTC) as primers. An amplification product of the expected size (1,356 bp) was cloned in the above pDL205 vector between the NheI and HindIII restriction enzyme sites within the MCS, yielding plasmid pDL229. This plasmid was verified by DNA sequencing of the insert across the *PrrnO-RBS-tetC* fusion junction.

(ii) pDL264. This plasmid is similar to pDL229 and carries the same *PrrnO-tetC* cassette but contained within a vector, pDG1664, that allows ectopic insertion at the *thrC* locus and selection for Erm^r transformants (17). The *PrrnO-PBS-MCS* segment from plasmid pDL180 [see "(i) pDL229" above] was PCR amplified, cleaved with PvuII, and inserted into plasmid pDG1664 cut with the EcoRI and BamHI sites using the same method as described above for pDL229, resulting in plasmid pDL242. pDG1664 is essentially identical to pDG364 but carries the left and right flanking segments of the *B. subtilis thrC* gene and an Erm^r marker with EcoRI-HindIII-BamHI cloning sites (17). The *tetC* gene was also amplified and cloned into plasmid pDL242 as described above, yielding plasmid pDL264.

General methods and preparation of spores. Methods for transforming *B. subtilis* competent cells, selection of antibiotic resistance, and measurement of heat-resistant spores were as described previously (6, 28). Spores prepared for immunization experiments were prepared by the exhaustion method using DSM (Difco sporulation medium) (9). Spore suspensions were lysozyme treated and then heat treated (68°C 1 h) to remove residual vegetative cells and stored as aliquots at −20°C prior to use. Spore coat proteins were extracted from concentrated suspensions of spores (>1×10^{10} spores/ml) using a sodium dodecyl sulfate-dithiothreitol extraction buffer (28). Western blotting was performed using 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels probed with a polyclonal antiserum to TTFC raised in rabbits, purified, and used at a dilution of 1/3,000. TTFC protein was expressed from a pET28b expression vector where the *tetC* gene was fused at its 3′ end to the poly-His tag. Bands were visualized using the ECL detection system (Amersham) and subjected to densitometric analysis using the ChemiDoc XRS System (Bio-Rad).

Immunizations and indirect enzyme-linked immunosorbent assay for detection of TTFC-specific IgG. Groups (6 to 8 animals, as indicated) of C57BL/6 (6 weeks old) mice (Harlan, United Kingdom) were dosed orally with suspensions of recombinant or nonrecombinant spores at 2×10^{10} per dose (0.2 ml) on days 1, 2, 3, 24, 25, 26, 49, 50, and 51. A naive group received sterile water. Serum samples were from tail bleeds collected on days 0, 23, 48, and 68. Immunoglobulin G (IgG) responses specific to TTFC were as described previously using indirect enzyme-linked immunosorbent assay (9). The end-point titer was calculated as the dilution of serum producing the same optical density as a 1/40 dilution of a pooled preimmune serum. Data are presented as arithmetic means, with error bars for standard deviations. Statistical comparisons between groups were made by the Mann-Whitney U test. A P value of >0.05 was considered nonsignificant.

Analysis of gene expression by reverse transcription (RT)-PCR. The basic strategy to examine expression of genes in the mouse GIT was as described previously (4). In brief, groups of mice (BALB/c, 6 weeks old; Harlan, United Kingdom) were dosed orally with suspensions of spores at 2×10^{10} per dose (0.2 ml) of DL291 (PY79 *amyE*::*cotB-tetC thrC*::*rrnO-tetC*), BT17 (*HU58 amyE*::*rrnO-tetC*), or BT47 (HU58 *amyE*::*cotB-tetC*). At appropriate times, groups were sacrificed and dissected, sections of the GIT were removed, and total RNA was extracted by treatment in Trizol (Life Technologies). RNA was DNase treated and quantified by spectroscopy (GeneQuant II; Pharmacia), and integrity was verified using mouse-specific β-actin PCR primers (4).

To detect *cotB-tetC* or *rrnO-tetC*, mRNA primers annealing to the *cotB* (FcotB1, 5′-AGCAGACGCCAGTTGGAGTTTTGG-3′) and *tetC* (RtetC5, 5′-GCCATTTATTCCGGGCACCAATTGAGC-3′) and *rrnO* (FrrnO4; 5′-CGTA GAGGATCGAGATCTGCAT GAC-3′) and *tetC* (RtetC5) segments, respectively, were used and are shown in Fig. 1. Using these primers, an amplification of *cotB-tetC* mRNA generated a cDNA product of 559 bp and 522 bp for *rrnO-tetC*.

Quantification of RNA. To quantify the level of *rrnO-tetC* mRNA, we used a competitive RT-PCR assay as described previously (4). The basic elements of this assay are to generate a competitive PCR template. To achieve this, a PCR is performed using two templates: total RNA extracted from the mouse gut section (above), used at a fixed concentration, and the competitor, which is used in a dilution series. The dilution where the two PCR products are equal enables extrapolation of the quantity of *rrnO-tetC* by regression analysis. Using a standard graph of the number of vegetative bacteria (CFU) versus the concentration of PCR-amplified *rrnO-tetC* (using competitor primers), the number of vegetative cells can be determined. In this assay, a new primer, FrrnO5 (5′-GAGGATC GAGATCTGCATGACAGTCAGTCAAACTAGGGCCTG-3′), that anneals to *rrnO* sequences downstream of those bound by FrrnO4 was used (Fig. 1). FrrnO5 also carried a 5′ tag sequence (underlined) that bound to the FrrnO4 primer. To generate the competitive template, FrrnO5 and RtetC5 were used to PCR amplify a 354-bp amplicon from DL291 chromosomal DNA. In conjunction with RtetC5, a 354-bp RT-PCR product is generated. This 354-bp competitive PCR product was gel purified, denatured, quantified, and used in a dilution series with a fixed concentration of total mRNA (1 μg). PCR primers for the competitive reaction were FrrnO4 and RtetC5. To quantify *cotB-tetC* mRNA, the primer FcotB2 (5′-AGACGCCAGTTGGAGTTTTGGTTGTAGATAATGCCGACG GCC-3′) was used in conjunction with RtetC5 using RH103 chromosomal DNA. Sequences in this primer annealed to *cotB* and also to FcotB1 (underlined). Use of FcotB2 and RtetC5 generated a competitive template of 466 bp.

