Journal of Clinical Microbiology

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J. Clin. Microbiol. **2005, 43(8):3925. DOI: 10.1128/JCM.43.8.3925-3931.2005.**

JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2005, p. 3925–3931 Vol. 43, No. 8
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.8.3925–3931.2005

Bifidobacterial Lipoglycan as a New Cause for False-Positive Platelia *Aspergillus* Enzyme-Linked Immunosorbent Assay Reactivity

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Received 11 November 2004/Returned for modification 24 January 2005/Accepted 19 April 2005

We previously hypothesized that a lipoglycan of *Bifidobacterium bifidum* subsp. *pennsylvanicum* cross-reacts with the Platelia *Aspergillus* (PA) enzyme-linked immunosorbent assay (ELISA) based on the presence of galactofuranosyl epitopes in the cell wall (M. A. S. H. Mennink-Kersten, R. R. Klont, A. Warris, H. J. M. Op den Camp, and P. E. Verweij, Lancet 363:325–327, 2004). We tested this hypothesis by testing bacterial suspensions of different bifidobacterial species and other gram-positive and -negative bacteria with the PA ELISA, which is used to detect circulating galactomannan for the serodiagnosis of invasive aspergillosis. Furthermore, neonatal fecal samples were enumerated for bifidobacteria by fluorescence in situ hybridization (FISH) and tested for PA ELISA reactivity. All bifidobacteria, except *B. infantis* and *B. adolescentis*, showed reactivity 6- to 600-fold higher compared to the controls (i.e., *Micrococcus luteus* and *Propionibacterium freudenreichii*, which contain a cell wall lipomannan). *Eggerthella lenta* showed a 25-fold-higher reactivity. ELISA reactivity was clearly shown to be associated with bacterial lipoglycans containing a β-1,5-galactofuranosyl chain. All neonatal feces showed PA ELISA reactivity and associated numbers of bifidobacteria. Since high concentrations of bifidobacteria are present in the human gut, these bacteria or excreted lipoglycan may cause false serum PA ELISA reactivity in selected patient groups, especially neonates.

Invasive aspergillosis (IA) has become a leading cause of death among immunocompromised patients. A commercial sandwich enzyme-linked immunosorbent assay (ELISA) (Platelia *Aspergillus* [PA] ELISA; Bio-Rad, Marnes-la-Coquette, France) that is widely used as a diagnostic tool in centers throughout the world detects a fungal antigen which is present in body fluids of patients with IA (5, 24, 44). The PA ELISA specifically detects circulating galactomannan (GM), a cell wall polysaccharide secreted by *Aspergillus*, by using the immunoglobulin M (IgM) monoclonal antibody EB-A2 as captor and detector (44). This EB-A2 binds to the β-1,5-linked galactofuranosyl (Gal*f*) chain of the GM molecule (45) and possibly to other fungal Gal*f*-containing antigens that circulate in sera of patients infected with aspergillus (22, 27).

A major difficulty in the serodiagnosis of IA is the occurrence of false-positive PA ELISA results, which varies from 5% in adults to as much as 83% in neonates in consecutive serum samples (38, 42, 46). At present no valid explanation has been found although several theories have been postulated. Cross-reactivity of the IgM monoclonal antibody with other molecules present in serum might occur although this could not be documented with blood products and antigens from bacteria that cause bacteremia (47). Cross-reactivity has been reported in vitro with the GM from *Penicillium* or *Paecilomyces* spp., but these molds very rarely cause invasive infection in humans (47). Besides resulting from IA, circulating *Aspergillus* GM could result from massive colonization of the gastrointestinal tract by *Aspergillus* (39) or from foods that are contaminated with *Aspergillus* (4) or contain its GM (1, 10). This fecal GM may reach the circulation in patients with dysfunction of the intestinal mucosal barrier. Furthermore, intravenous administration of the antibiotics piperacillin-tazobactam and amoxicillin-clavulanic acid, which show in vitro PA reactivity, has been shown to cause serum PA ELISA reactivity in patients without evidence of IA (26, 52). This PA reactivity is probably due to GM that originates from *Penicillium* that is used for antibiotic production (51).

After a review of the literature for microorganisms that contain galactofuranoses, it was found that a membrane-associated molecule of *Bifidobacterium bifidum* subsp. *pennsylvanicum* contained a terminal linear polysaccharide of more than seven β-1,5-linked Gal*f* residues which mimics the epitope recognized by EB-A2 (7). It was suggested that lipoteichoic acid (LTA)-like amphiphiles of bifidobacterial species might cause PA ELISA reactivity in neonates after translocation because of the immaturity of the intestinal mucosa (28). In addition, *Bifidobacterium* species are members of the normal gastrointestinal microflora of humans, comprising up to 6% of the total fecal microflora of adults and up to 91% and 75% of the total fecal microflora in breast-fed and formula-fed infants, respectively (15, 37).

