

Characterization of *Bacillus* Probiotics Available for Human Use

Le H. Duc,¹ Huynh A. Hong,¹ Teresa M. Barbosa,² Adriano O. Henriques,²
and Simon M. Cutting^{1*}

School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 OEX, United Kingdom,¹
and Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, 2781-901 Oeiras Codex, Portugal²

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***Bacillus* species (*Bacillus cereus*, *Bacillus clausii*, *Bacillus pumilus*) carried in five commercial probiotic products consisting of bacterial spores were characterized for potential attributes (colonization, immunostimulation, and antimicrobial activity) that could account for their claimed probiotic properties. Three *B. cereus* strains were shown to persist in the mouse gastrointestinal tract for up to 18 days postadministration, demonstrating that these organisms have some ability to colonize. Spores of one *B. cereus* strain were extremely sensitive to simulated gastric conditions and simulated intestinal fluids. Spores of all strains were immunogenic when they were given orally to mice, but the *B. pumilus* strain was found to generate particularly high anti-spore immunoglobulin G titers. Spores of *B. pumilus* and of a laboratory strain of *B. subtilis* were found to induce the proinflammatory cytokine interleukin-6 in a cultured macrophage cell line, and in vivo, spores of *B. pumilus* and *B. subtilis* induced the proinflammatory cytokine tumor necrosis factor alpha and the Th1 cytokine gamma interferon. The *B. pumilus* strain and one *B. cereus* strain (*B. cereus* var. *vietnami*) were found to produce a bacteriocin-like activity against other *Bacillus* species. The results that provided evidence of colonization, immunostimulation, and antimicrobial activity support the hypothesis that the organisms have a potential probiotic effect. However, the three *B. cereus* strains were also found to produce the Hbl and Nhe enterotoxins, which makes them unsafe for human use.**

Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance (10, 11). The potential benefits that are claimed include improved nutrition and growth and prevention of various gastrointestinal disorders. Probiotic-containing products are available for human nutrition, as animal feed supplements, and also for aquaculture (35, 36, 41, 43). In some countries probiotics are taken as prophylactic agents (for example, to prevent childhood diarrhea), while in southeast Asia they are also used as therapeutic agents (25). Products containing endospores of members of the genus *Bacillus* (in single doses of up to 10⁹ spores/g or 10⁹ spores/ml) are used commercially as probiotics, and they offer some advantages over the more common *Lactobacillus* products in that they can be stored indefinitely in a desiccated form (25). Originally, many commercial products were sold as products that carry *Bacillus subtilis* spores, but recent studies have shown that most products are mislabeled and carry other *Bacillus* species, including *Bacillus clausii*, *Bacillus pumilus*, and a variety of *Bacillus cereus* strains (13, 17). Product mislabeling raises a number of concerns about consumer confidence (15), as well as attendant safety issues, since some of the organisms found were strains of *B. cereus*, which is a major cause of gastrointestinal infections (12).

Continued ingestion of large quantities of *Bacillus* spores raises the question of what happens to the spores in the gastrointestinal tract (GIT). While no evidence of colonization has been found, it is possible that a spore can interact with the

gut-associated lymphoid tissue (GALT). Recent studies have shown that orally ingested *B. subtilis* spores are immunogenic and can disseminate to the Peyer's patches and mesenteric lymph nodes (MLN) (5, 6). Additional work has provided compelling evidence that ingested *B. subtilis* spores can germinate in the small intestine. This conclusion is based on three findings. First, when mice are given an oral inoculum, more spores are excreted than are ingested (18). Second, vegetatively expressed mRNA is detected in the GIT by reverse transcription (RT)-PCR following administration of spores to mice (2). Finally, systemic immunoglobulin G (IgG) responses are generated against vegetative *B. subtilis* following administration of suspensions carrying only spores to mice (5). Together, these studies show that spores may not be transient passengers in the gut or that if they are, they may still have an intimate interaction with the host cells or microflora that can enhance their potential probiotic effect.

The following three basic mechanisms have been proposed for how orally ingested nonindigenous bacteria can have a probiotic effect in a host: (i) immunomodulation (that is, stimulation of the GALT) (e.g., induction of cytokines), (ii) competitive exclusion of gastrointestinal pathogens (e.g., competition for adhesion sites), and (iii) secretion of antimicrobial compounds which suppress the growth of harmful bacteria (10). Few studies have demonstrated a direct probiotic effect of *Bacillus* spores, but preliminary studies with poultry have provided evidence that there is competitive exclusion of *Escherichia coli* 078:K80 by *B. subtilis* (24) and a number of studies have demonstrated that *Vibrio harveyi* in shrimp is suppressed by various *Bacillus* spore formers (34, 42). A recent study has described the characterization of an antibiotic produced by the *B. subtilis* strain (*B. subtilis* 3) found in the commercial product

* Corresponding author. Mailing address: School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 OEX, United Kingdom. Phone: 44 1784 443760. Fax: 44 1784 434326. E-mail: s.cutting@rhul.ac.uk.

TABLE 1. Bacteriocin production by probiotic strains^a

Indicator organism ^b	Enterogermina (<i>B. clausii</i>)	Biosubtyl ^{DL} (<i>B. cereus</i>)	Bactisubtil (<i>B. cereus</i>)	Biosubtyl ^{NT} (<i>B. pumilus</i>)	Subtyl (<i>B. cereus</i>)
<i>L. innocua</i>	– ^c	–	–	–	–
<i>P. aeruginosa</i> NCTC 12903	–	–	–	–	–
<i>B. megaterium</i> 899 (= BGSC 7A1)	+/-	–	–	+++	–
<i>B. cereus</i> T (= BGSC 6A1)	–	–	–	+/-	–
<i>B. licheniformis</i> 9945A (= BGSC 5A2)	–	–	–	–	–
<i>B. cereus</i> var. vietnami (Subtyl)	–	–	–	–	+/-
<i>B. cereus</i> (Biosubtyl ^{DL})	–	–	–	+/-	–
<i>B. pumilus</i> (Biosubtyl ^{NT})	–	–	–	–	+/-
<i>B. clausii</i> DSM 8716	–	–	–	–	+/-
<i>B. sphaericus</i> BGSC 13A5	–	–	–	++	+(+)
<i>B. sphaericus</i> BGSC 13A6	–	–	–	+(+)	+

^a +, clear halo of growth inhibition at least one time (results were determined after 5, 8, 24, and 48 h of incubation); +++, ++, and (+), different degrees of inhibitory activity, as assessed by decreased diameter of inhibition zone, respectively; –, no inhibition; +/-, clear reduction in growth but not complete inhibition.

^b All the strains were assayed two to four times.

^c After proteinase K treatment Enterogermina showed clear inhibitory activity with *L. innocua* but no activity with *B. megaterium* and *C. perfringens*. No other producers or indicators were tested under these conditions.

Biosporin, which has been shown to inhibit growth of *Helicobacter pylori* (31).

In this study we examined five commercially available *Bacillus* probiotic strains whose inoculum is in the spore form. These strains were Bactisubtil (*B. cereus* IP 5832) (17), Enterogermina (*B. clausii*) (13, 17, 39), Biosubtyl Nha Trang (referred to here as Biosubtyl^{NT}; a strain of *B. pumilus*) (13, 17), Biosubtyl Da Lat (referred to here as Biosubtyl^{DL}; a *B. cereus* strain) (17), and Subtyl (a strain similar to *B. cereus* spp. and designated *B. cereus* var. vietnami) (17). We looked for evidence of colonization and immune stimulation, and we determined potential pathogenic traits of the *B. cereus* products. Our results provide some interesting insights into a potential probiotic mechanism, and they also raise further concerns over the potential danger of using poorly characterized strains.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* wild-type strain used in this study was PY79, a Spo⁺ prototrophic derivative of type strain 168 (44). The commercial probiotics used have been described previously and were Enterogermina (*B. clausii*) (13, 17, 39, 40), Subtyl (*B. cereus* var. vietnami) (17), Biosubtyl^{DL} (*B. cereus*) (17), Biosubtyl^{NT} (*B. pumilus*) (13), and Bactisubtil (*B. cereus*) (17). The bacterial strains used as indicators for antimicrobial screening are listed in Table 1 and were obtained from the *Bacillus* Genetic Stock Center (Columbus, Ohio) (<http://bacillus.biosci.ohio-state.edu>), the National Collection of Type Cultures, or the Deutsche Sammlung von Mikroorganismen und Zellkulturen or from laboratory stocks.

