Identification and Quantification of *Bifidobacterium* Species Isolated from Food with Genus-Specific 16S rRNA-Targeted Probes by Colony Hybridization and PCR

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A *Bifidobacterium* genus-specific target sequence in the V9 variable region of the 16S rRNA has been elaborated and was used to develop a hybridization probe. The specificity of this probe, named lm3 (5′-CGG GTGCTI*CCCACTTTCATG-3′), was used to identify all known type strains and distinguish them from other bacteria. All of the 30 type strains of *Bifidobacterium* which are available at the German culture collection Deutsche Sammlung von Mikroorganismen und Zellkulturen, 6 commercially available production strains, and 34 closely related relevant strains (as negative controls) were tested. All tested bifidobacteria showed distinct positive signals by colony hybridization, whereas all negative controls showed no distinct dots except *Gardnerella vaginalis* DSM4944 and *Propionibacterium freudenreichii* subsp. *shermanii* DSM4902, which gave slight signals. Furthermore, we established a method for isolation and identification of bifidobacteria from food by using a PCR assay without prior isolation of DNA but breaking the cells with proteinase K. By this method, all *Bifidobacterium* strains lead to a DNA product of the expected size. We also established a quick assay to quantitatively measure *Bifidobacterium* counts in food and feces by dilution plating and colony hybridization. We were able to demonstrate that 2.1×10^6 to 2.3×10^7 colonies/g of sour milk containing bifidobacteria hybridized with the specific nucleotide probe. With these two methods, genus-specific colony hybridization and genus-specific PCR, it is now possible to readily and accurately detect any bifidobacteria in food and fecal samples and to discriminate between them and members of other genera.

Bifidobacteria are intestinal bacteria which play a major role in the most complex and diverse ecosystem of the intestinal tract of humans as well as of other warm-blooded animals and honeybees (5). Their role is still under investigation, and numerous studies have been done to investigate possible probiotic effects (17, 20, 25). In the food industry, bifidobacteria are widely used as food additives (6, 16, 34). However, it has been most difficult to monitor bifidobacterial counts in yogurts, cheese, butter, and other supposedly bifidobacterium-containing products since a truly selective medium which can discriminate between the genus *Bifidobacterium* and other genera does not exist (2, 22). Discrimination of this genus by classical physiological and biochemical methods is often time-consuming and takes a lot of effort and experience (14).

Bifidobacteria are phylogenetically grouped in the actinomycete branch of gram-positive bacteria (36, 37), within the high-G+C cluster (31), and are at present grouped into 32 species (11 species in humans, 15 species in warm-blooded animals, 3 species in honeybees, 2 species isolated from wastewater, and 1 species isolated from fermented milk) whose type strains are available at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) culture collection. In recent years, the attention of many taxonomically interested scientists has been drawn towards specific DNA probes (1, 3, 28) and rRNAs, whose genes contain highly conserved regions and have the advantage of being found in very large numbers within one single cell, exceeding the number of chromosomally encoded genes 10,000 fold (40), and thus being prime targets for an oligonucleotide probe. Many studies concerning 5S, 16S,

and 23S rRNA (26, 27, 38), as well as 16S to 23S rRNA internal transcribed spacer regions (24), have been done. Computational analysis of the specificity of 16S rRNA-targeted oligonucleotides and probe hybridization have become worthy tools for the elucidation of various taxonomic features such as genera, species, and strains, depending on the specificity of the probe (8, 15). Various studies of bifidobacteria have shown 16S rRNA-targeted probes which can detect different bifidobacterial species (13, 40) or the genus *Bifidobacterium* (21). However, none of these studies has compared and tested all available type strains, industrially used strains, and closely related species for the specificity of the probes.

PCR has been a promising method for detection of various bacterial species isolated from feces and food (9, 33). A recent study by Wang and coworkers (39) used a PCR-based method for detection and quantification of anaerobic bacteria in human and animal fecal samples. Herein, we describe a method for the use of direct PCR for genus-specific detection of bifidobacteria isolated from food without isolation of DNA.

