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Characterization of *Bacillus* Species Used for Oral Bacteriotherapy and Bacterioprophylaxis of Gastrointestinal Disorders

NGO THI HOA,[1] LOREDANA BACCIGALUPI,[2] ASHLEY HUXHAM,[1] ANDREI SMERTENKO,[1] PHAM HUNG VAN,[3] SERGIO AMMENDOLA,[4] EZIO RICCA,[2] AND SIMON M. CUTTING[1]*

School of Biological Sciences, Royal Holloway University of London, Egham, Surrey TW20 0EX, United Kingdom[1]; Section of Microbiology, Department of General and Environmental Physiology, University Federico II, 80134 Naples,[2] and Bioprogress S.p.A., Anagni (FR),[4] Italy; and Laboratory of Microbiology, Ho Chi Minh University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam[3]

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Bacillus subtilis **spores are being used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders in both humans and animals. Since *B. subtilis* is an aerobic saprophyte, how spores may benefit the gut microbiota is an intriguing question, since other probiotics such as *Lactobacillus* spp. which colonize the gut are anerobes. As a first step in understanding the potential effects of ingesting spores, we have characterized five commercial products. An extensive biochemical, physiological, and phylogenetic analysis has revealed that four of these products are mislabeled. Moreover, four of these products showed high levels of antibiotic resistance.**

Probiotics, or "friendly bacteria," are becoming increasingly available to the public as beneficial functional foods that purport to promote specific health benefits to consumers (2, 14, 18). In some countries probiotics are available for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders in humans. Often these disorders, many of which lead to diarrhea, are a direct result of antibiotic use, which produces an imbalance in the composition of the normal intestinal microbial flora. In the livestock industry the use of probiotics has potential as an alternative to antibiotics by competitive exclusion of pathogenic microorganisms (19), with some commercial products, such as Paciflor, already available. Bacteria most commonly used as probiotics include the lactic acid bacteria (e.g., lactobacilli, enterococci, streptococci, and bifidobacteria). Experimental evidence now suggests that the ingestion of substantial numbers of harmless bacteria does indeed provide a beneficial effect to the enteric flora (18). Precisely how this is achieved and whether the commercial claims are justified remains a contentious issue, though (14).

In addition to the lactic acid bacteria, *Bacillus* species are also sold as probiotics. These consist of preparations of bacterial spores, with the potential advantage that the spore can survive transit through the stomach intact. *Bacillus* species are substantially different from other probiotic bacteria, though, being primarily aerobic saprophytes found in the soil. If indeed they have any health benefit, then one obviously important question is how? Do spores germinate and colonize the gut, do they competively exclude colonization by potential pathogens, or does the dormant spore provide some unique stimulus to the gut microbiota, such as enhanced local immunity?

In an earlier study we have shown that one major *Bacillus* probiotic marketed in Europe contained spores of a taxonomically and phylogenetically unrelated *Bacillus* species (4). This was surprising, considering that in Europe probiotics must be licensed to be used as a functional or novel food.

In this work we have examined and characterized five commercial *Bacillus* spore probiotics as a first step in understanding the nature of spore probiotics.

MATERIALS AND METHODS

Bacterial strains. Bacteria were recovered by suspension of dried probiotic preparations in distilled water followed by dilution and serial plating on Difco sporulation agar (11). The commercial preparations and manufacturers were as follows: Lactipan *plus* (Istituto Biochimico Italiano S.p.A., Milan, Italy), Domuvar (Consorzio Farmaceutico e Biotecnologico Bioprogress a.r.i., Anagni-FR, Italy), Bactisubtil Marion Merrell S.A. Bourgoin-Jallieu, France), Biosubtyl, (National Institute of Vaccines and Biological Substances, Da Lat, Vietnam), and Subtyl (Pharmaceutical Factory 24, Ho Chi Minh City, Vietnam). A validated *Bacillus subtilis* strain, PY79 (20), a derivative of strain 168, was used as a *B. subtilis* type strain.

Strain depositions. All strains characterized in this work have been deposited with the *Bacillus* Genetic Stock Centre (BGSC), The Ohio State University, Columbus (http://bacillus.biosci.ohio-state.edu/). (BGSC designated names are given in Table 5.)

Biochemical tests. The API 50 CH kit (BioMerieux), comprising 49 unique biochemical tests appropriate for *Bacillus* spp., was used for diagnosis as described in the manufacturer's instructions. The complete test was performed five times for each strain. Hydrolysis of starch was as described previously (3). Growth at pH 10.1 was by the method of Horikoshi and Teruhiko (7), using Na_2CO_3 to adjust the pH. Sporulation was induced by nutrient exhaustion using Difco sporulation medium and measurements of heat-resistant spores were made as described previously (11).