Total DNA isolation. The DNAzol DNA extraction kit (Invitrogen) was used to isolate total DNA from mouse gut sections. The procedure is based on the use of a novel guanidine detergent lysing solution that hydrolyzes RNA and allows the selective precipitation of DNA from a cell lysate. Extracted DNA was dissolved in water, and the concentration was determined using a GeneQuant spectrophotometer (Pharmacia).

Quantification of DNA. For quantification of *B. subtilis* total DNA, competitive PCRs with an internal standard template were used. The primers, condi-

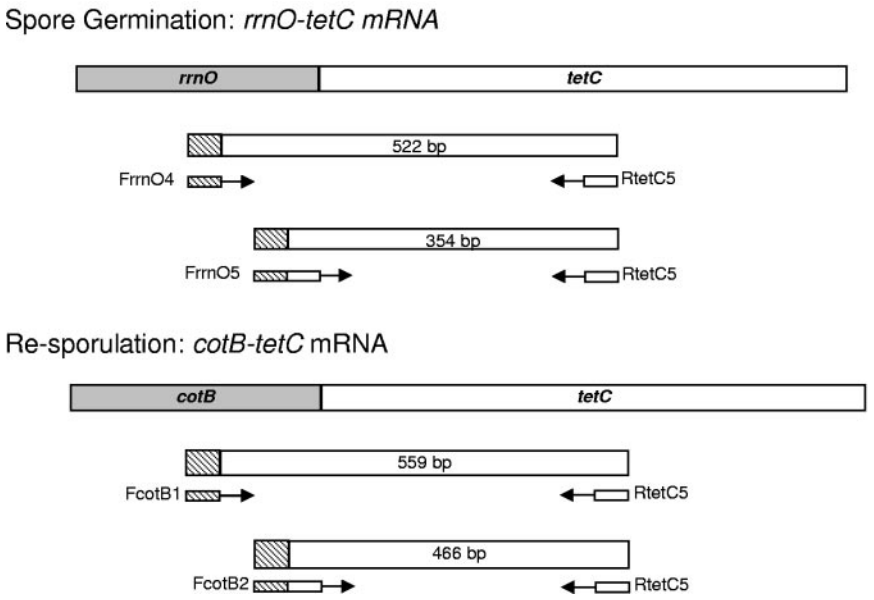

FIG. 1. RT-PCR primers. Physical map of the *cotB-tetC* and *rrnO-tetC* chimeric genes. The primer pairs FcotB1 and RtetC5 and FrrnO4 and RtetC5 were used to amplify a 559-bp segment across the *cotB-tetC* fusion junction and a 522-bp segment across the *rrnO-tetC* fusion junction. FcotB2 and RtetC5 and FrrnO5 and RtetC5 were, respectively, used to amplify 466- and 354-base competitor templates. The hatched boxes indicate the sequence included in the 5′ end of the FcotB2 primer, which is recognized by the FcotB1 primer, and the sequence included in the 5′ end of the FrrnO5 primer, which is recognized by the FrrnO4 primer.

tions, and regression analysis were the same as described above with competitive RT-PCR. Quantification was done using PY79 cells grown in LB medium, and supporting data are shown in the supplemental material. The number of cells was determined at the appropriate time points by serial dilution and plating for CFU/ml. At the same time points, the DNA concentration was determined by spectroscopy. Since during cell growth the number of chromosomes/cell would vary, we calculated their number as being maximal during logarithmic phase (10 chromosomes/cell) and lowest at stationary phase (2 chromosomes/cell). A total of 10^9 cells each carrying 2 chromosomes would equate to approximately 10 μg DNA, and 10^9 cells each carrying 10 chromosomes would be equivalent to 44 μg in total. Next, using DNA concentrations determined by PCR from intestinal samples, we could calculate the minimal and maximal number of cells using these values, with the caveat that the actual intestinal population of vegetative cells would be heterogeneous and would not carry all bacteria with an equal number of chromosomes.

Isolation and screening of spore formers from feces. Samples of freshly voided fecal material were collected from volunteers, diluted (1:10) in phosphate-buffered saline (PBS), and resuspended until a homogenous suspension was obtained. Next, 1 ml of the suspension was heated at 65°C for 1 h, and serial dilutions were made in PBS before plating on DSM agar. Volunteers were, in all cases, healthy and had not taken probiotic supplements for 12 months prior to sampling.

Persistence studies. Mice were housed in cages with gridded floors to prevent coprophagia. A single dose (0.2 ml) of 1×10^9 spores was given to mice by oral gavage. For sampling, individual mice were removed and held until a single fresh fecal pellet was collected, weighed by difference, and stored at −20°C before analysis of heat-resistant CFU/g as described previously (7).

Treatment in simulated intestinal conditions. Spores or vegetative cells were suspended in simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) and incubated at 37°C as described previously (7). To sample, the suspension was washed three times with water, serially diluted, and plated onto LB or DSM agar plates to determine CFU.

Anaerobic growth. *B. subtilis* strains grown in liquid culture to an optical density at 600 nm (OD_{600}) of 0.5 and plated on solid DSM agar plates. For anaerobic growth, potassium nitrate at a concentration of 5 mM or potassium nitrite at 2.5 mM was added to the medium as an electron acceptor as described previously (25, 30). After 72 h of incubation at 30°C in an anaerobic chamber, the entire bacterial lawn was recovered from each plate in 2 ml of PBS buffer. This suspension was immediately serially diluted and plated out for CFU or heat treated (68°C, 45 min) before serial dilution to determine spore counts. Serially diluted plates were incubated aerobically.

Biofilm formation. CMK agarose plates were used for biofilm formation (14). A fresh single bacterial colony was picked using a sterile wooden toothpick and dotted in the middle of the agarose plate. Plates were incubated at 37°C for 2 to 3 days.