The aim of this study is to develop a rapid and easy-to-handle identification system for the members of the genus *Bifidobacterium* isolated from food and feces. Therefore, we designed an oligonucleotide probe which is specific for the genus *Bifidobacterium*. In empirical studies we tested successfully this probe in hybridizations and developed a PCR-related technique for application to samples from food and feces.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table 1. *Bifidobacterium* strains were grown anaerobically at 37°C either in nonselective Bif medium (brain heart infusion [37 g/liter; Biolife] supplemented with yeast extract [5 g/liter] L-cysteine-hydrochloride [0.5 g/liter], and resazurine (2 mg/liter]) or in semiselective LP medium (22), also containing cysteine hydrochloride (0.5 g/liter) and nalidixic acid (30 mg/liter). Preparation of media and growth experiments involving *Bifidobacterium* strains on agar plates

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TABLE 1. Bacterial strains and their origins and reactions with specific probes as tested by colony hybridization and PCR

Number and strain	Origin[a]	Colony hybridization with[b]:		PCR lm26, lm3[c]
		lm3	pLME21	
Bifidobacterium type strains				
1. *B. adolescentis*	DSM 20083[T]	+	+	+
2. *B. angulatum*	DSM 20098[T]	+	+	+
3. *B. animalis*	DSM 20104[T]	+	+	+
4. *B. asteroides*	DSM 20089[T]	+	+	+
5. *B. bifidum*	DSM 20456[T]	+	+	+
6. *B. boum*	DSM 20432[T]	+	+	+
7. *B. breve*	DSM 20213[T]	+	+	+
8. *B. catenulatum*	DSM 20103[T]	+	+	+
9. *B. choerinum*	DSM 20434[T]	+	+	+
10. *B. coryneforme*	DSM 20216[T]	+	+	+
11. *B. cuniculi*	DSM 20435[T]	+	+	+
12. *B. dentium*	DSM 20436[T]	+	+	+
13. *B. gallicum*	DSM 20093[T]	+	+	+
14. *B. gallinarum*	DSM 20670[T]	+	+	+
15. *B. globosum*	DSM 20092[T]	+	+	+
16. *B. indicum*	DSM 20214[T]	+	+	+
17. *B. infantis*	DSM 20088[T]	+	+	+
18. *B. lactis*	DSM 10140[T]	+	+	+
19. *B. longum*	DSM 20219[T]	+	+	+
20. *B. magnum*	DSM 20222[T]	+	+	+
21. *B. merycicum*	DSM 6492[T]	+	+	+
22. *B. minimum*	DSM 20102[T]	+	+	+
23. *B. pseudocatenulatum*	DSM 20438[T]	+	+	+
24. *B. pseudolongum*	DSM 20099[T]	+	+	+
25. *B. pullorum*	DSM 20433[T]	+	+	+
26. *B. ruminatium*	DSM 6489[T]	+	+	+
27. *B. saeculare*	DSM 6531[T]	+	+	+
28. *B. subtile*	DSM 20096[T]	+	+	+
29. *B. suis*	DSM 20211[T]	+	+	+
30. *B. thermophilum*	DSM 20210[T]	+	+	+
Bifidobacterium production strains				
31. *B. longum* 2	Wiesby	+	+	+
32. *B. longum* 913	Wiesby	+	+	+
33. *B. thermophilum*	UFAG Lab	+	+	+
34. *B. animalis*	UFAG Lab	+	+	+
35. *B. pseudolongum*	UFAG Lab	+	+	+
36. *B. infantis*	Wiesby	+	+	+
Negative controls				
37. *Arthrobacter globiformis*	DSM 20124	−	+	ND
38. *Aureobacterium liquefaciens*	DSM 20638	−	+	ND
39. *Brevibacterium casei*	DSM 20657	−	+	ND
40. *Brevibacterium linens*	DSM 20425	−	+	ND
41. *Cellulomonas uda*	FM	−	+	ND
42. *Enterococcus faecalis* FO1	FM	−	+	−
43. *Enterococcus faecium* FJ2	FM	−	+	−
44. *Enterococcus* FO5	FM	−	+	−
45. *Enterococcus* RE9	FM	−	+	−
46. *Enterococcus* sp.	FM	−	+	−
47. *Escherichia coli* AC1	FM	−	+	−
48. *Escherichia coli* HB101	DSM	−	+	−
49. *Gardnerella vaginalis*	DSM 4944	+/−	+	−
50. *Lactobacillus acidophilus*	FM	−	+	−
51. *Lactobacillus casei*	FM	−	+	−
52. *Lactobacillus* GD1	FM	−	+	−
53. *Lactobacillus lactis*	FM	−	+	−
54. *Lactobacillus plantarum*	FM	−	+	−
55. *Lactobacillus rhamnosus*	FM	−	+	−
56. *Lactococcus* Bu2-60B	FM	−	+	−
57. *Microbacterium arborescens*	DSM 20754	−	+	ND
58. *Microbacterium lacticum*	DSM 20427	−	+	ND
59. *Micrococcus lylae*	DSM 20315	−	+	ND