Electron microscopy. Suspensions of sporulating bacteria (from 4-day-old plate cultures) were fixed in 3% glutaraldehyde plus 4% paraformaldehyde in 0.1, M PIPES [piperazine-*N,N′*-bis(2-ethanesulfonic acid] buffer (pH 7.2) according to method 1 of Page et al. (12). Silver sections of Spurr resin-embedded material were stained with uranyl acetate followed by Reynolds lead stain and viewed on a Zeiss EM 109 transmission electron microscope.

Antibiotic testing. Fresh plate cultures grown on Luria-Bertani (LB) medium were used to make bacterial suspensions with a density of approximately 0.5, adjusted using McFarland standards. Mueller-Hinton plates (Merck; NCCLS standard) were seeded using swabs. Antibiotic-inpregnated discs (6-mm diameter; Oxoid) were placed on the seeded plates, and following 18 h of growth at 37°C, zones of inhibition were measured. Those strains showing a zone of inhibition of less than 12 mm in diameter were characterized further for the MIC of antibiotic required to inhibit cell growth. MICs were determined using cultures grown in Mueller-Hinton broth.

Phylogenetic analysis. Two oligonucleotides were used to amplify the entire 16S rRNA (P1 [5′-GCGGCGTGCCTAATACATGC] anneals to nucleotides

* Corresponding author. Mailing address: School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, UK. Tel: 01784-443760; Fax: 01784-434326; E-mail: s.cutting@rhbnc.ac.uk.

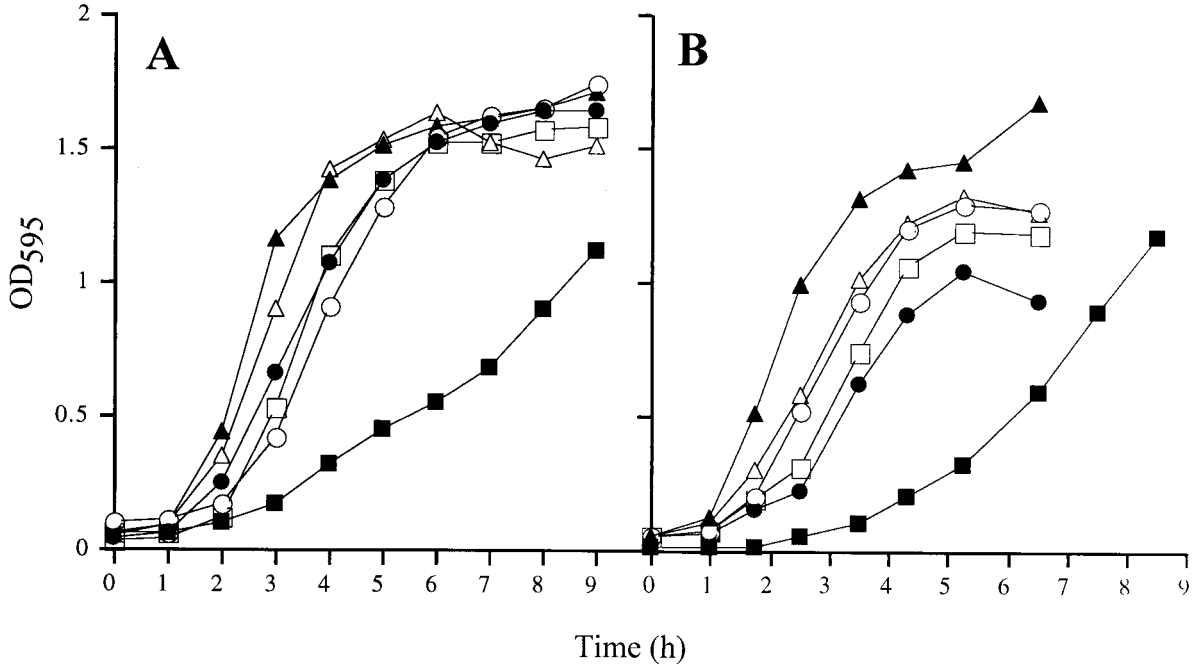

FIG. 1. Growth of probiotic strains. Bacterial strains were grown at 37°C in LB medium (A) or Difco sporulation medium (B). The strains were *B. subtilis* PY79 (●), Bactisubtil (○), Subtyl (▲), Biosubtyl "Dalat" (Δ), Domuvar (■), and Lactipan *plus* (❑). OD_{595}, optical density at 595 nm.