RESULTS

Evidence suggesting that spores germinate and sporulate within the GIT. In a mouse model, oral delivery of RH103 spores that express the *C. tetani* TTFC polypeptide on the spore coat has been shown to produce protective levels of anti-TTFC IgG antibodies (9). In these studies, peak antibody titers were achieved after the ninth dose on day 35. In addition to surface display of antigens, spores also offer an alternative route for delivery where the antigen is expressed only following germination of the spore in the gastrointestinal tract. In this approach, the antigen is expressed under the control of a *B. subtilis* vegetative promoter and was based on molecular studies showing in vivo germination of spores in the GIT (4) and studies where immune responses against a vegetatively expressed protein were achieved (1). In these immunological studies, though, there was one weakness in that the oral dose of spores was not verified as being free from vegetative cells, a common problem in the preparation of spores. More complete studies were made more recently, confirming that immune responses could be raised against a heterologous antigen expressed in a germinating spore (8). We constructed a strain (DL237) of *B. subtilis*, isogenic to RH103, which expresses the TTFC protein only in the vegetative cell. Here, the *C. tetani tetC* gene was fused to the *rrnO* gene of *B. subtilis*, enabling high levels of constitutive gene expression but only in the vegetative cell (8). Groups of inbred mice were dosed with 2 ×

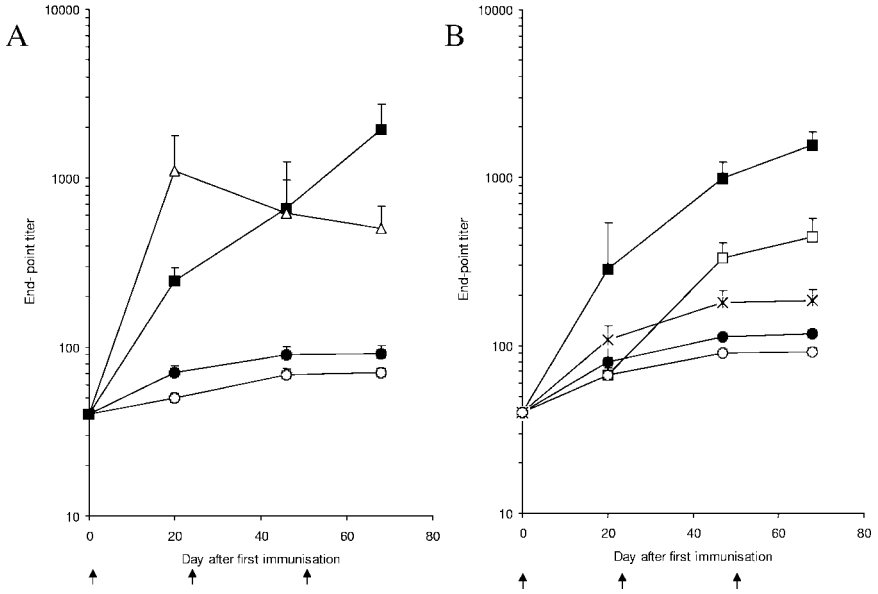

FIG. 2. Serum anti-TTFC-specific IgG responses. (A) Groups of 7 C57BL/6 mice were dosed orally with 2×10^{10} spores expressing a *C. tetani* antigen, TTFC, as follows: ■, spores expressing TTFC on the spore surface (strain RH103 *amyE*::*cotB-tetC*); △, spores expressing TTFC only when the spore germinates (strain DL237, *amyE*::*rrnO-tetC*). Control groups were groups immunized with nonrecombinant spores of strain PY79 (●; *spo*$^+$) and a naive group consisting of nonimmunized animals (○). The dosing strategy comprised three sets of three separate doses indicated by arrows. Serum samples were tested for anti-TTFC-specific IgG. (B) Groups of 7 C57BL/6 mice were dosed orally with 2×10^{10} spores as follows: ■, RH103 spores that express TTFC on the spore surface (*amyE*::*cotB-tetC*); ×, UL12 spores (*amyE*::*cotB-tetC gerD-cwlB*Δ::*neo*) that express CotB-TTFC on the spore surface but are unable to germinate; □, spores of RH103 that had been stripped of CotB-TTFC by treatment with SGF plus SIF. Control groups were mice immunized with nonrecombinant PY79 spores PY79 (●; *spo*$^+$) and a naive group (○). The dosing strategy comprised three sets of three separate doses indicated by arrows.

10^{10} spores of RH103 or DL237, and anti-TTFC IgG titers were determined postdosing (Fig. 2A). The kinetics of humoral responses showed a marked difference, with more immediate responses in DL237-immunized mice, peaking at day 23, and then declining. By contrast, anti-TTFC responses in mice dosed with RH103 spores, albeit more gradual, continued to rise. This result was unexpected, since DL237 spores must first germinate to deliver the TTFC antigen to the GALT, so we would have predicted a slower response.

We next constructed a strain, UL12, that displayed CotB-TTFC on the spore surface but that was unable to germinate by incorporating the *gerD-cwlB*Δ::*neo* allele on the chromosome. This allele reduces spore germination to levels less than 0.0015% (8). Mice dosed with 2×10^{10} spores of UL12 produced very low levels of anti-TTFC IgG when measured in parallel to mice dosed with RH103 spores (Fig. 2B). These were at levels significantly no different ($P > 0.05$) from control groups (naive and those dosed with nonrecombinant PY79 spores). These results suggest that, to generate anti-TTFC responses, the spore must germinate and it is resporulation and expression of CotB-TTFC that contributes to the anti-TTFC responses. In further support of this, we also ran in parallel an immunization experiment where we had dosed mice with 2×10^{10} spores of RH103 that had been preincubated in SGF (30 min) followed by SIF (90 min), which stripped over 95% of spore coat-associated TTFC (data not shown). This treatment had little effect on spore viability (measured over 3 days), though it did reduce the rate at which spores germinated. As shown in Fig. 2B, mice receiving these spores generated anti-TTFC responses that were delayed relative to untreated RH103 spores yet were significantly higher than control groups ($P < 0.05$). These experiments indicate that the majority of anti-TTFC IgG responses obtained when mice were immunized with RH103 spores may not have originated from the original inoculum of CotB-TTFC spores but instead from spores that had germinated and then resporulated.