Continued

TABLE 1—*Continued*

Number and strain	Origin[a]	Colony hybridization with[b]:		PCR lm26, lm3[c]
		lm3	pLME21	
60. *Micrococcus freudenreichii*	FAM1606b	−	+	ND
61. *Micrococcus kristinae*	DSM 20032	−	+	ND
62. *Micrococcus varians*	DSM 20033	−	+	ND
63. *P. freudenreichii* subsp. *shermanii*	DSM 4902	+/−	+	−
64. *P. freudenreichii*	DSM 20271	−	+	−
65. *Propionibacterium acidipropionici*	DSM 4900	−	+	−
66. *Propionibacterium jensenii*	DSM 20535	−	+	−
67. *Propionibacterium* sp.	FM	−	+	−
68. *Propionibacterium thoenii*	DSM 20276	−	+	−
69. *Rhodococcus chlorophenolicus*	FM	−	+	ND
70. *Streptococcus thermophilus* S3	FM	−	+	ND

[a] FM, Food Microbiology collection; UFAG Lab, UFAG Laboratories, Sursee, Switzerland; FAM, Eidgenössische Forschungsanstalt für Milchwirtschaft, Liebefeld-Bern, Switzerland.
[b] lm3, Dig-labeled *Bifidobacterium*-specific oligonucleotide; pLME21 (29), Dig-labeled 16S *rrna* gene from *B. lactis* cloned in pUC18 (35) which detects DNA from all bacteria. +, distinct signal; +/−, faint signal; −, no signal.
[c] lm26 and lm3, PCR primers, specific for the genus *Bifidobacterium*. See footnote b for symbols. ND, not done.

were carried out in an anaerobic chamber (COY Laboratories Products Inc., Ann Arbor, Mich.) with a gas atmosphere of 94% nitrogen and 6% hydrogen. Agar plates were incubated in anaerobic jars which were supplemented with a pad of Anaerocult C (Merck) that generates an oxygen-depleted and CO_2-enriched atmosphere. The incubation period was between 10 h and 3 days, depending on the strain. A stock of strains was kept at −72°C in Bif medium containing 43% glycerol. For colony hybridization experiments, frozen cultures were thawed on ice, inoculated in 10 ml of either Bif or LP medium, and grown until they reached the end of the logarithmic growth phase at an optical density at 600 nm of approximately 0.9.

Isolation of bifidobacteria from food. Dairy products supposedly containing bifidobacteria were purchased in drugstores mainly in Switzerland but also in Germany and France. Products consisted of yogurt, soft cheese, butter, and Bifidus-drink, a milk drink supplemented with bifidobacteria. Food samples (10 g each) were aerobically taken and dissolved in 90 ml of reduced (cysteine-containing) Bif medium, mixed until a homogenous suspension was obtained, and quickly brought into the anaerobic chamber. Serial dilutions were then performed and streaked out onto our modified LP agar. After anaerobic incubation as described above, agar plates were taken out of the anaerobic chamber and colony hybridization was performed as described below.