[nt] 40 to 59, and P2 [5′-CACCTTCCGATACGGCTACC] anneals to nt 1532 to 1513, of *B. subtilis rrnE*). The 1,400-base PCR product was sequenced in its entirety using an automated sequencer and oligonucleotides P1, P2, P3 (5′-AC GCCGCGTGAGTGATGAAG-3′) (anneals to nt 404 to 423 of *B. subtilis rrnE*), and P4 (5′-CATCTCACGACACGAGC-3′) (anneals to nt 1093 to 1076 of *B. subtilis rrnE*). The genes for 16S rRNA were aligned using the Clasl1W program (EMBL Outstation http://www2.ebi.ac.uk/clastlw/), and the alignment was used to construct phylogenetic trees with the Phylip software package from the Pasteur Institute (http://bioweb.pasteur.fr/seqanal/phylogeny/). The tree represents similarities between the 16S rRNA genes.

Nucleotide sequence accession numbers. The recovered 16S rRNA gene sequences have been deposited in GenBank with the following accession numbers: Domuvar, AJ277904; Lactipan *plus*, AJ277905; Subtyl, AJ277906; Biosubtyl "Dalat," AJ277907; Bactisubtil, AJ277908.

RESULTS

General characteristics of *Bacillus* probiotics. Five commercial probiotic preparations were characterized: Domuvar and Lactipan *plus*, originating from Italy; Bactisubtil, from France; and Subtyl and Biosubtyl "Dalat," from Vietnam. Bactisubtil was labeled as *Bacillus cereus*, Lactipan *plus* was labeled as *Lactobacillus sporogenes*, and the other three were labeled as *B. subtilis*. All were found to consist of homogenous populations. We examined growth of these bacteria as well as *B. subtilis* strain PY79, which is a derivative of the 168 type strain, in LB medium (Fig. 1A). All grew efficiently with similar growth rates, with the exception of Domuvar, which always produced a noticeable lag before exponential growth and which grew slower than the other strains. We found that Domuvar was the only strain which could support growth at pH 10.1 (Table 1). We also examined growth anerobically and found that Biosubtyl "Dalat" could grow efficiently. *B. subtilis* will not normally grow anerobically unless provided with glucose and nitrate as a terminal electron acceptor (10), so this result makes it highly unlikely that the species was *B. subtilis*. Similarly, the probiotic Subtyl was unable to hydrolyze starch, showing that it was not a *B. subtilis* strain, for which the capacity to hydrolyze starch is a diagnostic feature (15). Two probiotic strains, Bactisubtil and Subtyl, produced strong hemolysis when grown on either sheep or horse blood agar plates. Biosubtyl "Dalat" also produced weak levels of hemolysis when grown on sheep blood agar. Hemolysins are not generally associated with *B. subtilis* strains but are produced in strains of *B. cereus* (15).

Spore formation. We examined spore formation using *B. subtilis* strain PY79 as a control. When grown in Difco sporulation medium, Domuvar again produced a lag phase and exhibited a lower growth rate (Fig. 1B). We examined sporulating cultures at hour 18 of development for spore shape and position and for heat resistance (Table 1 and Fig. 2). Domuvar produced a very low percentage of spores and consisted primarily of long filamentous cells which had not broken down by septation into smaller cells. These spores appeared to be less resistant to higher temperatures, with a 100-fold reduction in viable spores at 75°C compared to 65°C (Table 1). Both Bactisubtil and Biosubtyl "Dalat" contained spores which varied in shape, being either ellipsoidal or spherical. The Subtyl strain appeared to sporulate very efficiently, and we found that following heat treatment at either 65 or 75°C, a higher viable count (CFU per milliliter) was produced than in the unheated cultures. This apparent paradox can be explained only if the spores are not able to germinate efficiently on nutrient agar plates, but when spores are heated this treatment activates germination and so increases the viable counts. It is known that *B. subtilis* spores germinate more efficiently when heat activated at 75°C (9), which would support this explanation. We also observed that Subtyl cultures plated on Difco sporulation agar plates always produced two colony types (white and translucent) at a 50:50 ratio. The white colony (Whi) type consisted almost entirely of mature released spores. In contrast, the translucent (Trn) colony type contained only 50% released spores and many long filamentous sporangial cells containing spores or no spores at all. When the Whi and Trn colony types were propagated, the Whi strain again generated two colony types (Whi and Trn) on Difco sporulation agar, showing that

TABLE 1. Characteristics of *Bacillus* probiotic strains

Characteristic	Lactipan *plus*	*Bacillus* strain:				
		Domuvar	Bactisubtil	Subtyl	Biosubtyl "Dalat"	*B. subtilis* PY79
Anerobic growth	−	−	−	−	+	−
Hydrolysis of starch	+	+	+	−	+	+
Hemolysis[a] on:						
Sheep blood	γ	γ	β	β	(β)	γ
Horse blood	γ	γ	β	β	γ	γ
Growth at pH 10.1	−	+	−	−	−	−
Spore shape[b]	E	E	E/S	E	E/S	E
Spore position[c]	ST	T	T/ST	C	T/ST	ST
Exosporium[d]	−	+	+	+	+	−
Sporulation[e] at:						
65°C	85	79	14	111	7.6	87
75°C	95	18	2	208	0.05	36