Molecular evidence that spores germinate and proliferate within the GIT. Groups of mice were given oral doses of 2×10^{10} spores of strains DL291 (*amyE*::*cotB-tetC thrC*::*rrnO-tetC*), BT17 (*amyE*::*rrnO-tetC*), or BT47 (*amyE*::*cotB-tetC*). DL291 is a derivative of the laboratory strain PY79, and BT17 and BT47 were derived from a natural *B. subtilis* isolate, HU58, that had been isolated from the human GIT (see below). Total RNA was recovered from dissected sections of the GIT at different times and evaluated for the presence of *rrnO-tetC* mRNA, which would indicate spore germination, and *cotB-tetC*, which would arise from sporulation of these germinated and growing cells. All experiments were performed in parallel and have been repeated in their entirety with similar results (Fig. 3). Using RT-PCR, we found that *rrnO-tetC* was expressed only in the jejunum of the murine gut. With PY79, expression occurred only between hours 18 and 30. By contrast, in the natural isolate, expression occurred earlier, beginning 12 h after dosing. With either strain, no signal was detected after 30 h. Quantification of the chimeric mRNA signal in jejunum samples showed high levels of germination at ~1 to 5% at hour 18 for PY79 and 1 to 4% for HU58 (Table 1). These levels of germination gradually declined thereafter.

Analysis of *cotB-tetC* mRNA showed that, for both PY79

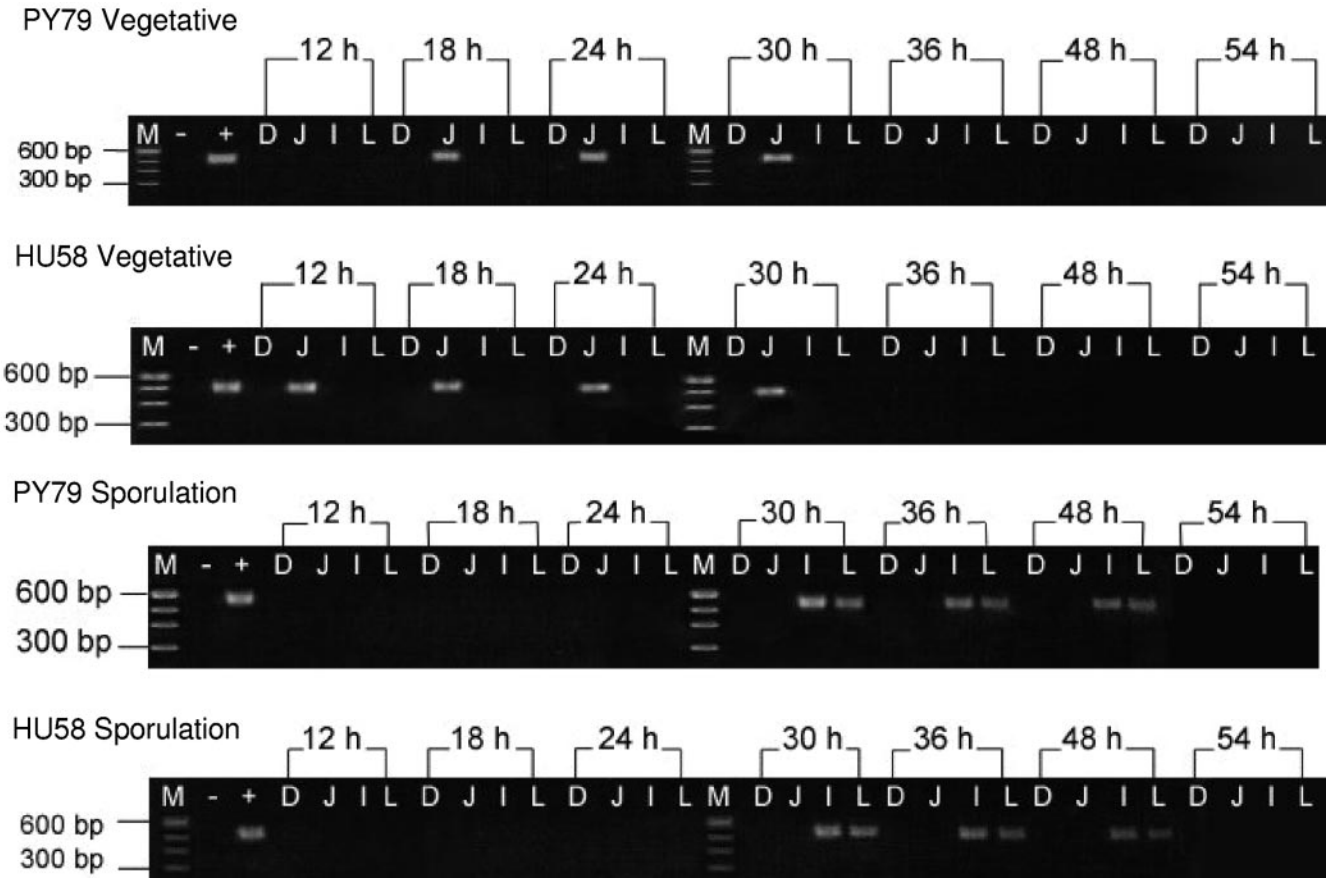

FIG. 3. RT-PCR analysis of germination and sporulation in the mouse gut. RT-PCR analysis of germination and sporulation genes in vivo. Groups of mice (4) were orally dosed with suspensions of spores (2×10^{10}) of DL291 (PY79 *amyE*::*cotB-tetC thrC*::*rrnO-tetC*), BT17 (HU58 *amyE*::*rrnO-tetC*), and BT47 (HU58 *amyE*::*cotB-tetC*). Mice were sacrificed at the indicated times, sections of the GIT were dissected, and total RNA was recovered. To detect germination gene expression, two primers (FrrnO4 and RtetC5) were used to amplify a 522-bp cDNA (Fig. 1). To detect sporulation gene expression, primers FcotB1 and RtetC5 were employed to amplify a 549-bp cDNA (Fig. 1). The marker (M) is a 100-bp ladder. The negative control (−) was from naive mice, and the positive control (+) was amplification from chromosomal DNA of either RH103 (*cotB-tetC*) or DL237 (*rrnO-tetC*). Reactions from one mouse in each group are shown, but all mice per group behaved identically. D, duodenum; J, jejunum; I, ileum; L, large intestine.