LTAs represent a group of structurally related lipid macroamphiphiles which are hydrophobically anchored to the cytoplasmic membrane, while the hydrophilic chain (mostly polyglycerophosphate) penetrates the peptidoglycan network and may be detectable as a surface antigen (Fig. 1) (54). In the

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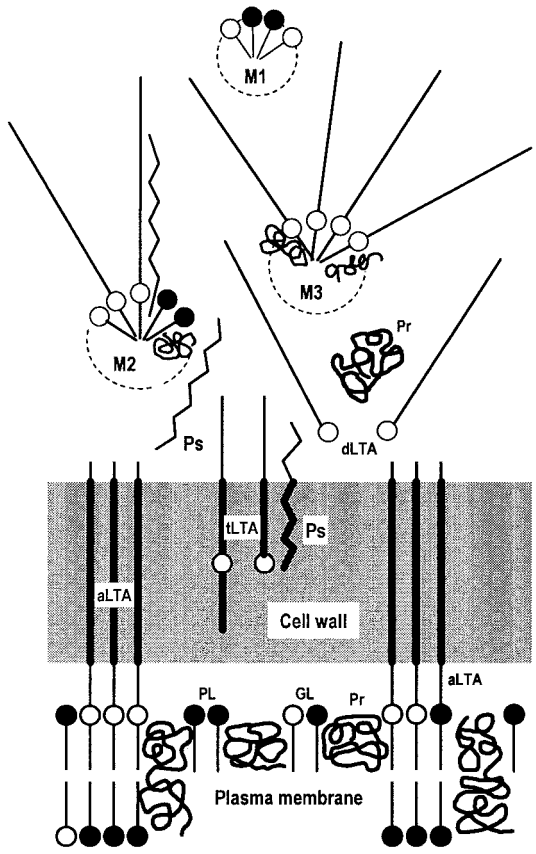

FIG. 1. Diagrammatic representation of a generalized gram-positive cell wall-plasma membrane complex. Pr, protein; PL, phospholipids; GL, glycolipid; Ps, polysaccharide; aLTA, acetylated lipoteichoic acid; dLTA, deacetylated lipoteichoic acid; tLTA, lipoteichoic acid molecules in the process of excretion. M1, M2, and M3 are various micellar complexes present in the external milieu. (Adapted from reference 54 with permission of the publisher.)

high-G+C subdivision of gram-positive bacteria, LTAs are functionally replaced by lipoglycans, which contain a linear or branched polysaccharide as a hydrophilic moiety; this polysaccharide may carry monoglycerophosphate side chains (*Bifidobacterium* species) or succinyl esters (lipomannan from *Micrococcus*, *Mycobacterium*, or *Propionibacterium*) (9). The macroamphiphiles are secreted even during normal growth both with and without their lipid anchor. Secretion is stimulated by exposure to β-lactam antibiotics (9, 12, 33, 35). In aqueous solutions, the acylated molecules form micellar aggregates (53).

The aim of our research was to test the in vitro reactivity of different bacteria including bifidobacterial species and other members of the gut microflora of humans with the PA ELISA. Any reactivity was correlated with the presence and structure of bacterial lipid macroamphiphiles based on review of the literature.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Reactivity with the PA ELISA was investigated in vitro by testing cell suspensions of a range of bacteria, including 11 bifidobacterial species and 15 other bacteria (gram-positive and -negative species), most known to be common inhabitants from the human gut (Table 1). For each bacterium the structure of the lipid macroamphiphile was documented based on review of the literature (7–9, 13, 16, 18, 30, 48). Bacteria were cultured anaerobically (80% N_2, 10% CO_2, 10% H_2) on agar plates containing fastidious anaerobic agar (Lab M) or aerobically on Columbia agar (BBL), at 37°C. *B. bifidum* subsp. *pennsylvanicum* was also cultured on reinforced clostridial medium (RCM; Oxoid). Because this liquid medium showed ELISA reactivity, it was hydrolyzed with acid (pH 2 with HCl for 3 h at 100°C) and neutralized with NaOH at pH 6.8 before inoculation. Acid hydrolysis removes Gal*f* chains that are present in the RCM, which originate from contaminated medium components like meat extracts.

Fecal sample collection and FISH analysis. Fecal samples from nine healthy neonates were obtained. Babies were fully breast-fed or formula fed and ranged in age from 3 days to 4.5 months. No baby had been on antibiotic treatment prior to fecal sampling. Fecal samples were stored at −80°C until shipment on dry ice to Groningen for fluorescence in situ hybridization (FISH) analysis. Samples were processed and FISH analysis was performed as described by Harmsen et al. (14). The bifidobacterial probe Bif164 (21), a 16S rRNA-based oligonucleotide probe, was used to enumerate total bifidobacteria in the fecal samples.

PA ELISA. Bacterial cells were scraped of the agar plates and suspended in 0.9% NaCl (wt/vol). Fresh fecal samples were suspended in saline to give a final concentration of 100 mg/ml (wet weight). The complete mixtures were serial diluted and used for detection of reactivity by the PA ELISA. The PA ELISA was performed according to the manufacturer's instructions. However, the pretreatment step intended to dissociate immune complexes was omitted, except for the R3, R4, and R5 calibration samples (serum spiked with GM) and the fecal samples. Briefly, 50 μl of a reaction mixture containing horseradish peroxidase-conjugated anti-GM monoclonal antibody EB-A2 was added to each well of a microtitration plate coated with the same monoclonal antibody EB-A2, followed by addition of 50 μl of the bacterial cell suspension or pretreated sample. After 90 min of incubation at 37°C, the plates were washed five times with washing buffer before 200 μl of buffer containing tetramethylbenzidine solution was added. Then the plates were incubated for another 30 min in the dark at room temperature, followed by the addition of 100 μl of 1.5 N sulfuric acid to stop the reaction. The optical density (OD) was read at 450 and 620 nm. A test sample was considered positive when the OD at 450 nm was higher than the cutoff sample (i.e., 1.0 ng GM). Pretreatment of the R3, R4, and R5 calibration samples and the fecal samples was done by mixing 300 μl of each sample with 100 μl of treatment solution (4% EDTA), and the mixture was subsequently boiled for 5 min. After centrifugation (10,000 × *g*, 10 min), the supernatant was used for further testing.