[a] Hemolysis was determined from growth on sheep or horse blood agar plates and defined as β (complete) or γ, (no hemolysis). Parentheses indicate a weak reaction.
[b] E, ellipsoidal; S, spherical.
[c] C, central; ST, subterminal; T, terminal.
[d] Determined from electron microscopic analysis.
[e] Sporulation was induced by exhaustion in Difco sporulation medium. At 24 h after the initiation of sporulation, 0.5-ml samples were measured for viable counts and incubated at either 65 or 75°C for 30 min prior to plating for heat-resistant survivors. Values given are percent survivors.

the Whi phenotype was genetically unstable. When grown in liquid culture, the Whi substrain of Subtyl produced a distinctive white, hydrophilic aggregate.

Examination of spores of each strain by electron microscopy revealed an exosporium in mature spores of Domuvar, Bactisubtil, and Biosubtyl "Dalat" (Table 1). The exosporium is a poorly defined structure which is often found loosely attached to the outer coat layer (1, 16). Subtyl spores had a most unusual appearance (Fig. 3) and did not stain well with uranyl acetate. Subtyl spores appeared, however, to contain an exosporial layer which was shed into the surrounding medium. This is shown in Fig. 3, and although highly speculative, it is possible that this shedded material produces the aggregate observed in liquid growth.

Antibiotic resistance. We examined the resistance of each of the probiotic strains to a selection of antibiotics using the disc

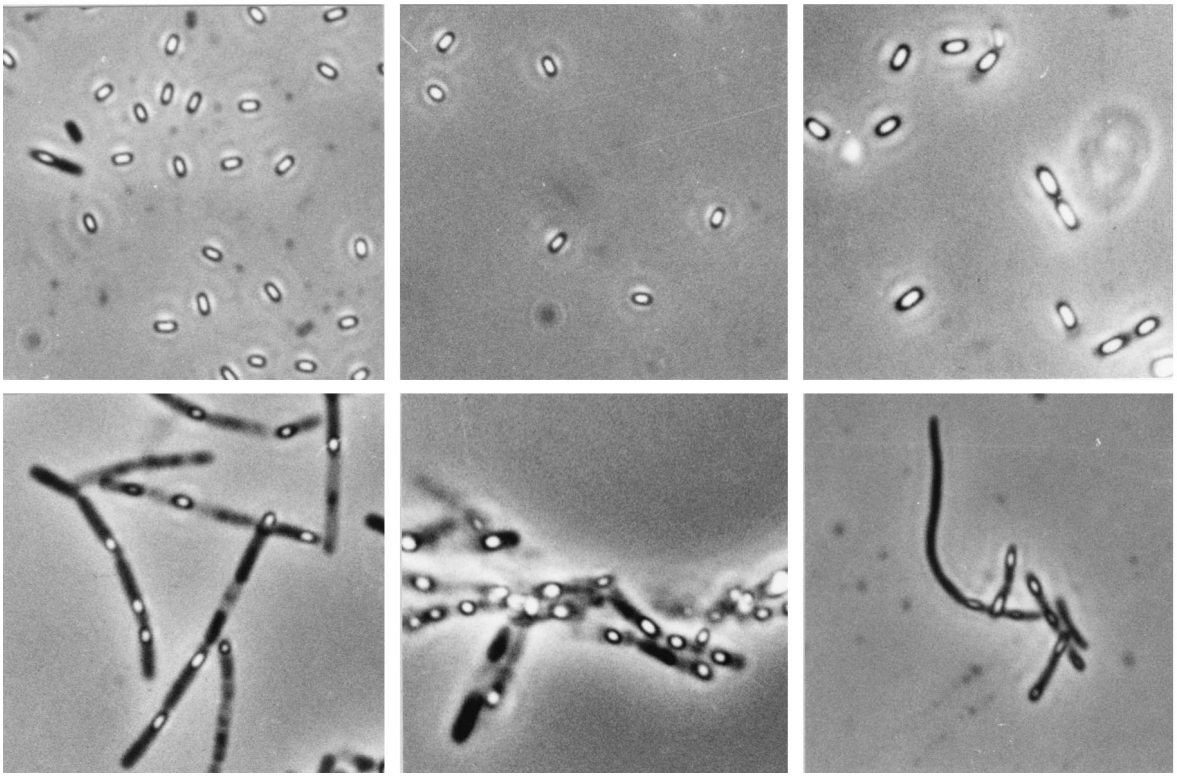

FIG. 2. Sporulation. Phase-contrast microscopy of cultures grown in Difco sporulation medium examined at approximately 12 h after the initiation of sporulation is shown. Clockwise from top left, *B. subtilis* PY79, Lactipan *plus*, Subtyl, Biosubtyl "Dalat," Bactisubtil, and Domuvar.