(DL291) and HU58 (BT47), sporulation occurred in the ileum and large intestine starting at hour 30 and ending 48 h postdosing. Using *cotB-tetC*-specific primers, we could determine the amount of RNA and DNA present in the GIT sections by competitive PCR. This analysis only measures live cells that are undergoing sporulation and not dormant spores, since the DNA and RNA extraction methods used here could not break endospores (data not shown). Therefore, competitive PCR revealed an estimate of the number of sporulating cells in the ileum and large intestine samples (Table 1). However, since we do not know precisely how much of the original inoculum germinated, we cannot determine the percentage that went on to sporulate.

These results show that all *B. subtilis* strains tested (natural and laboratory strains) must be able to sporulate in the mouse gut. To determine whether sporulation could occur under anoxic conditions, we examined growth and sporulation under anaerobic conditions of PY79 and two natural isolates, HU58 and HU78 (Table 2). PY79 was able to grow under anoxic conditions but was essentially unable to sporulate. By contrast, both HU58 and HU78 could sporulate under anaerobic conditions. Using medium enriched with nitrite or nitrate to provide an appropriate terminal electron acceptor (26) for anaerobic growth, sporulation efficiencies as high as 56% were achieved with these strains, in marked contrast to the laboratory isolate PY79.

Isolation of spore formers from the human GIT. We measured the numbers of spore formers that could be found in human feces from 30 volunteers. Our results shown in Table 3 demonstrate that spores could readily be recovered and that counts were in the range of 10^3 to 10^8 CFU/g of feces, with a figure of 10^4 being a realistic mean. Since these colonies grew aerobically, tested catalase positive, and carried spores, they were most likely to be species of *Bacillus* and could not be clostridia. In a separate study, we have pooled fecal samples from 34 volunteers and randomly isolated 82 colony isolates based on differences in morphology and 16S rRNA gene phylogenetic analysis representing 10 different *Bacillus* species, 24 isolates of which were *B. subtilis* (L. Duc and S. Cutting, unpublished data). Two of these *B. subtilis* isolates, HU58 and HU78, were both able to form biofilms, in contrast to the domesticated strain PY79 that cannot (Fig. 4) (3). We also

TABLE 1. Quantification of germination and sporulation[a]

Strain	Intestine section	Time point	RNA (μg/g)	DNA (μg/g)	Minimum no. of cells[b]	% of inoculum[c]	Maximum no. of cells[b]	% of inoculum[c]
PY79	Jejunum (*rrnO-tetC*)	12	—	—	—	—	—	—
		18	10.6 ± 3.4	79.4 ± 6.2	$(2.4 \pm 0.7) \times 10^8$	1.20	$(1 \pm 0.3) \times 10^9$	5
		24	1.0 ± 0.4	11.4 ± 1.1	$(2.3 \pm 0.9) \times 10^7$	0.12	$(9.6 \pm 3.8) \times 10^7$	0.48
		30	0.09 ± 0.04	0.6 ± 0.09	$(2.1 \pm 0.9) \times 10^6$	0.01	$(8.6 \pm 3.8) \times 10^6$	0.043
	Ileum (*cotB-tetC*)	30	2.0 ± 0.7	10.4 ± 1.9	$(4.6 \pm 1.5) \times 10^7$	NA	$(1.9 \pm 0.7) \times 10^8$	NA
		36	0.6 ± 0.1	2.7 ± 0.4	$(1.4 \pm 0.2) \times 10^7$	NA	$(5.8 \pm 1) \times 10^7$	NA
		48	0.03 ± 0.008	0.15 ± 0.02	$(6.8 \pm 1.8) \times 10^5$	NA	$(2.9 \pm 0.8) \times 10^6$	NA
	Large intestine (*cotB-tetC*)	30	0.6 ± 0.1	2.6 ± 0.3	$(1.4 \pm 0.2) \times 10^7$	NA	$(5.8 \pm 1) \times 10^7$	NA
		36	0.009 ± 0.006	0.05 ± 0.008	$(2.1 \pm 1.3) \times 10^5$	NA	$(8.8 \pm 5.8) \times 10^5$	NA
		48	0.007 ± 0.001	0.03 ± 0.007	$(1.6 \pm 0.2) \times 10^5$	NA	$(6.8 \pm 1) \times 10^5$	NA
HU58	Jejunum (*rrnO-tetC*)	12	7.6 ± 1.5	62.7 ± 6.0	$(1.7 \pm 0.3) \times 10^8$	0.85	$(7.3 \pm 1.4) \times 10^8$	3.65
		18	8.6 ± 2.8	70.8 ± 6.2	$(2.0 \pm 0.6) \times 10^8$	1.00	$(8.3 \pm 2.7) \times 10^8$	4.15
		24	0.7 ± 0.3	5.9 ± 1	$(1.6 \pm 0.7) \times 10^7$	0.08	$(6.8 \pm 3) \times 10^7$	0.34
		30	0.05 ± 0.02	0.5 ± 0.08	$(1.1 \pm 0.5) \times 10^6$	0.006	$(4.8 \pm 1.9) \times 10^6$	0.024
	Ileum (*cotB-tetC*)	30	1.6 ± 0.6	8.2 ± 1	$(3.6 \pm 1.4) \times 10^7$	NA	$(1.5 \pm 0.6) \times 10^8$	NA
		36	0.9 ± 0.5	4.2 ± 0.7	$(2.1 \pm 1.1) \times 10^7$	NA	$(8.8 \pm 4.8) \times 10^7$	NA
		48	0.07 ± 0.01	0.4 ± 0.07	$(1.6 \pm 0.2) \times 10^6$	NA	$(6.8 \pm 1) \times 10^6$	NA
	Large intestine (*cotB-tetC*)	30	0.6 ± 0.1	3.2 ± 0.3	$(1.4 \pm 0.2) \times 10^7$	NA	$(5.8 \pm 1) \times 10^7$	NA
		36	0.03 ± 0.008	0.18 ± 0.015	$(6.8 \pm 1.8) \times 10^5$	NA	$(2.9 \pm 0.8) \times 10^6$	NA
		48	0.01 ± 0.006	0.06 ± 0.008	$(2.3 \pm 1.4) \times 10^5$	NA	$(9.6 \pm 5.8) \times 10^5$	NA