Preparation of spores. Sporulation was induced in Difco sporulation medium (DSM) by using the exhaustion method as described elsewhere (28). Sporulating cultures were harvested 24 h after initiation of sporulation. Purified suspensions of spores were prepared as described by Nicholson and Setlow (28) by using a French press (40,000 lb/in²) to break any residual sporangial cells, followed by washing in 1 M NaCl, 1 M KCl, and water (twice). The French press was used instead of lysozyme treatment since some strains were known to exhibit sensitivity to lysozyme (17). Each spore suspension was titrated immediately to determine the number of CFU per milliliter before aliquots were frozen at –20°C.

Extraction of spore coat proteins. Spore coat proteins were extracted from suspensions of spores at high densities (>10¹⁰ spores/ml) by using a sodium dodecyl sulfate-dithiothreitol extraction buffer as described in detail elsewhere (28). The integrity of extracted proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the concentration was determined with a Bio-Rad DC protein assay kit (Bio-Rad).

Immunizations. Groups of eight female C57BL/6 mice that were 8 weeks old were immunized by the oral route with suspensions containing 10⁹ spores (in 0.15 ml) by intragastric gavage on days 0, 23, and 45. For oral administration mice were lightly anesthetized with halothane. A naïve, nonimmunized control group

(sterile water) was included. Serum samples were taken on days –1, 20, 44, and 72.

Whole-spore ELISA for detection of spore-specific serum antibodies. A whole-spore enzyme-linked immunosorbent assay (ELISA) technique was developed and optimized in our laboratory and was based on a number of whole-cell ELISA methods in which bacteria were used to coat plates at 4°C overnight (7, 20, 27) or at room temperature for 45 min (21). Spores were suspended in 0.03 M NaPO₄ buffer (pH 7.4) containing 4% (wt/vol) paraformaldehyde at a concentration of approximately 10⁶ spores/ml. Plates (MaxiSorp; Nunc) were coated with 50 µl of a spore suspension per well and left at room temperature for 2 h (the optimum incubation time). After three washes with phosphate-buffered saline (PBS), the plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Serum samples were subsequently applied by using a twofold dilution series starting with a 1/40 dilution in ELISA diluent buffer (0.1 M Tris-HCl [pH 7.4], 3% [wt/vol] NaCl, 0.5% [wt/vol] BSA, 10% [vol/vol] sheep serum [Sigma], 0.1% [vol/vol] Triton X-100, 0.05% [vol/vol] Tween 20). Every plate had replicate wells that contained a preimmune serum that was diluted 1/40. The plates were incubated for 2 h at 37°C before addition of anti-mouse IgG-horseradish peroxidase conjugates (Sigma) used at a dilution of 1:2,000 in PBS containing 1% BSA and 0.05% Tween 20. The plates were incubated for an additional 1 h at 37°C, washed three times in PBS containing 0.05% Tween 20, and then developed with the substrate 3,3',5,5'-tetramethylbenzidine (Sigma). Reactions were stopped with 2 M H₂SO₄. Dilution curves were drawn for each sample, and end point titers were calculated by determining the dilution that produced the same optical density as the 1/40 dilution of a pooled preimmune serum. Statistical comparisons between groups were performed by using the Mann-Whitney U test. A *P* value of >0.05 was considered nonsignificant.

Fecal analysis. Groups of six BALB/c female mice that were 6 weeks old were inoculated by using a plastic gavage with approximately 10⁹ spores suspended in 200 µl of sterile H₂O. The mice were housed individually in cages with grid floors to prevent coprophagia. Fresh fecal pellets were collected at appropriate times, weighed, and homogenized in PBS before serial dilutions were inoculated onto DSM agar (28) plates and incubated at 37°C for 2 days. Identification of probiotic strains from the normal flora was based on colony morphology and microscopic examination of spore size and shape as described previously (5). A control group of uninoculated mice was also included.

Simulated GIT conditions. Spores were suspended in simulated gastric juice (1 mg of pepsin [porcine stomach mucosa; Sigma] per ml; pH 2.0) or small intestine fluid (1 mg of pancreatin [porcine pancreas; Sigma] per ml and 0.2% bile salts [50% sodium cholate–50% sodium deoxycholate; Sigma]; pH 7.4) and incubated at 37°C. Samples were removed, serially diluted, and plated to determine the number of CFU per milliliter on DSM agar plates.

Enterotoxin genes. Chromosomal DNA was isolated from strains and tested for the presence of *B. cereus* enterotoxin genes by using PCR as described previously to profile food-poisoning *Bacillus* strains (14, 30).

Enterotoxin detection. Enterotoxins were detected by using two commercial immunoassay kits. A BCET-RPLA kit (Oxoid) was used to detect the HblC subunit of the Hbl enterotoxin in enrichment cultures, while a Tecra BDE kit (Tecra Diagnostics) was used to detect the NheA subunit of the Nhe enterotoxin.

Hemolysis and lecithinase detection. Each strain was streaked onto 5% sheep blood agar and *B. cereus* selective agar containing egg yolk and polymyxin B (Sigma) and incubated at 37°C for 24 to 48 h to detect patterns of hemolysis and lecithinase production, respectively.

Bacteriocin assays. A colony overlay assay was used to screen for bacteriocin-like activity (33). All the probiotic strains tested were found to grow well on Luria-Bertani (LB) medium. For this reason, cultures of the probiotic strains that were to be tested for bacteriocin-like activity were incubated overnight in LB medium. Then 5- μ l portions of the overnight cultures were inoculated as spots on LB medium plates, which were incubated at 37°C for 24 h before the cells were killed by exposure to chloroform vapor for 30 min. After exposure to air, the plates were overlaid with LB medium or brain heart infusion soft agar (according to the requirements of the indicator strain) that had been inoculated with an overnight culture of an indicator strain and reincubated. The presence of zones of growth inhibition around the spots at any of the times examined (5, 8, 24, and 48 h postinoculation) was considered a positive response. Proteinase K treatment in colony assays of the probiotic strain Enterogermina (*B. clausii*) was performed as described by Faye et al., with modifications (9). Portions (80 μ g total) of a proteinase K preparation (20-mg ml⁻¹ stock solution) were applied as spots around the producer colonies. The plates were incubated at 37°C for approximately 2 h before the chloroform treatment and overlay. Control plates without producer colonies were treated with proteinase K as described above.

In vitro cytokine analysis. The murine macrophage-like cell line RAW264.7 was cultured as monolayers in RPMI 1640 medium (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum, 50 μ g of penicillin ml⁻¹, and 50 μ g of streptomycin ml⁻¹ (complete medium) in an atmosphere with 90% humidity containing 5% CO₂ at 37°C. Two days before use, the cells were detached by gentle scraping and seeded into six-well disposable plates in the same medium. Two-day-old macrophages were cultured with probiotics at a ratio of 10 spores per macrophage in complete medium. At different times, the culture medium was removed, the macrophages were washed and lysed in situ and were homogenized by passing the cell extract five times through a 20-gauge needle, and the total RNAs were extracted and purified with a Qiagen RNeasy mini kit used as described by the manufacturer.