Colony hybridization with digoxigenin (Dig)-labeled probe. Colonies were transferred onto a nylon membrane (Hybond-N nylon membrane; Amersham Life Science) either by harvesting 500 μl of an end-log-phase culture (see above) and pipetting 10 μl of the resuspended pellet (in Bif medium) onto the membrane or by direct plaque lifts as described by the supplier (Amersham Life Science). As next steps, DNA had to be liberated, neutralized, washed, and fixed to the membrane. This was achieved by a series of solution-saturated 3MM paper filters (Whatman). First, lysis of the cells was performed on saturated 3MM paper (10 mM Tris-HCl, 250 mM sucrose, 5 mg of lysozyme per ml [pH 7.5]) by incubating the membrane for 60 min at 37°C. After this, each step was performed for 4 min on saturated 3MM paper with the following solutions: denaturation solution (1 M NaCl), neutralization solution (1 M Tris-HCl [pH 8]), and washing solution (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). Nylon membranes were dried at 80°C for 10 min, and DNA was fixed to the membrane by UV cross-linking (4 min at 302 nm).

Prehybridization and hybridization with a Dig-labeled probe were performed at 57°C as described by the manufacturer (Boehringer GmbH, Mannheim, Germany). The oligonucleotide probe lm3 was industrially 3′-end labeled by MWG-Biotech. Detection of the Dig-labeled DNA-DNA or DNA-RNA hybrids was performed with a Dig nucleic acid detection kit (Boehringer) as described by the manufacturer. The dots on the membranes were evaluated by scanning the membranes with a video copy processor (Mitsubishi) and analyzing these data with the Wincam densitometry package (Cybertech).

Colony hybridization with [α-^{32}P]ATP-labeled probe. Nylon membranes were prepared as described above, and after hybridization with the bifidobacterium-specific probe they were stripped as described by the manufacturer (Boehringer). DNA probes (approximately 0.5 μg) for radioactive hybridization were labeled with 40 to 50 μCi of [α-^{32}P]ATP (3×10^3 Ci/mmol) by using the random priming technique of Feinberg and Vogelstein (12). Prehybridization and hybridization with [α-^{32}P]ATP-labeled probe were done at 65°C in a Micro-4 hybridization

TABLE 2. Sequences of PCR primers and probes used in colony hybridization

Probe	Sequence or description	Direction	Target site[a]	Source or reference
lm26	5′-GATTCTGGCTCAGGATGAACG-3′	Forward	15–35	This study
lm3	5′-CGGGTGCTI[c]CCCACTTTCATG-3′	Reverse	1412–1432	This study
pLME21[b]	16S *rrna* gene of *Bifidobacterium lactis*			29
Bak11w	5′-AGTTTGATCMTGGCTCAG-3′	Forward	10–27	This study
Eub338	5′-ACTCCTACGGGAGGCAGC-3′	Reverse	321–338	1

[a] Corresponds to *E. coli* numbering of 16S rRNA gene (7).
[b] See Table 1, footnote *a*.
[c] Inosine, matches all four nucleotides (A, C, G, and T).

oven (Hybaid). The membrane filter was then washed and subjected to autoradiography by the method of Sambrook et al. (35).

Quantification of viable bifidobacteria. Viable bifidobacteria were enumerated after dilution plating, incubation, and hybridization of colony lifts by counting the number of positive signals on the membrane.

Selection of target sequences for colony hybridization. Total 16S rRNA sequences of 20 *Bifidobacterium* species and 23 partial 16S rRNA sequences were retrieved from the EMBL/GenBank data libraries (4, 30, 32). Multiple alignments with all the *Bifidobacterium* strains were performed by using the GCG sequence analysis software package (University of Wisconsin, Madison). Based on these findings, two target regions were chosen for PCR primers and *Bifidobacterium* species-specific oligonucleotide probes (Table 2). The oligonucleotide lm3 showed one or no mismatches with all available 16S rDNA sequences from *Bifidobacterium* species, which is a prerequisite for a genus-specific probe. Mismatches were due to unknown bases in the sequence.