[a] Samples of intestinal tissue from mice dosed with suspensions of spores expressing *rrnO-tetC* and *cotB-tetC* were removed, and total DNA and RNA were recovered as described in Materials and Methods and illustrated in Figure 3. RNA and DNA in jejunum samples were quantified for germination-specific gene expression using *rrnO-tetC* primers and ileum and large intestine samples for sporulation-specific gene expression with *cotB-tetC* primers. Using either RT-PCR for *B. subtilis*-specific RNA or PCR for *B. subtilis*-specific DNA, the quantity of each nucleic acid was determined by competitor and regression analysis and expressed in μg/g of sampled tissue. NA, not applicable. For PY79, the strain DL291 was used, and for HU58, strains BT17 and BT47 were used. A dash (—) indicates that no detectable PCR signal was apparent.

[b] Based on in vitro analysis of DNA extracted from cells growing in LB medium (see the supplemental material), the number of cells was approximated. Two estimates are given, a minimum estimate of the number cells based on 10^9 cells containing 10 chromosomes/cell and 44 μg DNA and a maximum estimate from 10^9 cells carrying 2 chromosomes/cell and containing 10 μg DNA.

[c] For samples showing germination, the number of cells based on DNA quantifications is expressed as a percentage of the original spore inoculum. Minimum and maximum estimated values are given.

examined the ability of spores of these strains to adhere to Caco-2 cells and found that both HU58 and HU78 as well as the laboratory strain PY79 could adhere (data not shown). For vegetative cells, though, the level of adhesion of all strains, including a *B. cereus* control, was reduced. We next examined the ability of HU58 and HU78 to persist in the mouse GIT in comparison to the laboratory strain PY79. We gave mice a single oral dose of 1×10^9 spores of HU58, HU78, and PY79 and then at appropriate time intervals counted heat-resistant CFU/g in the feces. Our results (Fig. 5A) showed that both HU58 and HU78 could persist significantly longer in the mouse GIT. The detection limit (the level at which counts were considered statistically significant) in feces was below 10^3/g, and using this criteria, PY79 was shed within 15 days. By contrast, HU58 and HU78 spores were still detectable at day 27, showing that these undomesticated strains could maintain themselves within the gut for almost twice as long as a domesticated strain.

Sporulation in human gut isolates of *B. subtilis*. Sporulation of HU58 and HU78 was measured in parallel to strain PY79

TABLE 2. Sporulation efficiency after 3 days of incubation at 30°C[a]

Growth conditions	Parameter (unit)	Result for strain and medium								
		PY79			HU58			HU78		
		DSM	DSM + NO_2	DSM + NO_3	DSM	DSM + NO_2	DSM + NO_3	DSM	DSM + NO_2	DSM + NO_3
Aerobic	Total count (CFU/plate)	2.1×10^{10}	2.21×10^{10}	1.05×10^{10}	2.07×10^{10}	2.18×10^{10}	1.88×10^{10}	1.46×10^{10}	1.19×10^{10}	1.31×10^{10}
	Spore count (CFU/plate)	1.82×10^{10}	2.22×10^{10}	1.04×10^{10}	1.96×10^{10}	2.02×10^{10}	1.82×10^{10}	1.40×10^{10}	1.16×10^{10}	1.30×10^{10}
	Sporulation efficiency (%)	87	100	99	95	93	97	98	99	100
Anaerobic	Total count (CFU/plate)	1.97×10^7	2.85×10^7	2.48×10^7	0.92×10^7	1.96×10^7	1.48×10^7	1.54×10^7	2.45×10^7	2.11×10^7
	Spore count (CFU/plate)	9	23	27	4.14×10^4	2.44×10^6	2.36×10^6	9.1×10^5	6.6×10^6	1.19×10^7
	Sporulation efficiency (%)	4.6×10^{-5}	8.1×10^{-5}	1.1×10^{-4}	0.5	12	16	6	27	56

[a] *B. subtilis* strains grown in liquid culture were plated on solid DSM agar plates. For anaerobic growth, potassium nitrate or potassium nitrite was added to the medium as an electron acceptor. After 72 h of incubation at 30°C, the entire bacterial lawn was recovered from each plate, and the suspension was serially diluted and plated out for CFU or heat treated (68°C, 45 min) before serial dilution to determine spore counts.