All bacterial isolates were tested twice and in duplicate. In vitro reactivity was quantified as ELISA index (EI) correlated to the total protein content of the tested cell suspension. Total protein concentrations were determined with a protein assay kit (Bio-Rad, Richmond, CA), with bovine γ-globulin as a standard, after boiling the cells for 15 min in 1 M NaOH and neutralizing with 1 M HCl. The reactivity of *Micrococcus luteus* and *Propionibacterium freudenreichii*, which are known to have a lipomannan in the cell wall (9), served as negative controls. Furthermore, 1 ng/ml of purified GM (22) was added to *M. luteus* samples with different total protein contents. Fecal samples were tested twice, and in vitro reactivity was expressed as EI per gram of feces.

Cell count. Direct microscopic counts of tested bacterial cell samples were obtained by using duplicate smears of 0.01 ml of a 10^2-fold dilution spread over 1 cm^2 of a glass slide. The smears were heat fixed and gently Gram stained. Ten fields were counted, and the counts were then correlated with the actual sample size.

Preparation and deacylation of cell extracts and cell pellet samples. After growth of *B. bifidum* subsp. *pennsylvanicum* on liquid RCM, samples were taken for PA ELISA. Part of the culture (1-ml samples) was centrifuged at 8,000 × *g* (10 min, room temperature) followed by filtration of the supernatant (0.2 μm). The clear supernatant was used as a cell-free culture fluid. The cell pellet was suspended in 200 μl 0.9% NaCl. The supernatant and suspended-pellet samples were serially diluted and analyzed for PA ELISA reactivity and compared with the total culture sample. Diluted samples were also deacylated by treatment with 0.1 M NaOH in ethanol for 30 min at 37°C as described before (34).

RESULTS

ELISA reactivities of different bacterial samples. *B. bifidum* subsp. *pennsylvanicum* bacterial suspensions showed reactivity when tested in the PA ELISA. Reactivity was tested with a range of sample dilutions and showed saturation kinetics (Fig.

TABLE 1. Origin and characteristics of tested bacterial species and strains

Bacterial species	Strain or source[a]	Origin	Characteristics	Reference(s)
Bifidobacterial species				
B. adolescentis	ATCC 15703	Human intestine	Soluble polysaccharide without β-1,5-Gal*f*	16
B. animalis	Winclove 53	Fermented milk	Lipoglycan	
	CUETM 89/13	Rat feces		
B. bifidum	CUETM 89/27	Breast-fed neonate intestine	Lipoglycan with 11–18 β-1,5-Gal*f* residues	7, 18
	Winclove 1	Human intestine		
subsp. *pennsylvanicum*	DSM20239	Human intestine		
B. breve	Winclove 9	Not specified		
	Gavini crohn 16	Neonate intestine	Lipoglycan with 11–18 β-1,5-Gal*f* residues	18
	ATCC 15700	Neonate intestine		
B. catenulatum	CUETM 89/96	Human intestine	Cell wall polysaccharide with 1 internal β-1,5-Gal*f* residue	30
	CUETM 89/29	Human intestine		
B. dentium	CUETM 89/20	Dental carries	Lipoglycan	
B. infantis	ATCC 15697	Infant intestine	Cell wall polysaccharide with 1 internal β-1,3-Gal*f* residue	48
B. lactis	Winclove 18	Fermented milk	Lipoglycan	
B. longum	Winclove 3	Not specified	Lipoglycan with 11–18 β-1,5-Gal*f* residues	18
	Winclove 16	Not specified		
	Gavini BS 175	Neonate intestine		
B. pseudocatenulatum	Gavini BS 82	Human intestine	Lipoglycan	
B. pseudolongum				
subsp. *pseudolongum*	Gavini MB7	Animal feces	Lipoglycan	
subsp. *globosum*	Gavini RU 224	Animal feces	Lipoglycan	
Other gram-positive bacteria				
Actinomyces viscosus	AZN	Human infection	Lipoglycan	
Bacillus subtilis	AZN	Human infection	LTA	8
Clostridium perfringens	ATCC 13124	Not specified	LTA	13
Enterococcus faecalis	ATCC 29212	Urine	LTA	8
Eggerthella lenta	ATCC 25559	Rectal tumor	Lipoglycan	
Lactobacillus acidophilus	AZN	Human infection	LTA	
Lactobacillus casei	AZN	Human infection	Unglycosylated LTA	8
Listeria monocytogenes	AZN	Human infection	LTA with αGal*p* monomeric residues	8
Micrococcus luteus	AZN	Human infection	Lipomannan	9
Nocardia farcinica	AZN	Human infection	Lipoglycan	
Propionibacterium acnes	ATCC 6919	Facial acne	Lipoglycan	
Propionibacterium freudenreichii	ATCC 6207	Dairy	Lipomannan	9
Staphylococcus epidermidis	ATCC 12228	Not specified	LTA	
Streptococcus pyogenes	ATCC 43202	Not specified	Unglycosylated LTA	8
Gram-negative bacteria				
Bacteroides fragilis	AZN	Human infection	LPS	
Escherichia coli	ATCC 25022	Human infection	LPS	
Klebsiella pneumoniae	ATCC 27736	Human infection	LPS with 1 internal β-1,3-Gal*f* residue	
Pseudomonas aeruginosa	ATCC 10145	Not specified	LPS	