These two probes were compared to all available bacterial 16S rDNA sequences (33,231 sequences on 3 December 1996) in the two gene banks stated above.

PCR. Fast DNA extraction from bifidobacteria was performed by a modified procedure described earlier (18), using proteinase K and sodium dodecyl sulfate (SDS). The procedure was as follows. Bif medium (10 ml) was inoculated with a single colony and grown under anaerobic conditions to 10^8 CFU/ml. Then, 0.5 ml was harvested and diluted in 0.2 ml of digestion buffer (50 mM Tris-HCl [pH 8], 1 mM EDTA, 0.5% SDS, 1 mg of proteinase K [Sigma] per ml). After incubation at 55°C for 3 h, proteinase K was inactivated at 95°C for 10 min and cell debris was removed by centrifugation. Then, 10 μl of the supernatant was applied for PCR, along with 2% Tween 20 (final concentration) and Dynazyme II F-501L polymerase (Finnzymes Oy). A 50-μl amplification mix contained the following chemicals: a sample of the above test solution (10 μl), optimized Dynazyme buffer (5 μl) (Finnzymes Oy), polymerase (2 U), deoxynucleoside triphosphates (300 μM), and primer pairs (1 μM each). PCR parameters in the Perkin-Elmer thermal cycler were the following: denaturation: 94°C, 1 min; annealing: 57°C, 3 min; and elongation: 72°C, 4 min. After 35 cycles the reaction mixture was cooled down to 4°C or frozen at −20°C until further use. DNA was fractionated by horizontal electrophoresis on 0.8% agarose gels and stained with ethidium bromide.

RESULTS

Genus specificity of selected target region. The proposed target region in the 16S *rrna* gene for *Bifidobacterium* genus-specific probe lm3 (Table 2) was aligned with all bacterial sequences obtained from EMBL/GenBank. This alignment revealed that the 16S *rrna* genes of all the *Bifidobacterium* species

showed no mismatch with the lm3 sequence (Table 3). The position of inosine (I in lm3) is covered by a cytosine (C) in the 16S rRNA sequences of 22 subspecies belonging to 12 species, whereas a thymine (T) takes this place among 8 subspecies belonging to 6 species. However, the 16S ribosomal DNA (rDNA) sequence of *Bifidobacterium angulatum* showed an ambiguity at the first position of the target sequence. *B. dentium* and *Gardnerella vaginalis* had an ambiguity at the inosine site of the primer. All other bacteria including lactic acid bacteria had two or more mismatches at the position of the target site.

Colony hybridizations with *Bifidobacterium*-specific probe. After colony hybridization and colorimetric detection with the Dig-nucleic acid detection kit, typical images, as shown in Fig. 1 and 3, were obtained by a video camera. All of the *Bifidobacterium* type strains and all of the *Bifidobacterium* production strains listed in Table 1 showed distinct dots after hybridization with the genus-specific probe lm3, whereas none of the control strains hybridized with the specific probe lm3 (Table 1 and Fig. 1A), and the bacteria showed dots with the universal probe pLME21 (Fig. 1B). The genus specificity of probe lm3 was further investigated for bacterial species which are closely related to *Bifidobacterium* (Table 2). In colony hybridizations no distinct dots can be seen except at the positions of *G. vaginalis* and *Propionibacterium freudenreichii* subsp. *shermanii* on the membrane (data not shown), which showed weak signals. In order to prove that there was actually DNA at the dots of the negative controls, the universal probe pLME21 (Table 2), which detects all DNA, was used as hybridization probe. The results of these hybridization experiments are presented in Table 1. The positive results indicate that rDNA or rRNA, respectively, was present in all *Bifidobacterium* species as well as in all other tested strains.