In vivo cytokine analysis. Specific-pathogen-free female BALB/c mice that were 8 weeks old were inoculated with 10¹⁰ spores. A naive, nonimmunized group of mice was also included. Spleens, livers, MLN, and submandibular glands (SMG) were removed from sacrificed mice at different times and frozen immediately at -80°C until they were needed. To extract total RNAs, organs and tissues were thawed, disrupted by pressing them between two glass slides, lysed in RLT buffer (Qiagen) containing 1% β -mercaptoethanol, and homogenized by passing them twice through a QIAshredder column (Qiagen). Total RNAs were extracted from the lysates and purified with a Qiagen RNeasy mini kit used as described by the manufacturer.

RT-PCR for cytokine detection. Total RNAs were quantified with a GeneQuant spectrophotometer (Amersham Biosciences). RT-PCR was carried out by using 1 μ g of total RNA per reaction mixture as described by the manufacturer (Amersham Biosciences Ready-To-Go RT-PCR beads). Primers specific for β -actin and various cytokines have been described elsewhere (32). The reaction conditions were as follows: first-strand cDNA synthesis at 42°C for 15 min, reverse transcriptase inactivation at 95°C for 5 min, and PCR at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. RT-PCR products were electrophoresed on a 2% agarose gel and subjected to UV visualization and densitometric analysis with a Bio-Rad Gel Doc system.

RESULTS

Persistence of *Bacillus* probiotic strains in GIT in a mouse model. Groups of inbred mice were inoculated with 10⁹ spores of a wild-type *B. subtilis* strain (PY79) and the five commercial products. Fresh fecal pellets were collected, and the numbers of CFU per gram were determined (Fig. 1) (this assay differed from that used in a previous study in which total feces were collected and assayed at individual times [18]). This assay provided measurements of both spores and vegetative cells since some of the probiotic strains have been shown to have different sensitivities to heat treatment (17). PY79 was found to be rapidly cleared from the mouse gut; no measurable counts were detectable after only 6 days, in agreement with a similar study in which strain PY79 was used (18). The other strains

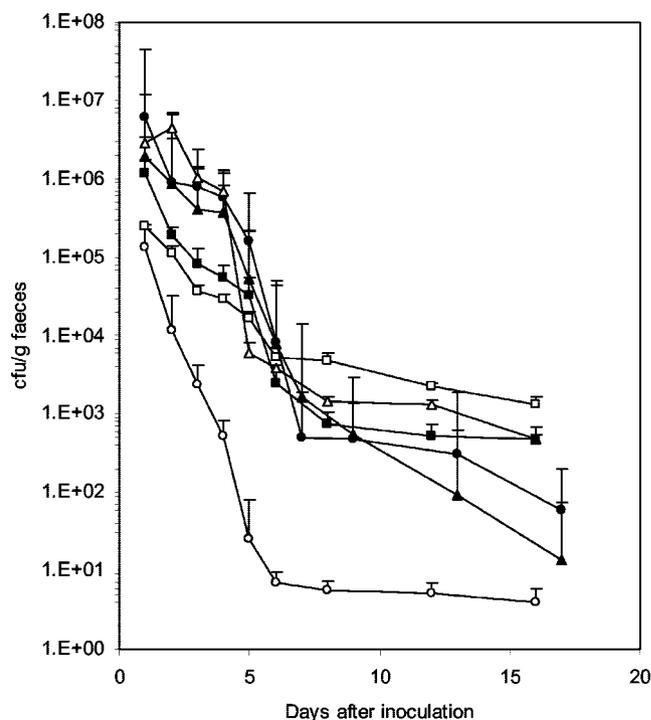


FIG. 1. Persistence of *Bacillus* probiotic strains in the mouse GIT. Groups of six mice were inoculated with 10⁹ spores, fecal pellets were collected, and spores were counted at different times. The data are arithmetic means, and the error bars indicate standard deviations. ○, *B. subtilis* PY79; ●, Biosubtyl^{NT}; □, Subtyl; ■, Biosubtyl^{DL}; △, Bactisubtil; ▲, Enterogermina.

persisted longer; the concentrations of three *B. cereus* strains (Subtyl, Biosubtyl^{DL}, and Bactisubtil) appeared to level off, and measurable values of 10³ CFU/g were obtained for 18 days.

Resistance of spores in simulated GIT conditions. We measured the survival of spore suspensions in simulated gastric fluid (SGF) (Fig. 2) and simulated intestinal fluid (SIF) (Fig. 3). Three of the probiotics, Biosubtyl^{NT} (Fig. 2A), Subtyl (Fig. 2C), and Bactisubtil (Fig. 2D), exhibited sensitivity to SGF. In the case of Subtyl only 0.02% of the spore suspension was able to survive 1 h of incubation in the gastric fluid, and even in PBS alone more than 50% of the suspension was destroyed. In SIF, spores of two *B. cereus* probiotics, Subtyl (Fig. 3C) and Bactisubtil (Fig. 3D), exhibited sensitivity to the bile salts contained in SIF. Again, there was substantial killing of the Subtyl strain under these conditions, with 0.2% survival after 3 h of incubation.

Humoral responses to orally administered spores. Groups of eight inbred mice were inoculated orally three times with suspensions of spores (10⁹ spores) at 23-day intervals. As shown in Fig. 4, oral immunization of mice with Biosubtyl^{NT} spores resulted in titers of more than 10³ CFU/g by day 72, which were significantly greater ($P < 0.05$) than the titers for the naive immunized mice. Immunization with the other probiotic strains, including strain PY79, resulted in more modest systemic responses, and the spore-specific IgG titers were between 1.5×10^2 and 3×10^2 CFU/g by day 72. Although modest, these levels were significantly greater than those in naive, unimmunized mice ($P < 0.05$).