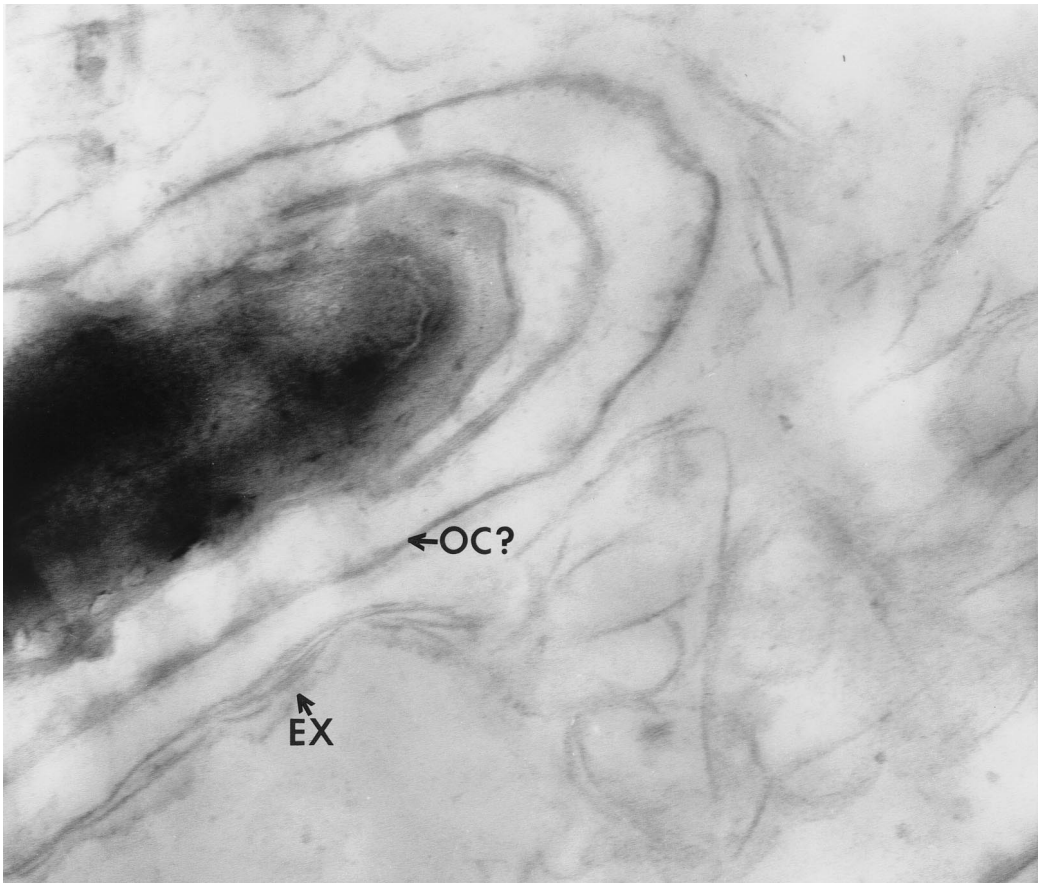

FIG. 3. Electron microscopy of a mature Subtyl spore. The material shed from the spore is thought to have come from the exosporial layer associated with the outer coat layer. Bar, 0.2 μm. EX, exosporium; OC, outer coat.

diffusion test (Table 2). Those strains which showed small zones of inhibition (less than 12 mm) were considered resistant and evaluated further to establish the MIC. Substantial levels of resistance to several antibiotics were identified, as shown in Table 3. All strains, including *B. subtilis* PY79, were found to have a basal level of resistance to spectinomycin (30 to 60 μg/ml). Although the results were inconclusive, we failed to identify any plasmids in the antibiotic-resistant strains, suggesting that drug resistance may be chromosome borne (data not shown).