PCR of bifidobacterial 16S rDNA. Another method to detect and identify bifidobacteria was PCR with lm26 and lm3 as primers (Table 2). The target DNA for these primers was obtained by a simple lysis procedure (see Materials and Meth-

TABLE 3. Sequence alignment of *Bifidobacterium* species in the position of probe lm3

Organism[b]	Alignment relative to target sequence[a]: 5′-CATGAAAGTGGGI[c]AGCACCCG-3′
Bifidobacterium species	
22 subspecies of 12 different species (3, 4, 6, 8, 11, 16, 17, 20, 21, 24, 29, 30)	5′-•••••••••••C•••••••-3′
8 subspecies of 6 different species (1, 9, 10, 13, 15, 23)	5′-•••••••••••T•••••••-3′
Bifidobacterium dentium	5′-•••••••••••N[d]•••••••-3′
Bifidobacterium angulatum	5′-N•••••••••••T•••••••-3′
Gardnerella vaginalis	5′-•••••••••••N•••••••-3′
All other bacteria	Two or more mismatches

[a] According to the GenBank/EMBL data bank as of 6 June 1996.
[b] Numbers in parentheses correspond to *Bifidobacterium* species listed in Table 1.
[c] I, inosine, matches all four nucleotides.
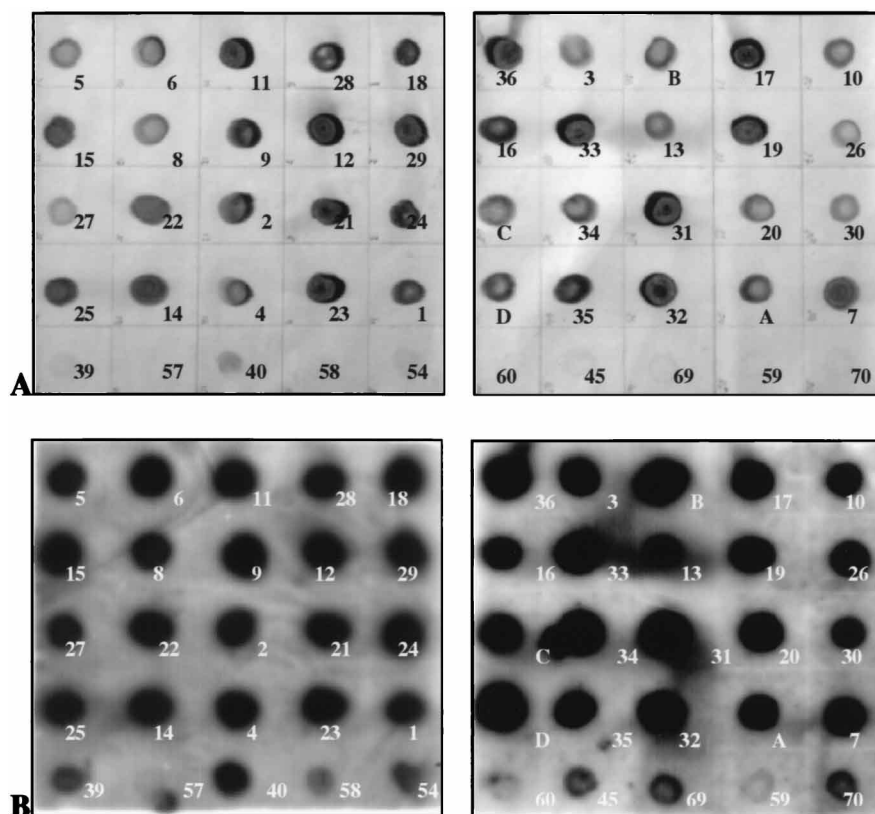[d] N, nondetermined nucleotide.