[a] CUETM, Collection de 1'Unité d'Ecotoxicologie Microbienne, Institut National de la Santé et de la Recherche Médicale, 59651 Villeneuve d'Ascq, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1B, D-38124 Braunschweig, Germany; Gavini, strains received from F. Gavini, INRA, Villeneuve d'Ascq, France; Winclove, Collection from Winclove BioIndustries BV, P.O. Box 37239, 1030 AE Amsterdam, The Netherlands; AZN, Collection of the Department of Medical Microbiology, Radboud University Nijmegen Medical Center, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

2). Reactivity was determined in the linear part of the graph and was expressed as EI per milligram of protein. Protein concentrations of the undiluted samples ranged from 0.1 to 0.5 mg/ml. Some cell samples reacted so strongly that even a 40-fold dilution gave a positive PA ELISA result (EI > 1.0) as shown for *Bifidobacterium lactis* (Fig. 2). The reactivity of all tested bacteria is shown in Fig. 3. Most bifidobacteria (from human, animal, or food origin) showed cross-reactivity with the PA ELISA. Reactivities ranged from 6-fold (*Bifidobacterium breve*) to 600-fold (*B. lactis*) higher than the controls. Some of these species are known to contain a lipoglycan with more than 7 β-1,5-linked Gal*f* residues (*B. bifidum*, *B. breve*, and *Bifidobacterium longum*; Table 1). *Bifidobacterium infantis* and *Bifidobacterium adolescentis*, which do not seem to have β-1,5-

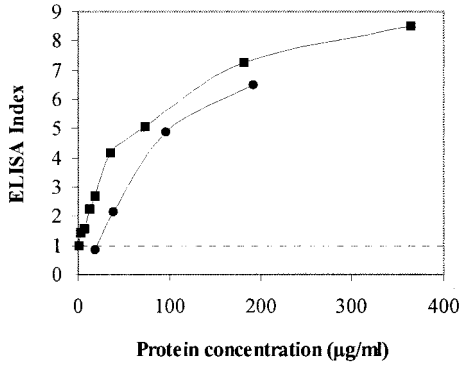

FIG. 2. PA EI related to the protein concentration of the total bifidobacterial cell sample. ●, *B. bifidum* subsp. *pennsylvanicum*; ■, *B. lactis*. The detection limit of the assay is indicated as a dotted line.

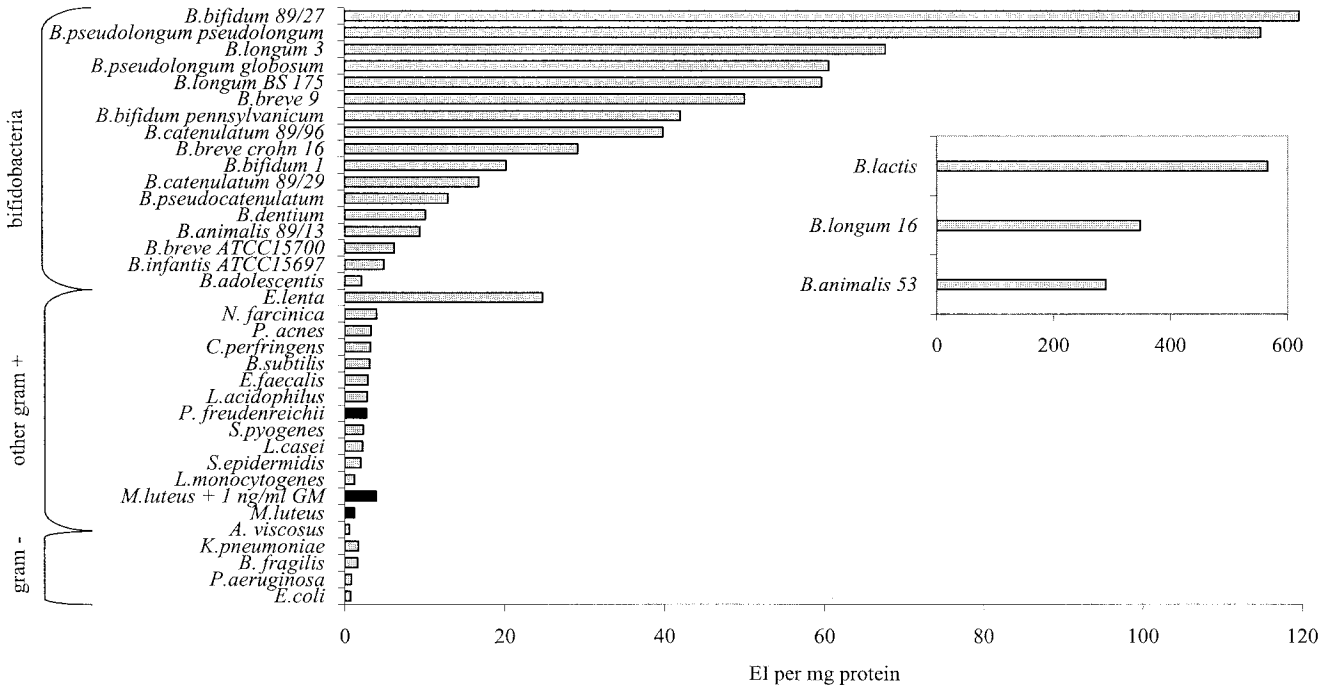

FIG. 3. Cross-reactivity of different bacterial species and strains with the PA ELISA. Reactivity is quantified as EI correlated to the total protein content of the cell suspension tested. Black bars indicate the control samples. Total protein contents of cell samples ranged from 0.1 to 0.5 mg/ml. Control samples include 1 ng/ml GM (clinical cutoff value) added to *M. luteus* samples with different total protein contents.

linked Gal*f* residues in their cell wall, showed low reactivity. Furthermore, other gram-positive bacteria containing LTA or lipoglycan showed low reactivity, including the negative controls containing a lipomannan. Gram-negative bacteria containing lipopolysaccharide (LPS) also reacted negatively. The only nonbifidobacterial species that showed PA ELISA reactivity was *Eubacterium lentum*. This species, which is one of the predominant microorganisms from the human intestine, has been reclassified as *Eggerthella lenta* (19). In contrast to other eubacteria, which belong to the low-G+C group, this bacterium has a high G+C content and by definition a lipoglycan instead of an LTA (31).