Phylogenetic analysis and species identification. Initial examination of the probiotic strains showed substantial heterogeneity and indicated that at least some were unlikely to be *B. subtilis*. As a first step, we used the API CH 50 test to identify

TABLE 2. Antibiotic resistances[a]

Antibiotic (amt)	Zone of inhibition (mm)[b]					
	B. subtilis PY79	Bactisubtil	Subtyl	Biosubtyl "Dalat"	Domuvar	Lactipan *plus*
Neomycin (30 μg)	27–30	25–26	23–26	25	43–46	15–23
Streptomycin (10 μg)	15–16	14–16	19–21	16–18	19–24	16–19
Rifampin (5 μg)	19–24	16–18	15–20	18–26	36–40	30–31
Kanamycin (30 μg)	31	23–26	21	26–30	45	26–27
Penicillin G (1 U)	25–29	26–32	<9	<8	15–20	20–26
Compound sulfonamides (300 μg)	24–28	15–27	21	25	32–40	28–31
Tobramycin (10 μg)	28	24–27	19–21	25–30	35	24–30
Novobiocin (30 μg)	24–26	20–24	22–23	18–25	14	24–25
Tetracyline (10 μg)	15–18	6	21–26	22–30	18–20	15
Chloramphenicol (50 μg)	27–32	12–14	29–30	28–33	14–15	29–30
Spectinomycin (10 μg)	13–15	10–14	<12	9–10	27–29	7–9
Erythromycin (15 μg)	29	19–23	25–29	31	13	27–28
Ampicillin (25 μg)	30–31	33–38	10–14	11–15	30–32	29–31
Lincomycin (15 μg)	16–17	22–27	16–20	19–21	6	25–27

[a] Antibiotic-immpregnated discs (6 mm in diameter) were used to evaluate antibiotic sensitivities as described in Materials and Methods
[b] Zones of inhibition (diameter) from three individual experiments are given, with a value of 6 mm representing no zone of inhibition.

TABLE 3. MICs

Strain	Antibiotic, MIC[a]
Biosubtyl "Dalat"	Penicillin G, >800 U/ml
	Ampicillin, >300 μg/ml
Subtyl	Ampicillin, >300 μg/ml
	Penicillin G, >800 U/ml
Bactisubtil	Chloramphenicol, 120 μg/ml
	Tetracycline, 60 μg/ml
Domuvar	Erythromycin, 1600 μg/ml
	Lincomycin, 800 μg/ml
	Novobiocin, 320 μg/ml
	Penicillin G, 160 U/ml
	Chloramphenicol
	60 μg/ml
PY79	Penicillin G, <5 U/ml
	Chloramphenicol, <5 μg/ml
	Ampicillin, <25 μg/ml
	Erythromycin, <1 μg/ml
	Lincomycin, <45 μg.ml
	Tetracycline, <10 μg/ml
	Novobiocin, <20 μg/ml

[a] MIC of antibiotic required to inhibit cell growth in liquid medium.

species. This test generates a species identification as a percent probability, and the accuracy increases the more times the test is repeated. Our results shown in Table 4 identified Lactipan *plus* as most likely to be *B. subtilis* and Subtyl, Bactisubtil, and Biosubtyl "Dalat" as most likely to be *B. cereus*. Domuvar, though, proved to be unrecognizable using this test.

To determine the relatedness the strains at the genetic level, we sequenced the entire 16S rRNA gene from each strain. Our analysis (Fig. 4) revealed that Lactipan *plus* was 99% identical to *B. subtilis* strain PY79 (GenBank accession no. AF142577). Both Dalat and Bactisubtil were members of the *B. cereus* subgroup. Bactisubtil was 99% conserved with *Bacillus thuringiensis* (D16281) and 98% identical to Biosubtyl "Dalat," while Biosubtyl "Dalat" was 98% identical to *B. thuringiensis* (D16281). Domuvar was found to be 95% homologous with another probiotic strain, Enterogermina (AF142576). Subtyl did not show strong homology with any *Bacillus* species in GenBank.

DISCUSSION

The purpose of this study was to establish the identity of *Bacillus* bacteria available in commercial spore probiotics used for human consumption. In turn, this would provide an important and necessary first step in understanding how bacterial endospores might serve as "friendly bacteria" or probiotics. Our results were somewhat unexpected and have proven intriguing, with only one commercial product correctly labeled. For those products marketed or produced in Europe this is of particular concern, since, as a novel food, probiotics must be licensed and satisfy European Union regulations (e.g., EC Regulation 258/97).

We have used both 16S rRNA analysis and biochemical tests to establish species identity. Our final conclusions are shown in Tables 4 and 5. Lactipan *plus* is clearly *B. subtilis* and is closely related to PY79. Strain PY79, derived from the 168 type strain, is used extensively as an isogenic strain for the genetic analysis of spore development (20). What is surprising with this commercial product is that it is labeled as *Lactobacillus sporogenes*. Neither *Bergey's Manual* nor the *Approved List of Bacterial Names* cites this as a species.