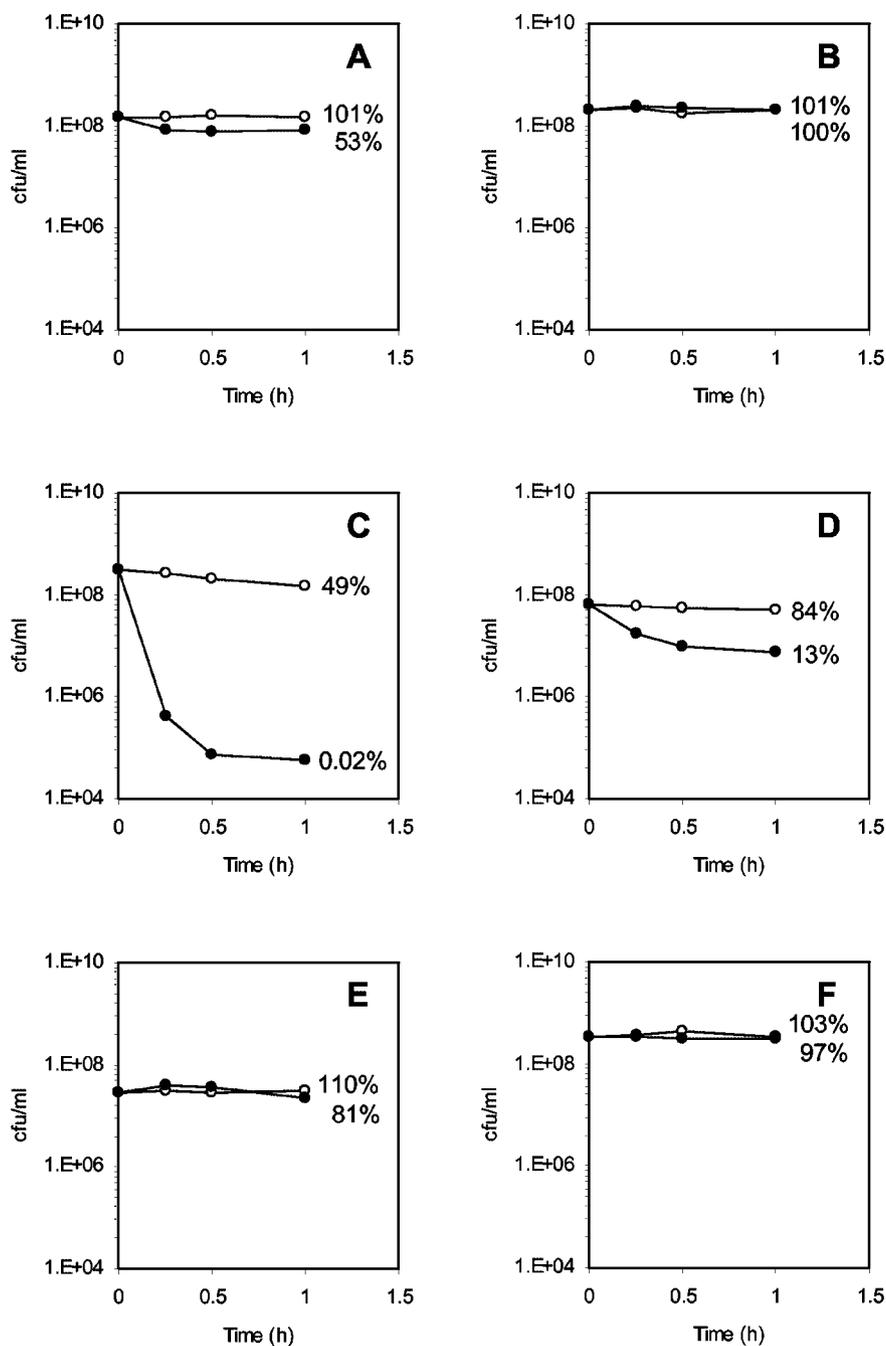


FIG. 2. Survival of spores of probiotic strains in SGF. Spores of the probiotic strains Biosubtyl^{NT} (A), Enterogermina (B), Subtyl (C), Bactisubtil (D), and Biosubtyl^{DL} (E) and the laboratory strain PY79 (F) were treated in simulated gastric conditions (solid symbols), and viability was assessed at different times and compared with the viability of untreated samples (open symbols). The percentages were based on the original inocula. The data are arithmetic means of duplicate independent experiments.

Cytokine responses. The immunogenicity of Biosubtyl^{NT} spores was striking, and accordingly, we examined selective cytokine responses elicited in vitro and in vivo with this strain by using PY79 as a control. First, RT-PCR was used to examine the expression of the proinflammatory cytokines tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and IL-1 α in RAW264.7 macrophages cocultured with spores of wild-type laboratory strain PY79 and Biosubtyl^{NT} (Fig. 5). The most significant induction for both strains was the induction of IL-6,

which reached maximum levels 5 to 10 h after infection of macrophages, after which the level of IL-6 began to decline. The IL-1 α and TNF- α responses were very small, and peak levels were reached at hour 5 for PY79 and around hour 10 for Biosubtyl^{NT}.

An in vivo analysis of cytokine mRNA from mice immunized orally with PY79 (Fig. 6A) and Biosubtyl^{NT} (Fig. 6B) spores was performed as described in Materials and Methods. We examined seven cytokines, IL-1 α , IL-2, IL-4, IL-5, IL-6,

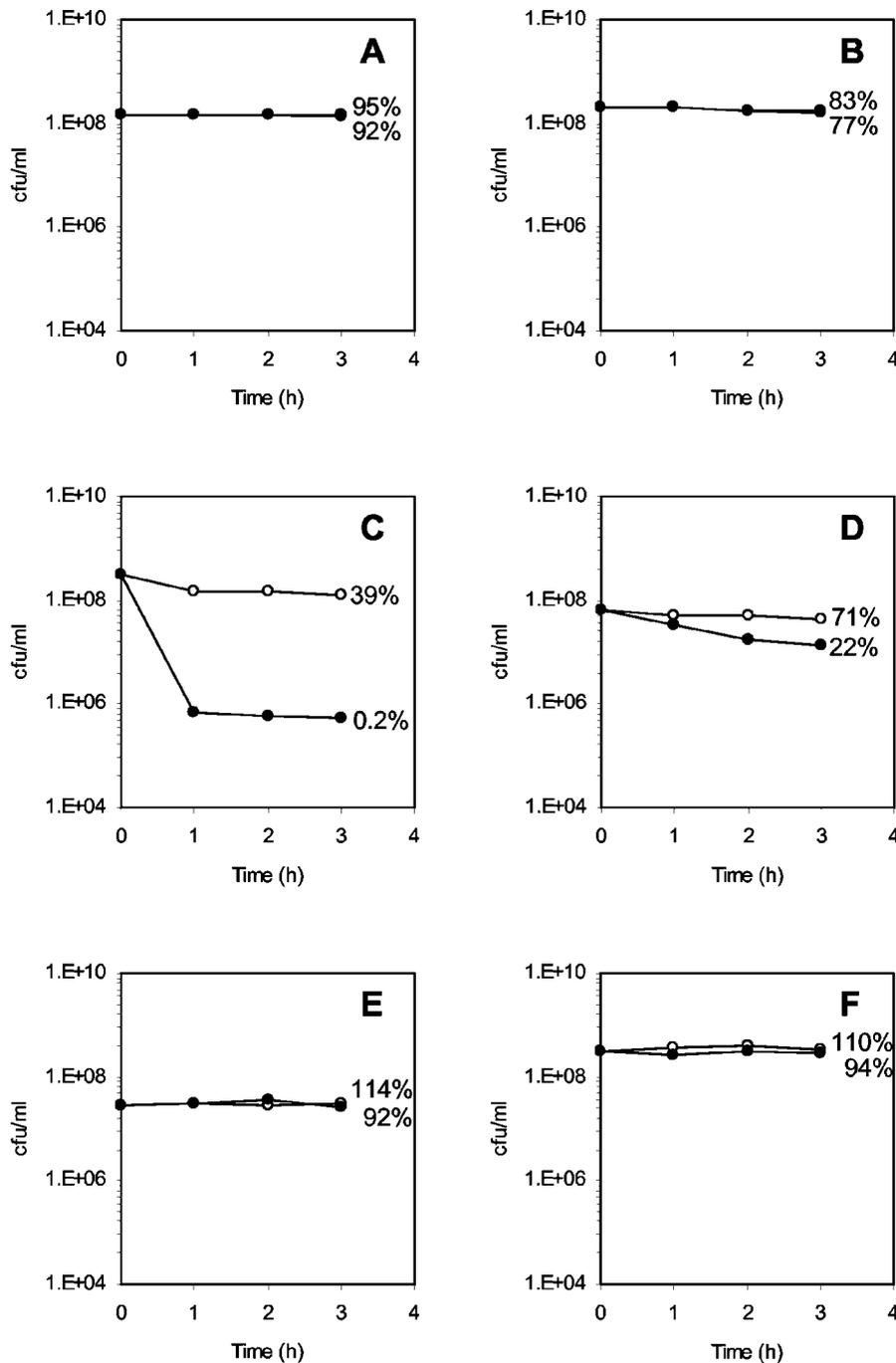


FIG. 3. Survival of spores of probiotic strains in SIF. Spores of the probiotic strains Biosubtyl^{NT} (A), Enterogermina (B), Subtyl (C), Bactisubtil (D), and Biosubtyl^{DL} (E) and the laboratory strain PY79 (F) were treated in simulated intestinal conditions (solid symbols), and viability was assessed at different times and compared with the viability of untreated samples (open symbols). The percentages were based on the original inocula. The data are arithmetic means of duplicate independent experiments.