FIG. 1. Colony hybridization of *Bifidobacterium* type strains with *Bifidobacterium* genus-specific Dig-labeled probe lm3 (A) and with [α-^{32}P]ATP-labeled universal probe pLME21 (B). For each panel: first four lanes, *Bifidobacterium* type strains; last lane, negative controls. Numbers correspond to species listed in Table 1. Panels A to D contain *Bifidobacterium* species isolated from food.

ods). An example of this PCR approach is shown in Fig. 2. In addition, representatives of all *Bifidobacterium* species produced a distinct band of 1.35 kb, which represents the size of the 16S rDNA fragment amplified by the PCR, whereas bacteria other than bifidobacteria gave no band at all (data not shown). In order to prove the lysis of the negative controls, a second PCR approach was performed using, in addition to the above-mentioned primers lm3 and lm26, another primer pair, Eub338 and Bak11w (Table 2), which are universal primers and amplify a fragment of 0.3 kb. In this assay, *Bifidobacterium*
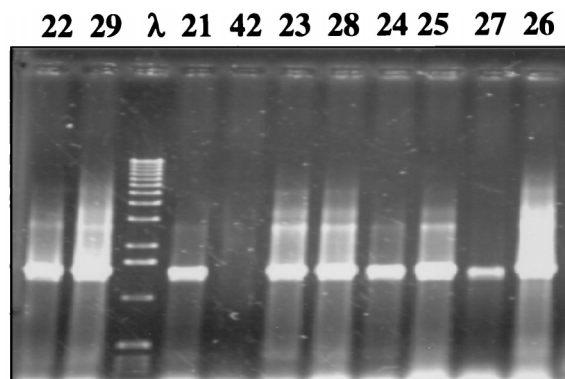
species produced two bands (1.35 and 0.3 kb) whereas negative strains gave only one fragment of ca. 0.3 kb (data not shown).

Quantification of viable bifidobacteria isolated from food. After dilution plating of different food samples (cheeses, yogurts, butter, and probiotic drinks containing bifidobacteria), colony lift, and hybridization with lm3, distinct dots were observed on the membrane, representing colonies of bifidobacteria (Fig. 3). These colonies could be counted manually, and CFU per gram of food sample or feces were calculated. By analyzing eight different sour milk products we were able to find 2.1×10^6 to 2.3×10^7 CFU of bifidobacteria per g which hybridized with the specific nucleotide probe. Yogurt without bifidobacteria showed no signals after hybridization (data not shown). By analyzing human feces, we detected 10^8 CFU of bifidobacteria per g (data not shown). These findings demonstrated the suitability of this method.

DISCUSSION

A useful tool for taxonomic and phylogenetic relationships is the comparison of the 16S rRNA, especially from the level of domains to genera (13, 38). This method is less suitable for differentiation between different species; DNA-DNA hybridization is more precise and reliable. The genus *Bifidobacterium* seems to be a closely related phylogenetic cluster, considering the 16S rRNA similarities of >93% (24). An EMBL/GenBank search revealed 30 partial or total 16S rRNA sequences from bifidobacteria. We aligned these sequences and selected a stretch of 21 nucleotides (Table 3) which was highly conserved within the genus *Bifidobacterium* and which showed only one
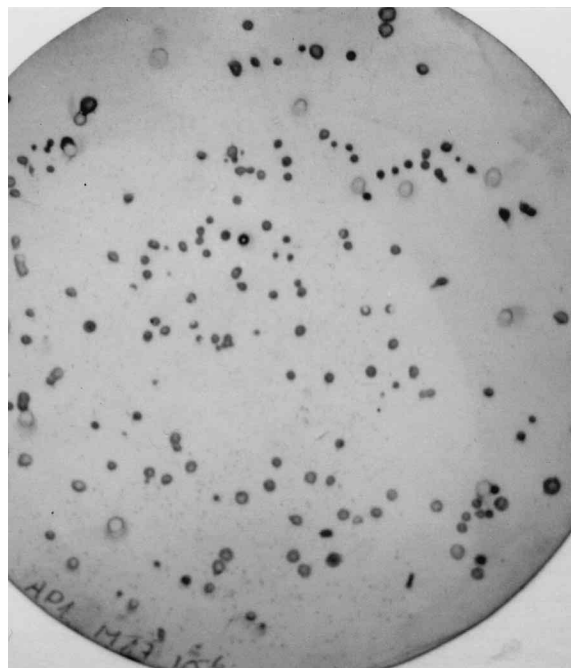
22 29 λ 21 42 23 28 24 25 27 26

FIG. 2. Agarose gel electrophoresis of PCR products with PCR primers lm26/lm3. Numbers correspond to the strains listed in Table 1. Lane λ, size markers generated by *Hin*dIII-digested λ DNA.