TABLE 4. Species designations[a]

Bacillus strain	API score		16S rRNA identification
	% ID[b]	T[c]	
Lactipan *plus*	61.6 *B. subtilis*	0.73	*B. subtilis*
Domuvar	UR[d]		*B. clausii*
Bactisubtil	97.9 *B. cereus*	0.62	*B. cereus*
Subtyl	79.2 *B. cereus*	0.98	*B. vietnami*
Biosubtyl "Dalat"	97.9 *B. cereus*	0.62	*B. cereus*
PY79	88.8 *B. subtilis*	0.9	*B. subtilis*

[a] Species identification using either the API 50 CH kit, as described in Materials and Methods, or sequencing of the entire 16S rRNA genes and analysis for homologous sequences (Fig. 4).

[b] Percent similarity, i.e., how closely the profile corresponds to the taxon relative to all the other taxa in the database.

[c] The T index, i.e., how closely the profile corresponds to the most typical set of reactions for each taxon.

[d] UR, unrecognizable using the API 50 CH kit.

Domuvar was found in this work to tolerate growth at high pH. This was a distinguishing feature of this strain, and we have observed this previously with another commercial probiotic, Enterogermina (4). 16S rRNA analysis revealed Domuvar to be homologous with Enterogermina, suggesting a common origin. Enterogermina has since been shown to be most closely related to *Bacillus clausii* (17), and our sequence analysis also confirmed Domuvar to be most closely related to this species. Enterogermina is a mixture of three antibiotic-resistant derivatives, giving resistance to chloramphenicol (strain O/C), novobiocin and rifampin (strain N/R), and streptomycin and neomycin (strain SIN). All derivatives also have chromosomeborne resistance to penicillin G, erythromycin, and lincomycin. Domuvar was found to be resistant to novobiocin and chloramphenicol as well as penicillin G, erythromycin, and lincomycin, suggesting that it may have been derived from one of the Enterogermina strains. Although it is highly probable that Domuvar and Enterogermina have the same origin, it is curious that the sequences are not more conserved. Both strains have been subject to a history of mutagenesis in the process of creating antibiotic resistances, and this may, in part, account for the sequence variation.

The Vietnamese probiotic Biosubtyl "Dalat" was found to be highly conserved with the French probiotic Bactisubtil. Bactisubtil is labelled as *B. cereus* strain IP 5832, and our analysis would confirm this if based only on biochemical data, notably, the production of hemolysins and an exosporium which are characteristic of *B. cereus* strains. Based on 16S rRNA analysis, though, this strain was clearly most similar to *B. thuringiensis. B. thuringiensis* strains nearly always produce parasporal crystal toxins, and we were unable to identify such structures by microscopy. We conclude, then, that this strain is most probably

TABLE 5. Status of *Bacillus* probiotics[a]

Commercial name	BGSC strains	Indicated species	Actual species
Lactipan *plus*	3A16	*L. sporogenes*	*B. subtilis*
Domuvar	17A1	*B. subtilis*	*B. clausii*
Bactisubtil	6A8	*B. cereus*	*B. cereus*
Enterogermina	15A1, 15A2, 15A3	*B. subtilis*	*B. clausii*
Subtyl	6A6	*B. subtilis*	*B. vietnami*
Biosubtyl "Nha Trang"	14A1	*B. subtilis*	*B. pumilus*
Biosubtyl "Dalat"	6A7	*B. subtilis*	*B. cereus*

[a] Enterogermina and Biosubtyl "Nha Trang" have been characterized elsewhere (4).