PA ELISA reactivity per gram of feces. Table 2 shows a calculation of the EI per gram of neonatal feces for the most frequently found bifidobacterial species, i.e., *B. longum*, *B. bifidum*, and *B. breve*. Matsuki et al. (25) studied the distribution of bifidobacterial species in the intestinal tract with 16S rRNA gene-targeted species-specific primers and found about one to three species per neonate (25). The EI was corrected for the dilution factor. This reactivity was then correlated to the

TABLE 2. Calculated and measured PA ELISA reactivity per gram of neonatal feces

Tested sample	Occurrence (%)[a]	EI × dilution factor	No. of cells (10^7)[b]	No. present in feces g^{-1}	EI g feces^{-1}
B. longum	37	80.7	1.0	$10^{9.8–10.1}$[c]	51,000–102,000
B. bifidum	22	10.2	0.7	$10^{10.3}$[c]	29,000
B. breve	70	4.8	3.8	$10^{10.5–10.7}$[c]	4,000–6,000
Fecal samples from neonates					
1		5.6		1.40×10^7[d]	1,120
2		72.0		1.90×10^7[d]	14,400
3		1.0		2.50×10^9[d]	200
4		11.5		1.20×10^7[d]	2,300
5		181.6		3.00×10^9[d]	36,320
6		54.8		1.90×10^7[d]	10,960
7		125.8		3.60×10^9[d]	25,160
8		45.1		2.10×10^9[d]	9,020
9		57.6		5.10×10^9[d]	11,520

[a] Expressed as % of the total number of fecal samples that were tested, taken from reference 25.
[b] Present in 50 μl undiluted cell sample.
[c] Taken from reference 29.
[d] Determined by FISH analysis.

TABLE 3. PA ELISA reactivity of liquid RCM cultures of *B. bifidum* subsp. *pennsylvanicum*

Sample	EI[a] ($n = 2$)	Recovery (%)
Total culture	62.1	100
Cell-free supernatant	23.5	37.8
Suspended cell pellet	16.0	25.8

[a] Sample EI was corrected for dilution (20 × to 40×), and cell pellet values are also corrected for the five-fold concentration step (from 1,000 μl to 200 μl)

number of cells present in 50 μl undiluted cell sample. So knowing the reactivity per cell, the reactivity per gram of feces can be calculated by multiplying it with the amount of cells present per gram of feces. These fecal numbers were taken from the literature but may vary depending on the method used for quantification (15, 29). Table 2 also shows the EI per gram of feces as measured in the nine collected neonatal fecal samples. All fecal samples exhibited PA ELISA reactivity. Furthermore, all fecal samples contained bifidobacterial species as determined by FISH.

ELISA reactivity of liquid cultures. The result of the ELISA reactivity of the *B. bifidum* subsp. *pennsylvanicum* RCM cultures is shown in Table 3. When the undiluted total cell culture was tested in the PA ELISA, no reactivity was observed. However, 40-fold dilution of the samples gave a positive reaction (EI = 1.553). Part of this reactivity was associated with the cells (25.8%) and another part (37.8%) with secreted products. Blank media showed no reactivity. Deacylation of the same samples completely removed ELISA reactivity in contrast to deacylation of GM samples containing 1.5, 3, and 5 ng GM (purified GM was a kind gift from Marc Tabouret, Bio-Rad, Steenvoorde, France) per ml water, which showed a 50% decrease in reactivity upon this treatment (results not shown).

DISCUSSION

Most bifidobacteria showed significant reactivity with the PA ELISA in contrast to other gram-positive bacteria containing LTA or lipomannan or gram-negative bacteria containing LPS (Table 1; Fig. 3). ELISA reactivity was clearly shown to be associated with bacterial lipoglycans containing a β-1,5-galactofuranosyl chain. Only a few bifidobacterial lipoglycans have been structurally analyzed, but also other bifidobacterial species seem to contain reactive residues in their cell wall (7, 18, 30). This specific epitope can be detected as a surface antigen, shown by the reactivity of the suspended cell pellet, but also as an exoantigen, shown by the reactivity of the cell-free supernatant of a liquid culture (Table 3). Secretion of lipoglycans during normal growth was already shown by Op den Camp et al. with *B. bifidum* subsp. *pennsylvanicum* (33). The complete removal of PA ELISA reactivity after deacylation of the supernatant, compared to the 50% decrease with GM, suggests that only the acylated micellar form reacts in the PA ELISA.