FIG. 3. Colony hybridization of bifidobacteria after dilution plating (10^{-5}) from sour milk to a nylon membrane. The probe was Dig-labeled lm3.

mismatch at position 1224 (*Escherichia coli* numbering) containing either C or T. *G. vaginalis* was the sole representative among all bacteria which had a homologous sequence with an undetermined nucleotide (N) at this position. Since the taxonomic positioning of this strain, which represents the only species of the genus *Gardnerella*, is still in discussion, *Gardnerella* might be regarded as a bifidobacterium, as it was assumed by Embley and Stackebrandt (11). In addition, other workers stated that *Gardnerella* and *Bifidobacterium* exhibit close phylogenetic proximity (23) and that 16S rRNA similarity is consistent with placing *Gardnerella* and *Bifidobacterium* in the same genus (24, 29). However, a separate genus status of *Gardnerella* was established by comparing its low G+C content (42 to 44 mol% for *Gardnerella* versus 55 to 67 mol% for bifidobacteria) (5) and on the basis of DNA-DNA hybridization experiments (19). Since DNA-DNA hybridization experiments are not suitable for the distinction of genera (38), the question regarding the genus still remains to be answered. All other strains in the EMBL/GenBank, however, had two or more mismatches within this sequence.

In order to approve the theoretically found specificity of the selected probe lm3 for *Bifidobacterium*, it had to be shown that this approach also functioned in empirical tests. Table 1 and Fig. 1 show colony hybridization experiments with all bifidobacterium type strains available from the DSM culture collection except both recently published new species *B. inopinatum* and *B. denticolens* (10). All of the *Bifidobacterium* type strains showed distinct signals in colony hybridization experiments with the *Bifidobacterium*-specific probe lm3. Negative controls, including closely related bacteria and bacteria which are important in the dairy industry (Table 1 and Fig. 1), showed no signal after detection with primer lm3 and demonstrated the requested specificity. Weak signals could be seen for *G. vaginalis*, consistent with our theoretical findings of only one mismatch in the target sequence (Table 3), and for *P. freudenreichii* subsp. *shermanii*, whose 16S rRNA is now known

(10a). A weak signal could be detected with *Brevibacterium linens*, but on the original membrane it was distinguished from the other dots by the reddish color which originates from the pigment produced by this strain. Colony hybridization experiments with [α-^{32}P]ATP-labeled probe showed more distinct signals than with chemically labeled probe. This finding implies that radioactivity is still useful for this kind of difficult experiment despite the problem with its waste.

Another highly efficient way of detecting bifidobacteria is the PCR technique. Using the primers lm26 and lm3 (Table 2), we were able to amplify a segment of the 16S rRNA of the expected size of 1.35 kb in bifidobacteria but in no other bacteria tested (Table 1 and Fig. 2). Thus, it is possible to rapidly and accurately distinguish bifidobacteria by means of PCR.

Isolation of bifidobacteria from food out of a complex bacterial flora was possible by plating serial dilution steps in semiselective agar medium and incubating these agar plates in an anaerobe chamber. Colony counts of bifidobacteria were performed after plaque lift and colony hybridization with the *Bifidobacterium*-specific probe lm3 (Fig. 3). We were able to find 2.1×10^{6} to 2.3×10^{7} CFU per g in commercially available dairy products.

We developed a rapid identification and quantification system for viable bifidobacteria isolated from food or feces with either a genus-specific oligonucleotide probe or a PCR-based method. Therefore, our system is a worthy tool for monitoring bifidobacterial counts in the industry as well as in governmental laboratories.

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