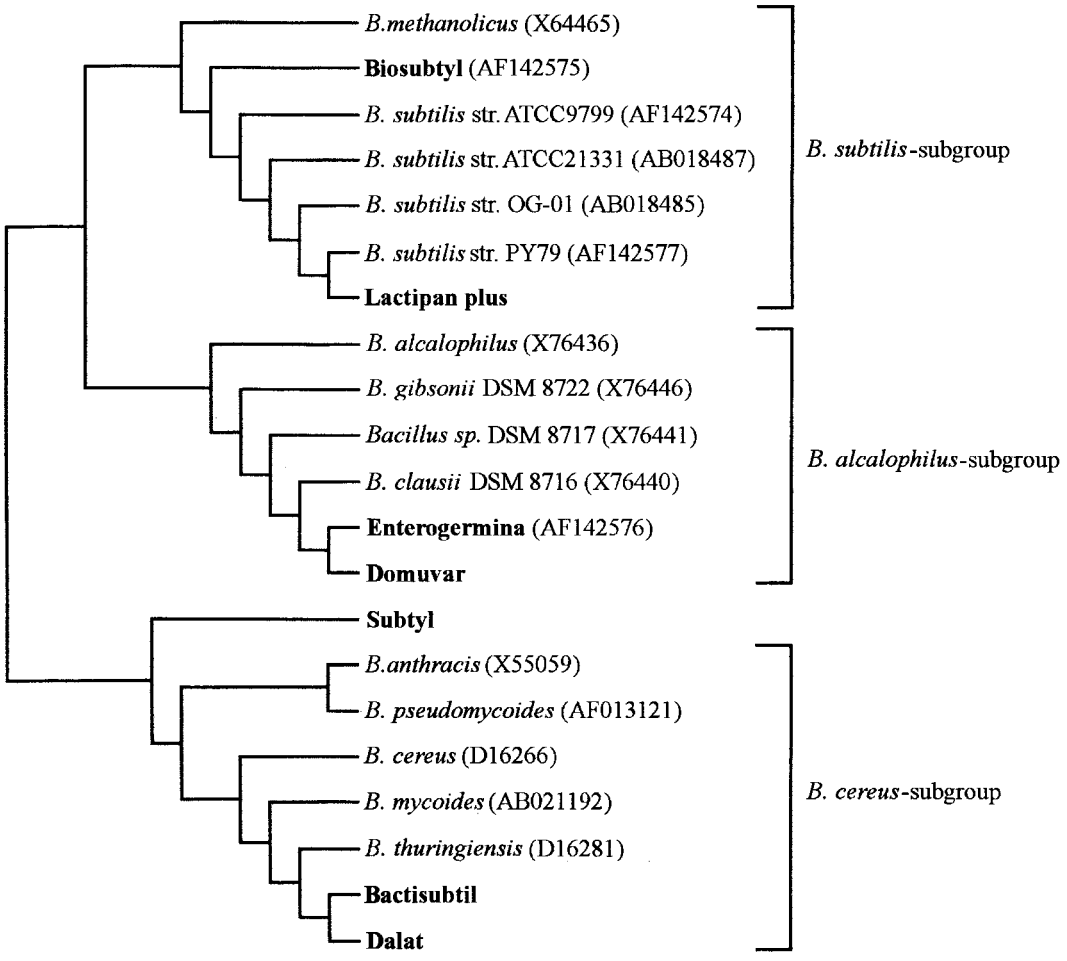

FIG. 4. Phylogenetic relationship of *Bacillus* probiotic bacteria. The relatedness of commercial *Bacillus* probiotics based on analysis of 16S rRNA is shown. Enterogermina and Biosubtyl (Biosubtyl "Nha Trang") are commercial *Bacillus* probiotics and have been characterized elsewhere (4).

correctly labeled as *B. cereus*. Interestingly this product, which is the same as that found in the livestock probiotic Paciflor, was originally labeled as *B. subtilis* (8). The Vietnamese probiotic Biosubtyl "Dalat," produced at the Pasteur Institute in Vietnam, was found to be virtually identical to Bactisubtil both at the genetic level and using the API test. Although the similarity of these strains suggests a common origin, there were some notable differences. First, Biosubtyl "Dalat" grew reasonably well under anerobic conditions, which is found in some *B. cereus* strains. A second difference was in the antibiotic resistance profile. Biosubtyl "Dalat" could grow at high concentrations of ampicillin and penicillin G. In contrast, Bactisubtil was resistant to both chloramphenicol and tetracycline. *B. cereus* strains are known to contain a chromosomal β-lactamase (13), although we are unable to comment on whether any drug resistance is plasmid encoded.

The last probiotic we examined was Subtyl. 16S rRNA analysis of this strain did not show any significant homology with any known *Bacillus* species, and in fact, it was so divergent that it should be considered a new species. It was related to *B. cereus* based on biochemical tests, although at the genetic and biochemical levels this species was the most diverged from existing *Bacillus* species. We believe it should be considered a new species, and we propose the name *Bacillus vietnami*. Another feature of note with this strain was the high level of resistance to penicillin and ampicillin. It is curious that both Vietnamese probiotics should exhibit such high levels of resistance to these antibiotics, and it is worrying that in southeast Asia, where the overuse of antibiotics is widespread, ampicillin and penicillin-resistant bacteria are being consumed on a daily basis.

In conclusion, although our original goal of characterizing spore probiotics has been achieved, we have also revealed the poor state of species classification being applied in the licensing of products for human consumption (Table 5). In a previous study we found two other probiotics to be mislabeled (4), and, as has been stressed previously, this should be considered a public health issue (5, 6). The fact that some of these species have high levels of drug resistance is of particular concern. It is not clear to us how spores may exert a beneficial effect for oral bacteriotherapy. Only one strain, Biosubtyl "Dalat," was found to support anerobic growth, so in principle spores of this strain could germinate and colonize the intestine as has been suggested previously (8). However, the fact that the other strains were aerobic suggests another mechanism for their action.

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