TABLE 3. Counts of *Bacillus* spore formers in human feces[a]

Subject no.	CFU/g feces for:					
	Infant		Adult		Elderly	
	Male	Female	Male	Female	Male	Female
1	6.88×10^3	6.80×10^3	8.10×10^3	3.05×10^3	4.50×10^4	1.92×10^4
2	8.6×10^4	9.70×10^4	6.20×10^4	9.60×10^4	5.35×10^3	1.50×10^4
3	2.34×10^5	2.30×10^3	1.92×10^5	1.53×10^8	2.60×10^4	8.60×10^3
4	4.30×10^5	2.70×10^4	2.51×10^5	2.00×10^4	2.30×10^4	1.40×10^4
5	5.00×10^3	2.00×10^4	6.30×10^4	3.80×10^4	1.67×10^4	1.40×10^4
Avg	7.50×10^4	3.48×10^4	1.15×10^5	1.54×10^8	2.32×10^4	1.41×10^4

[a] Infant, <10 yr; adult, 20 to 60 yr; elderly, >60 yr. Colonies were grown aerobically, and all tested positive for catalase and were found to contain phase-bright spores after 3 days of incubation at 37°C.

using the exhaustion method. The number of heat-resistant spores was measured at time points following the initiation of sporulation (Fig. 5B). Remarkably, both HU58 and HU78 showed strikingly different kinetics of spore formation. Specifically, following the transition to stationary phase (T_0), heat-resistant counts could be detected at T_4, whereas for PY79, heat-resistant counts were first detectable at significant levels at T_7. This experiment has been repeated twice and shows that the natural gut isolates of *B. subtilis* are able to form a spore in as little as 4 h at 37°C.

The resistance of PY79, HU58, and HU78 spores as well as vegetative cells to SGF at pH 2 to 4 and SIF were measured as described previously (8). Our results (not shown) demonstrated that spores of all *B. subtilis* strains were unaffected by treatment with either SGF or SIF. However, for vegetative cells, all strains were acutely sensitive to SGF at pH 2 to 3 showing, at best, 0.008% survival after 30 min of incubation in SGF at pH 3 (strain PY79). HU58 and HU78, though, were both noticeably more sensitive than PY79 under these conditions by up to 1 log. Treatment with SGF at pH 4 was essentially tolerated with between 25 to 55% survival for the three strains. Vegetative cells were also sensitive to SIF showing essentially no survival (0.0001 to 0.001%) after 90 min of incubation. Again, HU58 and HU78 were 1 log more sensitive than strain PY79.

DISCUSSION

Immunological data obtained in this work provided the first clues that *B. subtilis* spores not only germinate in the mouse gut but appear to be able to grow and resporulate. First, mice that were dosed with suspensions of spores expressing the TTFC antigen on their surface but unable to germinate could only generate a much reduced anti-TTFC response. If the anti-TTFC response was due to the TTFC carried on the spore surface in the inoculum, then a response would be expected even with spores unable to germinate. This was not the case and was supported by experiments showing that, even if the majority of TTFC has been removed from the spore surface, anti-TTFC responses can still be generated (data not shown). These treated spores were found to retain viability but germinated more slowly than wild-type spores. Despite these deficiencies, they were able to generate statistically significant anti-TTFC responses. Finally, we have shown that responses to surface-exposed TTFC (fused to the spore coat protein CotB) are slower than those to spores expressed in the vegetative cell, i.e., in the germinated spore. If indeed TTFC is susceptible to stomach or intestinal juices in vivo (and this would depend on a number of physiological factors, such as food intake, etc.), it is possible that a proportion of spore-displayed TTFC is degraded, reducing the effective dose and delaying the response.

The molecular proof comes from RT-PCR analysis that clearly showed the presence of vegetative and sporulation-specific gene expression in the murine GIT. Germination was localized to the jejunum, which agrees with a previous study (4). Expression of the *cotB-tetC* fusion occurred between hours 30 and 48 but was confined to the ileum and large intestine. One explanation for these results could be that the administered dose of spores carried some level of either *cotB-tetC* or *rrnO-tetC* mRNA species within the spore. We believe this not to be the case for several reasons. First, we have shown that Trizol treatment used to extract total mRNA fails to break the spore, and it is impossible to recover either *cotB-tetC* or *rrnO-tetC* mRNA from Trizol-treated spores. Second, in the case of *cotB*, expression is confined to the mother cell compartment (32) after the immature forespore has been formed, so it would not be expected that any *cotB-tetC* mRNA would be sequestered to the spore. Finally, in previous studies using the *rrnO* promoter for heterologous gene expression, we have observed declining and extremely low levels of *rrnO*-directed gene expression during sporulation (10). Therefore, the only realistic explanation is that the mRNA signals we observed originate from either germinating spores in the case of *rrnO-tetC* or from germination and then resporulation in the case of *cotB-tetC*.

It appears then, that upon exiting the stomach, *B. subtilis* spores can germinate, proliferate, and then resporulate. We know cells must proliferate, since at least one cell division is

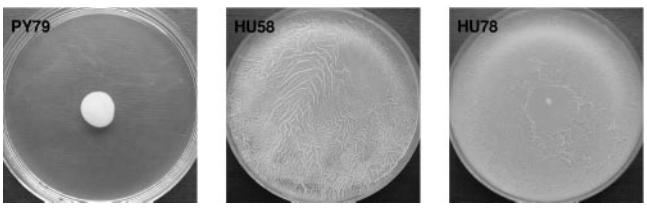

FIG. 4. Biofilm formation. Pellicle formation in two undomesticated strains (HU58 and HU78) of *B. subtilis* versus no pellicle formation in the laboratory strain PY79 grown on CMK medium.