TNF- α , and gamma interferon (IFN- γ), in the spleens, livers, MLN, and SMG from mice that had been given one oral dose of PY79 spores (Fig. 6A) or Biosubtyl^{NT} (Fig. 6B). Induction of only two cytokines was apparent during the time that we investigated; these cytokines were the proinflammatory cytokine TNF- α and the T-helper type 1 (Th1) cytokine IFN- γ . Both cytokine mRNAs were induced early (days 1 to 3) in the liver, SMG, and MLN, and the levels of IFN- γ were slightly

higher. Expression of both cytokines was highest in the MLN and liver, and in the MLN IFN- γ expression was maintained at a steady level for mice immunized with PY79. Low but detectable levels of expression were observed in the spleen. In control experiments with naïve mice no cytokine mRNA was detectable (data not shown).

Enterotoxins and potential virulence factors. PCR was used to test chromosomal DNA from the *B. cereus* probiotic strains

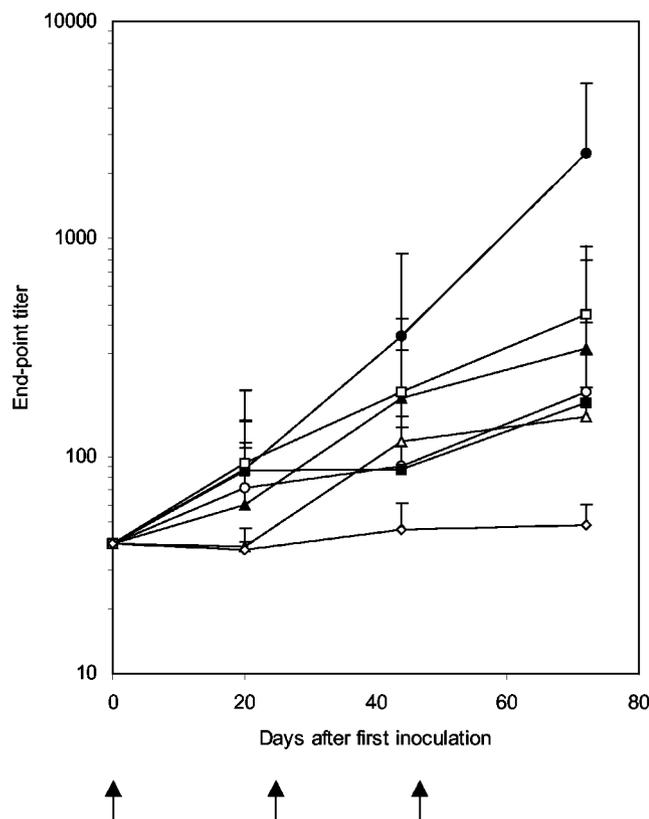


FIG. 4. Systemic anti-spore IgG responses. Groups of six mice were inoculated (arrows) orally with 10^9 spores of probiotic strains of *Bacillus*, including Biosubtyl^{NT} (●), Enterogermina (▲), Subtyl (△), Bactisubtil (■), and Biosubtyl^{DL} (□). A laboratory strain of *B. subtilis* (PY79) was also included (○). Individual samples were tested by indirect whole-spore ELISA for spore-specific serum total IgG. Naïve groups gave basal responses for all strains, and a representative line (anti-PY79) (◇) is shown. For each animal, the end point IgG titer was calculated by determining the dilution of serum that produced the same optical density as the 1/40 dilution of the preimmune serum. The data are arithmetic means, and the error bars indicate standard deviations.

for the presence of enterotoxin genes as described previously for profiling of food-poisoning *Bacillus* strains (14, 30). The three *B. cereus* probiotics were all found to carry enterotoxin genes (Table 2). Subtyl and Biosubtyl^{DL} carried all three genes (*nheA*, *nheB*, and *nheC*) encoding the nonhemolytic enterotoxin (Nhe), and when the Tecra test for detection of expressed toxin was used, both strains were clearly positive. Bactisubtil was found to carry the *nheB* and *nheC* genes but not *nheA*. Accordingly, no enterotoxin could be formed, and this strain was negative when the Tecra test was used. Both Biosubtyl^{DL} and Bactisubtil were found to carry three genes (*hblA*, *hblC*, and *hblD*) that encode hemolysin BL (Hbl), the primary virulence factor in *B. cereus* diarrhea (12). Using a *B. cereus* enterotoxin reverse passive latex agglutination test kit (Oxoid), we detected Hbl enterotoxin in both strains. Neither strain carried the *hblB* gene, whose function is not yet known, but in any event this gene is not essential for toxicity (12). Both Bactisubtil and Biosubtyl^{DL} carried the *bceT* gene, which encodes the single-component toxin enterotoxin T, for which no in vivo toxin production test is yet available. No strain carried

the *cytK* gene, which encodes the single-component toxin cytotoxin K. All three *B. cereus* probiotics produced complete or partial hemolysis on sheep blood agar (Table 2). We also tested for lecithinase production. On *B. cereus* selective agar, all three *B. cereus* probiotics, Subtyl, Biosubtyl^{DL}, and Bactisubtil, were lecithinase positive (Table 2).

Screening for bacteriocin-like inhibitory substances. We used a colony overlay assay (see Materials and Methods) to screen for antimicrobial activity in Enterogermina, Biosubtyl^{DL}, Bactisubtil, Biosubtyl^{NT}, and Subtyl. Twenty-three indicator strains, including both gram-positive and gram-negative organisms, were used. The strains used included strains of *B. cereus*, *B. subtilis*, *B. cereus* var. vietnami, *B. pumilus*, *Bacillus licheniformis*, *Bacillus megaterium*, *B. clausii*, *Bacillus sphaericus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria innocua*, *Listeria monocytogenes*, *Clostridium perfringens*, *E. coli* O78:K80, *E. coli* O157:H7, *Salmonella enterica* serotype Enteritidis, *Salmonella enterica* serotype Bareilly, *Citrobacter rodentium*, *Pseudomonas aeruginosa*, and *Enterobacter aerogenes*. Under our assay conditions both Biosubtyl^{NT} and Subtyl exhibited measurable activity (Table 1) (the results were negative for all other indicator strains not shown in Table 1). Biosubtyl^{NT} clearly inhibited *B. megaterium* 899 (= BGSC 7A1) and *B. sphaericus* strains ATCC 33203 and ATCC 14577 (obtained from the *Bacillus* Genetic Stock Center as strains BGSC 13A5 and BGSC 13A6, respectively). Subtyl, on the other hand, inhibited the growth of only the *B. sphaericus* strains. In both cases limited antimicrobial activity was observed with a few other indicator strains. However, inhibition was never complete and resulted only in a reduction in the growth intensity. The remaining probiotic strains had little or no effect on the growth of indicator strains under these experimental conditions. Importantly, we observed in parallel studies that *B. clausii* strain Enterogermina, which had no effect in the previous screening, became active if the plate area surrounding its growth spot was treated with proteinase K before addition of the indicator strains (9). Of the three indicator strains tested, the inhibitory effect was seen with *L. innocua* but not with *B. megaterium* 899 or *C. perfringens*. Note that addition of proteinase K to plates without producer colonies had no antagonist effect on the *L. innocua* indicator strain, indicating that proteinase K per se had no inhibitory effect.