When our results were extrapolated to the neonatal host, a calculation was made to estimate the PA ELISA reactivity of feces as potential source of cross-reactivity in the neonate. The bifidobacterial numbers found in feces (15, 29) are very high, and the EI per gram of feces is also high, ranging from 4,000 to 100,000. In order to confirm this calculation, FISH analysis was performed on neonatal fecal samples with a genus-specific probe for bifidobacteria. This 16S rRNA hybridization technique is a fast method to quantify bifidobacteria in the human gut (21). The presence of bifidobacteria was associated with PA ELISA reactivity of the fecal samples, but reactivity showed some variation, which probably depends on the bifidobacterial species present, as shown by the calculated examples. The results clearly show that the bifidobacterial community in the gut is a significant source of PA ELISA reactivity and that, considering the dilution in the blood volume, the serum concentration could become high enough after transmucosal passage to be detected with the PA ELISA. Furthermore, false-positive PA reactivity in fully breast-fed neonates could not come from galactomannan since breast milk reacts negatively in the PA ELISA (results not shown).

E. lenta is, like *Bifidobacterium* species, a gram-positive, obligatory anaerobic, non-spore-forming rod and has been found in adult feces at concentrations of $10^{8.5}$ cells/g, in 4 out of 12 subjects that were tested (detection limit, 10^7 cells/g) (41). Furthermore, the number increased in the intestinal microflora of healthy subjects after administration of antibacterial agents (32). Comparable species have also been found in feces of children at $10^{9.6}$ cells/g, but the bacteria were not identified to species level (15, 29). So, in addition to *Bifidobacterium*, this species might also form an important source of PA ELISA reactivity in the intestine. Since *E. lenta* is present in the fecal flora of adults, it might also be a cause of false PA ELISA serum reactivity in adults. Recently developed species-specific oligonucleotide probes might be helpful with future studies of *E. lenta* in the human intestine (19, 41).

Positivity of fecal samples has already been shown (1) but was always correlated with consumption of food containing fungal GM from contaminated sources (1, 23). In addition to the host's own microflora as a source of bifidobacteria, ELISA reactivity of certain food products is more likely to result from bifidobacterial lipoglycan than from fungal GM. Because of their health-promoting effects, bifidobacteria are widely used as probiotics and food additives and are present in fermented foods like milk, olives, sauerkraut, yogurt, butter, and cheese (3). Especially *B. lactis* and *Bifidobacterium animalis* are often used, and amounts of 10^7 to 10^8 of bifidobacteria per gram of food can be found (20, 40, 50). As can be seen in Fig. 3, *B. lactis* (recently reclassified as a subspecies of *B. animalis* [50]) and *B. animalis* show high reactivities and could act as a source of ELISA serum reactivity after consumption of food products. Furthermore, bifidobacteria are used as fecal indicator organisms and have been found on meat and meat products and in raw milk (2, 11). Especially meat products are often GM positive (23) but are more likely contaminated with bifidobacteria instead of fungal GM.

The frequent isolation of bifidobacteria from clinical infections in recent years has raised debate whether the bacteria are actually infective (3, 17, 36). However, even if there's a lack of pathogenicity in immunocompromised patients (3), bifidobacteria can invade the host by bacterial translocation (17). This phenomenon is caused by a diminished intestinal barrier, resulting in the passage of bacteria or bacterial components or products across the mucous membrane and epithelium. Not only intestinal mucosal injury by for instance cytotoxic chemotherapy or an immature intestinal mucosa but also immuno-

deficiency in the host, overgrowth of intestinal bacteria, and treatment with antibiotics and/or immunosuppressive agents have been shown to promote translocation of intestinal bacteria (i.e., bifidobacteria) (6, 17). The actual translocation of reactive components across the intestinal wall remains to be proven but seems even more likely for an exocellular lipoglycan molecule of 10 kDa. Penicillin treatment of *B. bifidum* subsp. *pennsylvanicum* resulted in an increase of lipoglycan excretion (33). This was not the result of bacteriolysis, as was also observed for acylated or deacylated LTAs of other bacteria and for other inhibitors of cell wall synthesis (33, 35, 43, 49). Consequently, antibiotic treatment of immunocompromised patients will increase the free lipoglycan pool in the intestine which may easily translocate to the blood.

In the blood, lipoglycan might bind to different components, resulting in a mixture of Gal*f*-containing molecules that react in the PA ELISA, as is the case with GM (27). LTAs and lipoglycans spontaneously bind to mammalian cell membranes. In a study on *B. bifidum* subsp. *pennsylvanicum* it was shown that its lipoglycan bound reversibly to human colonocytes (8.3×10^8 binding sites/cell) and erythrocytes (2.1×10^6 binding sites/cell) (34). The lipid part of the molecule was shown to be responsible for binding. Furthermore, the lipoglycan of *B. bifidum* subsp. *pennsylvanicum* exhibited strong binding to the macrophage scavenger receptor, comparable to the LTA of *Staphylococcus aureus* (13). In order to analyze the nature and structure of the cross-reacting molecule(s) present in (false-) positive serum samples, a method is needed that specifically isolates reactive components because of their small amounts present. Several techniques (28) can then be used for further analysis to discriminate between false- and true-positive PA ELISA results and thereby prevent unnecessary preemptive treatment of patients.

The *Bifidobacterium* lipoglycan offers an explanation for the occurrence of a high rate of false PA ELISA reactivity among neonates and infants. Furthermore, together with *E. lenta*, these bacteria might also be the cause of false-positive reactivity in adult patients.

ACKNOWLEDGMENTS

We thank Marc Tabouret, Bio-Rad (Steenvoorde, France), for kindly providing the purified GM. We also thank Arie Oosterhof (Dept. Biochemistry, Radboud University, Nijmegen, The Netherlands), Ger Bongaerts (Dept. Medical Microbiology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands), and Winclove BioIndustries BV (Amsterdam, The Netherlands) for kindly providing some of the bifidobacterial species. We thank Gjalt Welling (Dept. Medical Microbiology, University of Groningen) for performing the FISH analysis.