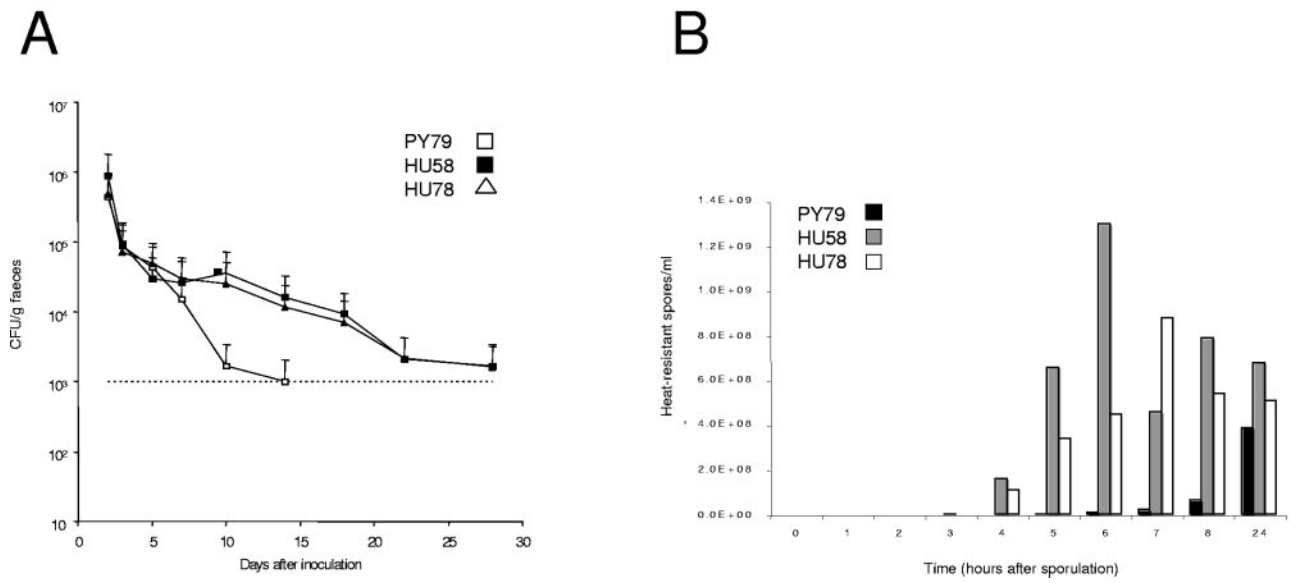

FIG. 5. Behavior of domesticated and undomesticated strains. (A) Counts of spores excreted in the feces of mice following a single fixed oral dose (1×10^9) of spores of PY79 and two undomesticated strains (HU58 and HU78). Heat-resistant (65°C, 1 h) counts were determined from fresh feces collected from individual mice as described previously (7). The detection limit was 1×10^3 and is indicated by the dashed line. Counts shown are means of results from 6 animals. (B) Development of heat-resistant spores during sporulation in PY79, HU58, and HU78. Cells were induced to form spores by the exhaustion method, and the number of heat-resistant spores was determined (80°C, 20 min) at time points following the initiation of spore formation (T_0).

thought to be required before sporulation can commence (11). Our experiments examine gene expression at discrete times and are subject to obvious experimental difficulties in accurate recovery of total RNA. However, persistence experiments demonstrate that spores were still being shed from the mouse gut for up to 27 days postdosing, although for *B. subtilis*, this does not appear to be a permanent existence (at least within detectable limits), so this bacterium cannot be colonizing. Interestingly, natural gut isolates of *B. subtilis* recovered from human feces persist in the murine gut for almost twice as long as the laboratory strain PY79, with shedding still detectable up to 27 days after administration. These isolates were shown to form biofilms and were able to adhere to Caco-2 cells. Interestingly, spores of these isolates also germinated earlier in the mouse gut, indicating they may be better able to respond to nutritional signals within the upper reaches of the GIT that might trigger germination.

We have found that these natural isolates can sporulate efficiently in anaerobic conditions, which supports the sporulation we observed in the GIT. While growth of vegetative *B. subtilis* under anaerobic conditions is well known (26), we believe this is the first confirmation that spores of *B. subtilis* can be formed in anoxic conditions. As has been noted before, the efficiency of growth under anaerobic conditions for *B. subtilis* is substantially reduced compared to aerobic growth and demonstrates the preference of this organism for growth under oxic conditions (5). The ability to form biofilms has been observed with undomesticated isolates of *B. subtilis* (3), and while biofilm formation in the GIT has not been shown, here it would seem that this attribute might enable germinated *B. subtilis* to establish itself alone or in association with other gut microbes in the small intestine. Biofilms can create an anaerobic microenvironment, and it is perhaps not surprising that, coupled with the anoxic environment of the GIT, sporulation can occur under these conditions. We have also shown using RT-PCR analysis that the domesticated strain PY79 could also sporulate in the GIT, so either the GIT is not completely anoxic or the proper nutritional signals required to induce anaerobic sporulation in plate culture are not present. Another remarkable finding was that the natural gut isolates could form heat-resistant spores in as little as 4 h compared to 7 h for the domesticated strain. We could imagine that rapid sporulation is an adaptation that enhances survival of vegetative cells that, at least in planktonic growth, are acutely sensitive to intestinal fluids.

If the animal gut is not an appropriate environment for *B. subtilis*, we would predict that either all of the ingested spores would be excreted in the feces or spores would germinate and then be destroyed. That we observe quite significant levels of germination and sporulation indicates that *B. subtilis* has adapted to the GIT for use as a natural habitat. This is supported by not only the increasing number of studies showing that *Bacillus* species can be recovered from the GIT of animals (20) but also by our study here which showed that 30 volunteers all carried *Bacillus* spores in their feces. While the numbers may be small compared to *Bifidobacteria* and *Lactobacillus* species (that can reach as many as 10^{11} CFU/g of feces), the importance of *Bacillus* should not be overlooked, since *B. subtilis* has been shown to be of primary importance in development of the GALT (29). This study also showed that it was specifically sporulation that influenced GALT development, supporting our findings here and underlining the use of one *Bacillus* species (*Bacillus clausii*) as a licensed probiotic drug (20). The intestinal life cycle of *B. subtilis* is probably repre-

sentative of most species of *Bacillus* spores that are ingested. *B. cereus* and *B. anthracis* are two notable examples that exploit the GIT for pathogenesis (22). Interestingly, in the pathogenic life cycle of *B. anthracis*, it is vegetative cells that are released from the dying host that are thought to be responsible for dissemination, yet it is the spore that survives in the environment (23). We wonder whether germination and sporulation of *B. anthracis* spores in the GIT can also play some role in dissemination. Our inference from this work is that *B. subtilis* is probably representative of many spore formers that can use the GIT for growth and proliferation. Although it cannot yet be considered a gut commensal, it is certainly autochthonous. This seems reasonable for an organism that is going to be ingested and raises the interesting question of whether the spore evolved to enable survival in the environment or to enable survival in the GIT.

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