DISCUSSION

Previous studies with the laboratory strain PY79 have provided evidence that germination, growth, and resporulation occur in the GIT (2, 18). This is based on the finding that when mice are inoculated orally, on some occasions more spores are excreted than are inoculated (18). Probiotic strains may have a beneficial effect by persisting in the GIT, perhaps by association with the mucosa, and we addressed this possibility by examining the persistence of different probiotics in the mouse GIT (note that these experiments are different from those described previously [18] since in this study we examined spore counts at distinct times rather than counts recovered in the total feces collected between time points). We found that all *B. cereus* probiotics appeared to persist longer in the GIT and that after 15 days significant numbers were still present. In contrast, the *B. subtilis*, *B. clausii*, and *B. pumilus* strains ap-

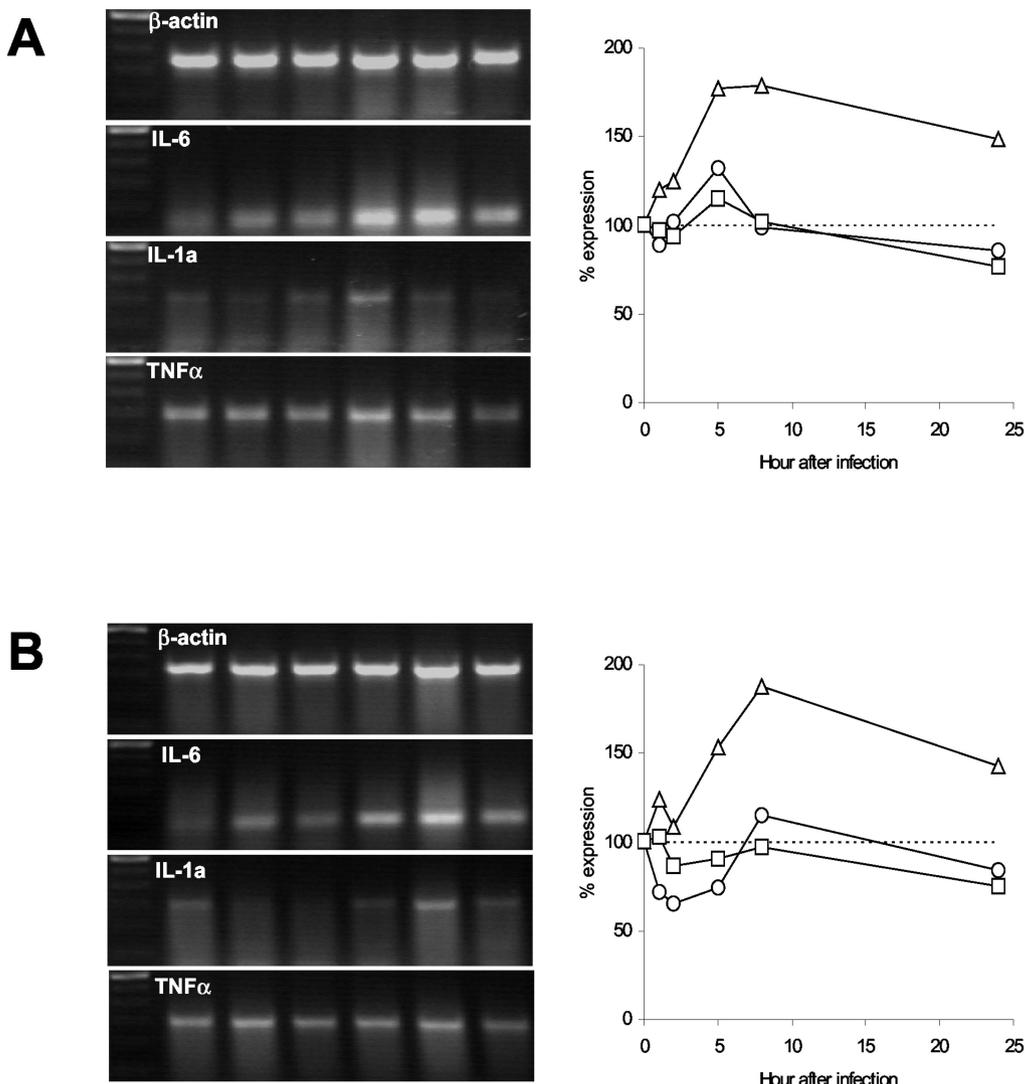


FIG. 5. Expression of cytokines in spore-infected macrophages. The murine macrophage cell line RAW264.7 was infected with spores of *Bacillus* strain PY79 (A) or Biosubtyl^{NT} (B). The results are representative of three independent experiments. Specific mRNAs for β -actin (348 bp), IL-1 α (308 bp), IL-6 (154 bp), and TNF- α (307 bp) were detected by RT-PCR with macrophage lysate samples taken at zero time and 1, 2, 5, 8, and 24 h after infection with spores. The results of a densitometric analysis of the gels for expression of IL-1 α (○), IL-6 (△), and TNF- α (□) compared to the expression of the housekeeping β -actin gene at each time is shown in panel A for PY79 and in panel B for Biosubtyl^{NT}.

peared to be cleared from the gut. This corresponds with studies showing that *B. cereus* spores can efficiently adhere to human epithelial cells (Caco-2), an attribute related to the hydrophobicity of spores (1). This feature, of course, may be important for germination and infection within the small intestine. Interestingly, the laboratory strain (PY79) was rapidly cleared from the GIT within just 5 days, and it is possible that as a frequently passaged laboratory strain this bacterium has lost one or more of its natural traits which would otherwise promote persistence within the GIT. We noted that the exosporium, which is the outermost layer of the spore, is absent from the laboratory strains of *B. subtilis* (16). Interestingly, the exosporium also seems to be absent from at least three newly discovered fecal isolates of *B. subtilis* (Barbosa and Henriques, unpublished results) but is found in the other *Bacillus* probiotic strains (17). It should be interesting to determine whether the presence of this structure correlates with increased persistence

of spores in the GIT. One of the *B. cereus* strains, Biosubtyl^{DL}, has been shown to be facultatively anaerobic (17). This strain did not appear to persist longer in the mouse GIT, so we assume that the ability of this strain to grow in anoxic conditions does not enable enhanced persistence.

One surprising result of this work was that spores of some of the probiotic strains were apparently sensitive to SGF and SIF. In the case of Subtyl this sensitivity was acute, and only a tiny fraction of the spores survived incubation in SGF and SIF. We do not believe, however, that the spores are themselves sensitive to acid; rather, we provide two explanations to account for these results. First, spore germination is activated by the low pH since acid activation of spore germination (as opposed to the more common heat-dependent activation) is known to promote germination of spores of *B. cereus* (8, 22) and rapid synchronized germination in the SGF and SIF could account for this rapid loss of viability. Second, Subtyl spores are intrin-