REFERENCES

1. **Ansorg, R., R. van den Boom, and P. M. Rath.** 1997. Detection of *Aspergillus* galactomannan antigen in foods and antibiotics. Mycoses **40:**353–357.
2. **Beerens, H., H. B. de la Perriere, and F. Gavini.** 2000. Evaluation of the hygienic quality of raw milk based on the presence of bifidobacteria: the cow as a source of faecal contamination. Int. J. Food Microbiol. **54:**163–169.
3. **Borriello, S. P., W. P. Hammes, W. Holzapfel, P. Marteau, J. Schrezenmeir, M. Vaara, and V. Valtonen.** 2003. Safety of probiotics that contain lactobacilli or bifidobacteria. Clin. Infect. Dis. **36:**775–780.
4. **Bouakline, A., C. Lacroix, N. Roux, J. P. Gangneux, and F. Derouin.** 2000. Fungal contamination of food in hematology units. J. Clin. Microbiol. **38:** 4272–4273.
5. **Denning, D. W.** 2000. Early diagnosis of invasive aspergillosis. Lancet **355:** 423–424.
6. **Duffy, L. C.** 2000. Interactions mediating bacterial translocation in the immature intestine. J. Nutr. **130:**432S–436S.
7. **Fischer, W.** 1987. 'Lipoteichoic acid' of *Bifidobacterium bifidum* subspecies *pennsylvanicum* DSM 20239. A lipoglycan with monoglycerophosphate side chains. Eur. J. Biochem. **165:**639–646.
8. **Fischer, W.** 1988. Physiology of lipoteichoic acids in bacteria. Adv. Microb. Physiol. **29:**233–302.
9. **Fischer, W.** 1994. Lipoteichoic acids and lipoglycans, p. 199–215. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Science B.V., Amsterdam, The Netherlands.
10. **Gangneux, J. P., D. Lavarde, S. Bretagne, C. Guiguen, and V. Gandemer.** 2002. Transient *Aspergillus* antigenaemia: think of milk. Lancet **359:**1251.
11. **Gavini, F., and H. Beerens.** 1999. Origin and identification of bifidobacteria strains isolated from meat and meat products. Int. J. Food Microbiol. **46:** 81–85.
12. **Ginsburg, I.** 2002. Role of lipoteichoic acid in infection and inflammation. Lancet Infect. Dis. **2:**171–179.
13. **Greenberg, J. W., W. Fischer, and K. A. Joiner.** 1996. Influence of lipoteichoic acid structure on recognition by the macrophage scavenger receptor. Infect. Immun. **64:**3318–3325.
14. **Harmsen, H. J., G. C. Raangs, T. He, J. E. Degener, and G. W. Welling.** 2002. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Appl. Environ. Microbiol. **68:**2982–2990.
15. **Harmsen, H. J., A. C. Wildeboer-Veloo, G. C. Raangs, A. A. Wagendorp, N. Klijn, J. G. Bindels, and G. W. Welling.** 2000. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J. Pediatr. Gastroenterol. Nutr. **30:**61–67.
16. **Hosono, A., J. Lee, A. Ametani, M. Natsume, M. Hirayama, T. Adachi, and S. Kaminogawa.** 1997. Characterization of a water-soluble polysaccharide fraction with immunopotentiating activity from *Bifidobacterium adolescentis* M101-4. Biosci. Biotechnol. Biochem. **61:**312–316.
17. **Ishibashi, N., and S. Yamazaki.** 2001. Probiotics and safety. Am. J. Clin. Nutr. **73:**465S–470S.
18. **Iwasaki, H., Y. Araki, E. Ito, M. Nagaoka, and T. Yokokura.** 1990. Structure of macroamphiphiles from several *Bifidobacterium* strains. J. Bacteriol. **172:** 845–852.
19. **Kageyama, A., Y. Benno, and T. Nakase.** 1999. Phylogenetic evidence for the transfer of *Eubacterium lentum* to the genus *Eggerthella* as *Eggerthella lenta* gen. nov., comb. nov. Int. J. Syst. Bacteriol. **49:**1725–1732.
20. **Kaufmann, P., A. Pfefferkorn, M. Teuber, and L. Meile.** 1997. Identification and quantification of *Bifidobacterium* species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. Appl. Environ. Microbiol. **63:**1268–1273.
21. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. Wilkinson, and G. W. Welling.** 1995. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61:** 3069–3075.
22. **Latge, J. P., H. Kobayashi, J. P. Debeaupuis, M. Diaquin, J. Sarfati, J. M. Wieruszeski, E. Parra, J. P. Bouchara, and B. Fournet.** 1994. Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. Infect. Immun. **62:**5424–5433.
23. **Letscher-Bru, V., A. Cavalier, and E. Pernot-Marino.** 1998. Recherche d'antigène galactomannane aspergillaire circulant par Platelia *Aspergillus*: antigènèmies positives persistantes en l'absence d'infection. J. Mycol. Med. **8:**112–113.
24. **Maertens, J., J. Van Eldere, J. Verhaegen, E. Verbeken, J. Verschakelen, and M. Boogaerts.** 2002. Use of circulating galactomannan screening for early diagnosis of invasive aspergillosis in allogeneic stem cell transplant recipients. J. Infect. Dis. **186:**1297–1306.
25. **Matsuki, T., K. Watanabe, R. Tanaka, M. Fukuda, and H. Oyaizu.** 1999. Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl. Environ. Microbiol. **65:**4506–4512.
26. **Mattei, D., D. Rapezzi, N. Mordini, F. Cuda, N. C. Lo, M. Musso, A. Arnelli, S. Cagnassi, and A. Gallamini.** 2004. False-positive *Aspergillus* galactomannan enzyme-linked immunosorbent assay results in vivo during amoxicillin-clavulanic acid treatment. J. Clin. Microbiol. **42:**5362–5363.
27. **Mennink-Kersten, M. A., J. P. Donnelly, and P. E. Verweij.** 2004. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. Lancet Infect. Dis. **4:**349–357.
28. **Mennink-Kersten, M. A., R. R. Klont, A. Warris, H. J. Op den Camp, and P. E. Verweij.** 2004. *Bifidobacterium* lipoteichoic acid and false ELISA reactivity in aspergillus antigen detection. Lancet **363:**325–327.
29. **Mitsuoka, T.** 1992. The human gastrointestinal tract, p. 69–114. *In* B. J. B. Wood (ed.), The lactic acid bacteria, vol. 1. Elsevier Applied Science, London, United Kingdom.
30. **Nagaoka, M., S. Hashimoto, H. Shibata, I. Kimura, K. Kimura, H. Sawada, and T. Yokokura.** 1996. Structure of a galactan from cell walls of *Bifidobacterium catenulatum* YIT4016. Carbohydr. Res. **281:**285–291.
31. **Nakazawa, F., and E. Hoshino.** 1994. Genetic relationships among *Eubacterium* species. Int. J. Syst. Bacteriol. **44:**787–790.
32. **Nord, C. E., L. Meurling, R. L. Russo, A. Bello, D. M. Grasela, and D. A. Gajjar.** 2003. Effect of garenoxacin on eubacteria in the normal intestinal