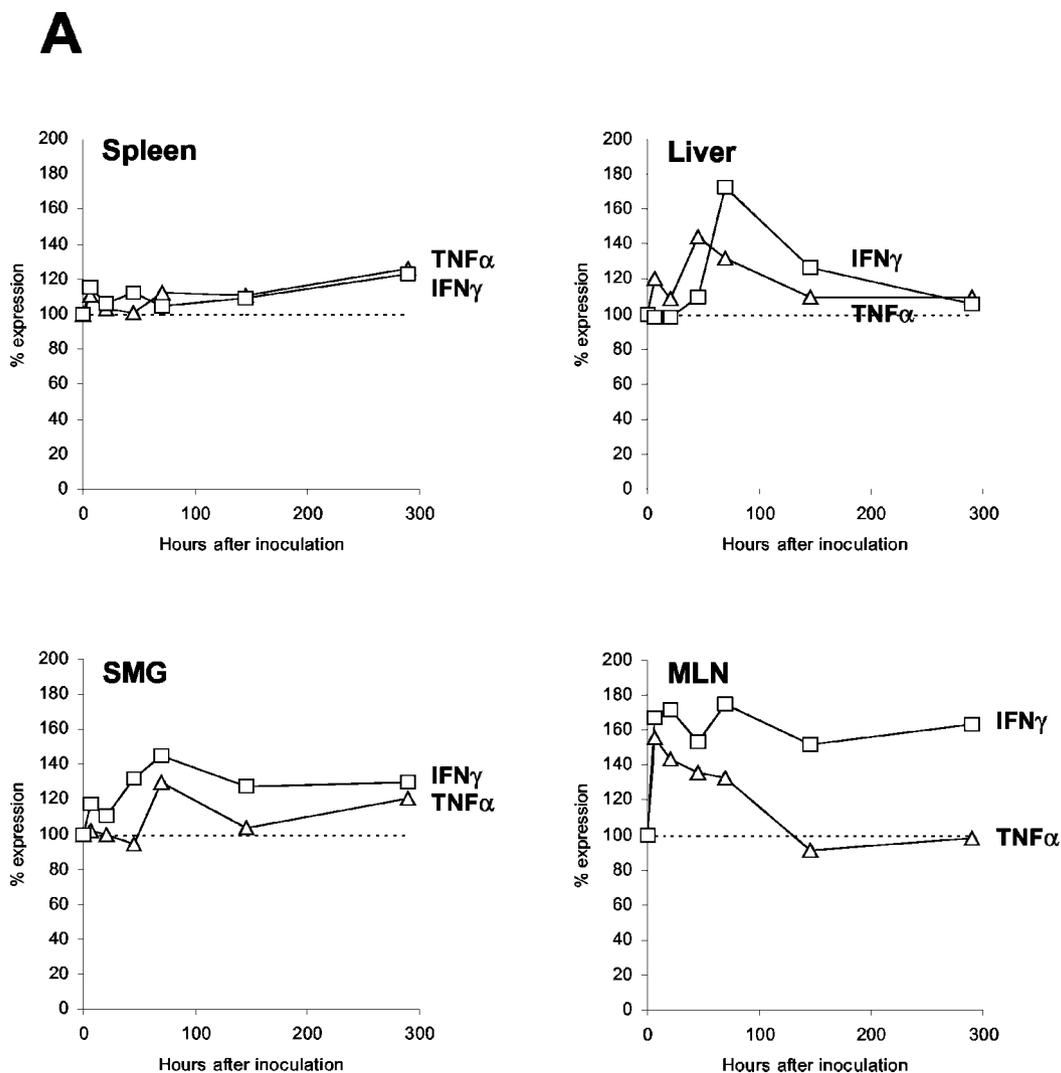


FIG. 6. Expression of cytokines in various organs of mice. Inbred BALB/c mice were inoculated with 10^{10} spores of *Bacillus* strain PY79 (A) or Biosubtyl^{NT} (B). At different times, cytokine expression in various organs and tissues of the animals was detected with total RNAs by using RT-PCR. A graph showing the results of densitometric analyses (typical of the entire group) of RT-PCR data for one mouse is shown in each case; the percent expression represents the relative abundance of each cytokine compared to the abundance of β -actin. The dotted lines indicate the relative abundance of each cytokine at zero time (100%). No expression was detected in naïve, nonimmunized mice at any time (data not shown).

sically more sensitive to the extraction procedure which we used to prepare spores (most probably the high-pressure treatment with the French press), which makes them sensitive to the SGF. The second explanation is supported by the drop (50%) in viability of spores suspended only in water. If the former hypothesis is correct, however, and germination of Subtyl spores is acid sensitive, then in a natural environment physiological conditions (food composition and fluctuations in pH, etc.) should ensure that a greater number of spores survive, and our analysis of fecal counts did appear to show that in vivo Subtyl can escape cell death, which supports this explanation. Interestingly, in other work it has been shown that germination of spores of the laboratory strain *B. subtilis* PY79 is partially inhibited in the presence of SIF (5), so clearly conditions within the small intestine can have opposing effects on spores.

The most important finding of this work is that the three *B. cereus* probiotics, Bactisubtyl, Biosubtyl^{DL}, and Subtyl, produce

enterotoxins. Both Biosubtyl^{DL} and Bactisubtyl produce the Hbl enterotoxin, which is the primary virulence factor of *B. cereus* food poisoning (12, 23), while Biosubtyl^{DL} and Subtyl produce the Nhe enterotoxin. Although no commercial test is yet available to detect enterotoxin T, the presence of the structural gene of this toxin in Biosubtyl^{DL} and Bactisubtyl suggests that the toxin could be produced under favorable conditions. One additional enterotoxin found in some *B. cereus* strains, the 1.2-kDa emetic toxin (cereulide), was not tested for, primarily because strains producing this preformed toxin cause emesis at a low dose (total dose, 10^3 to 10^4 CFU/g), so it is not possible that an emetic strain is being used for human probiotics. The typical dose of spore probiotics is between 10^7 and 10^9 spores, and the total infective dose of pathogenic *B. cereus* required to produce diarrhea is between 10^5 and 10^7 spores (12). Food-poisoning *B. cereus* spores adhere to the mucosal epithelium of the small intestine, germinate, and are able to produce ente-

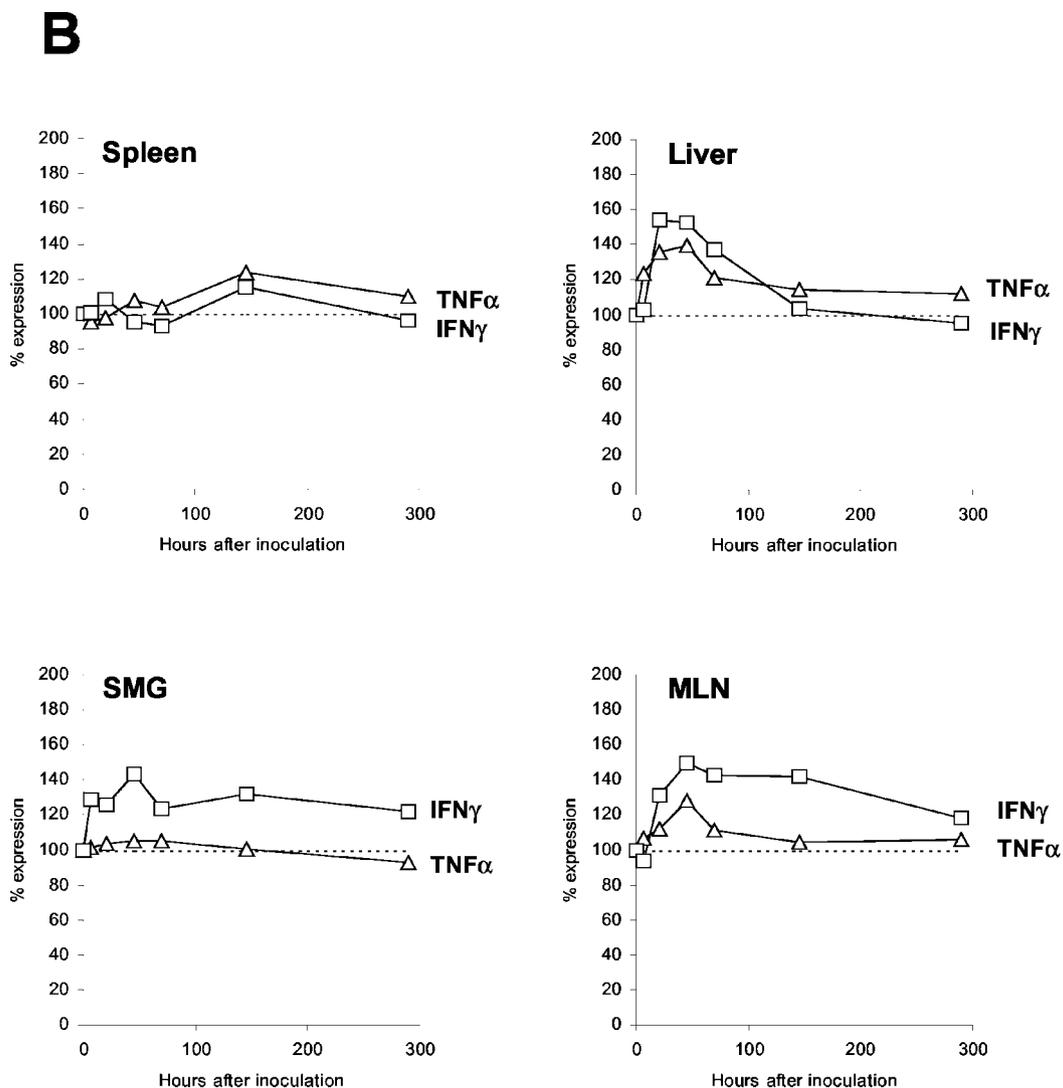


FIG. 6—Continued.

rotoxins that induce diarrhea (12, 23). It seems probable, then, that the three *B. cereus* probiotics tested here behave similarly. This appears to be a paradox, but there are several points that should be clarified. First, enterotoxins are not always pro-

duced, and the microenvironment (adhesion and competition with other commensal bacteria, food intake, luminal pH, etc.) within the GIT may affect enterotoxin production, as well as adhesion to the mucosa. In the case of Subtyl it is possible that

TABLE 2. Potential virulence traits of commercial strains

Strain or product	Taxon ^a	Hemolysis ^b	Lecithinase ^c	Hbl complex genes ^d				Oxoid kit test index ^e	Nhe complex genes ^d			Tecra kit test index ^f	cytK ^d	bceT ^d
				hblA	hblB	hblC	hblD		nheA	nheB	nheC			
PY79	<i>B. subtilis</i>	γ	—	—	—	—	—	0	—	—	—	2	—	—
Subtyl	<i>B. cereus</i> var. vietnami	β	+	—	—	—	—	0	+	+	+	5	—	—
Biosubtyl ^{DL}	<i>B. cereus</i>	α	+	+	—	+	+	128	+	+	+	4	—	+
Bactisubtil	<i>B. cereus</i>	β	+	+	—	+	+	256	—	+	+	2	—	+

^a Species were assigned by 16S rRNA analysis (13, 17). Subtyl has also been referred to as *Bacillus vietnami* pending approval of a new species (17).

^b α, brownish zone around colonies; β, complete hemolysis, with clear zone around each colony; γ, no change. In a previous study it was reported that Biosubtyl^{DL} produces beta hemolysis (17).

^c +, blue precipitation of hydrolyzed lecithin around peacock blue colonies (typical of *B. cereus*); —, no change.

^d +, a PCR product of the expected size was observed; —, no PCR product was observed.

^e For the Oxoid test, the indices corresponded to the last supernatant dilution (in twofold serial dilutions) for which enterotoxin remained detectable. Strains with an index of 0 were considered negative, and the sensitivity of the test is 2 ng/ml.

^f For the Tecra test, indices from 1 to 5 corresponded to the intensity of coloration. According to the manufacturer's instructions, strains with an index of <3 are considered negative, and the sensitivity of the test is 1 ng/ml.

germination of spores in the stomach and small intestine significantly reduces the infective dose, presumably explaining why food poisoning does not result. Although *B. cereus*-based food poisoning is short-lived, an interesting and controversial concept is whether exposure to repeated doses of enterotoxin-producing *B. cereus* strains actually immunizes and offers some level of protection (vaccination) from subsequent infection with an infectious food-poisoning strain of *B. cereus*. If this is the case, protection would be afforded only against *B. cereus*-induced food poisoning, so such a treatment cannot provide universal protection against other enteric infections that lead to diarrhea. Although this is not a rational reason for using the strains, the generation of enterotoxin-specific IgG could provide a mechanism that protects against subsequent *B. cereus* food poisoning. Interestingly, the Bactisubtil *B. cereus* strain is listed as IP 5832 and is apparently identical to the strain used in the animal feed additive labeled Paciflor. Paciflor was recently withdrawn from production because of an assessment by the Scientific Committee on Animal Nutrition of the European Commission. In this assessment the presence of both the Hbl and Nhe enterotoxins was demonstrated, and it was concluded that this was a risk to human health, primarily because of the risk of infection of humans in the slaughtering process (assessment by the Scientific Committee on Animal Nutrition on the Safety of the Product Paciflor for use as a feed additive, adopted 16 May 2003; http://europa.eu.int/comm/food/fs/sc/index_en.html). It is somewhat surprising that Bactisubtil is still listed as a product for human use by Aventis Pharma Portugal. In our work, we detected only the Hbl enterotoxin in Bactisubtil, suggesting that despite the identical strain designation (IP 5832) the strains most probably represent derivatives of a common ancestor and the loss of the Nhe enterotoxin can be attributed to spontaneous or deliberate inactivation of the *nheA* gene.

Germination of the spore could allow production of antimicrobial agents, such as bacteriocin-like inhibitory substances, thereby contributing to the competitive exclusion of pathogens, and it is one factor that could support the probiotic effect. A number of *Bacillus* species produce antimicrobial agents, and more than 80 different types have been reported (25). These antimicrobial agents are active mostly against gram-positive bacteria, but some are active against gram-negative bacteria. Recently, an antibiotic compound isolated from a strain of *B. subtilis* found in the probiotic Biosporin with activity against *H. pylori* has been reported (31). We show here that at least two probiotic strains, Biosubtyl^{NT} and Subtyl, produce antimicrobial agents (or bacteriocin-like inhibitory substances) that are active against other *Bacillus* species. We note that certain *Bacillus* species have been associated with infection of the GIT, but it is also possible that these agents are active against a broader group of species. In any case, production of the antimicrobial agents could be an element in the probiotic effect.

Immune stimulation as a mechanism for a probiotic effect is difficult to define, but this must result from induction of proinflammatory cytokines that increase phagocytosis (by macrophages or dendritic cells) and perhaps also stimulation of cytotoxic cells. We show here that when given orally to mice, all probiotic strains generate systemic IgG responses. This shows that spores are immunogenic and are not treated as a food. To generate humoral responses, spore antigens could interact with

the GALT. As reported elsewhere, there is strong evidence that *B. subtilis* spores enter the Peyer's patches and MLN, and presumably they do this by translocation across M cells (6). In the case of Biosubtyl^{NT} the spore-specific IgG responses were almost 10-fold higher than the responses to the other strains, showing that this strain is particularly immunogenic. Analysis of cytokine expression in vivo showed that there is early production of IFN- γ and TNF- α in the secondary lymphoid organs and GALT following oral inoculation of mice with PY79 and Biosubtyl^{NT} spores. Using a coculture with macrophages in vitro, we failed to detect significant levels of TNF- α or IL-1 α (IFN- γ was not tested), but clear production of IL-6 was observed; we have no explanation for these results. Interestingly, in a recent study (29) performed with human monocytes (isolated from peripheral blood mononuclear cells) stimulated with *B. subtilis* spores, significant levels of IL-1 β and TNF- α were found to be produced. IFN- γ is an activator of cellular responses, particularly the Th1 response that, in turn, is responsible for stimulating phagocytosis. IFN- γ is also produced during inflammation (as opposed to a specific immune response), as is TNF- α , whose production by macrophages has been linked with chronic infections (4, 26). These early responses suggest that there is an innate immune response and secretion of IFN- γ by peripheral blood mononuclear cells. Examination of macrophages cultured in vitro with PY79 and Biosubtyl^{NT} also showed that there was potent induction of the proinflammatory cytokine IL-6. Proinflammatory responses should not necessarily be considered a beneficial feature of a probiotic since these responses show been linked to autoimmune diseases, such as inflammatory bowel diseases, including ulcerative colitis and Crohn's disease (37). Together, these inflammatory responses show the complexity of immunomodulatory responses that can result from oral consumption of bacterial spores. While in this study we examined cytokine responses elicited from the GALT, it should be emphasized that nonprofessional antigen-presenting cells (e.g., epithelial cells) could also play an important role in immunomodulation. The importance of these responses in a potential probiotic effect remains to be determined, but the responses may well play a role in increasing resistance to infection by recruitment and activation of immune and inflammatory cells (neutrophils and mast cells). Similarly, oral administration of various probiotic *Lactobacillus* species has been shown to enhance the innate immune system and to enhance macrophage phagocytosis (38), NK cell functions (3), and production of macrophage lysosomal enzymes (19).

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