microflora when administered concomitantly with digoxin. J. Chemother. **15:**244–247.
33. **Op den Camp, H. J., A. Oosterhof, and J. H. Veerkamp.** 1985. Cell surface hydrophobicity of *Bifidobacterium bifidum* subsp. *pennsylvanicum*. Antonie Leeuwenhoek **51:**303–312.
34. **Op den Camp, H. J., A. Oosterhof, and J. H. Veerkamp.** 1985. Interaction of bifidobacterial lipoteichoic acid with human intestinal epithelial cells. Infect. Immun. **47:**332–334.
35. **Pollack, J. H., A. S. Ntamere, and F. C. Neuhaus.** 1992. D-alanyl-lipoteichoic acid in *Lactobacillus casei*: secretion of vesicles in response to benzylpenicillin. J. Gen. Microbiol. **138**(Pt. 5)**:**849–859.
36. **Pulverer, G., H. Schutt-Gerowitt, and K. P. Schaal.** 2003. Human cervicofacial actinomycoses: microbiological data for 1997 cases. Clin. Infect. Dis. **37:**490–497.
37. **Requena, T., J. Burton, T. Matsuki, K. Munro, M. A. Simon, R. Tanaka, K. Watanabe, and G. W. Tannock.** 2002. Identification, detection, and enumeration of human *Bifidobacterium* species by PCR targeting the transaldolase gene. Appl. Environ. Microbiol. **68:**2420–2427.
38. **Rohrlich, P., J. Sarfati, P. Mariani, M. Duval, A. Carol, C. Saint-Martin, E. Bingen, J. P. Latge, and E. Vilmer.** 1996. Prospective sandwich enzyme-linked immunosorbent assay for serum galactomannan: early predictive value and clinical use in invasive aspergillosis. Pediatr. Infect. Dis. J. **15:**232–237.
39. **Sarfati, J., H. E. Jensen, and J. P. Latge.** 1996. Route of infections in bovine aspergillosis. J. Med. Vet. Mycol. **34:**379–383.
40. **Satokari, R. M., E. E. Vaughan, H. Smidt, M. Saarela, J. Matto, and W. M. De Vos.** 2003. Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. Syst. Appl. Microbiol. **26:**572–584.
41. **Schwiertz, A., G. Le Blay, and M. Blaut.** 2000. Quantification of different *Eubacterium* spp. in human fecal samples with species-specific 16S rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **66:**375–382.
42. **Siemann, M., M. Koch-Dorfler, and M. Gaude.** 1998. False-positive results in premature infants with the Platelia *Aspergillus* sandwich enzyme-linked immunosorbent assay. Mycoses **41:**373–377.
43. **Stuertz, K., H. Schmidt, H. Eiffert, P. Schwartz, M. Mader, and R. Nau.** 1998. Differential release of lipoteichoic and teichoic acids from *Streptococcus pneumoniae* as a result of exposure to beta-lactam antibiotics, rifamycins, trovafloxacin, and quinupristin-dalfopristin. Antimicrob. Agents Chemother. **42:**277–281.
44. **Stynen, D., A. Goris, J. Sarfati, and J. P. Latge.** 1995. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J. Clin. Microbiol. **33:**497–500.
45. **Stynen, D., J. Sarfati, A. Goris, M. C. Prevost, M. Lesourd, H. Kamphuis, V. Darras, and J. P. Latge.** 1992. Rat monoclonal antibodies against *Aspergillus* galactomannan. Infect. Immun. **60:**2237–2245.
46. **Sulahian, A., F. Boutboul, P. Ribaud, T. Leblanc, C. Lacroix, and F. Derouin.** 2001. Value of antigen detection using an enzyme immunoassay in the diagnosis and prediction of invasive aspergillosis in two adult and pediatric hematology units during a 4-year prospective study. Cancer **91:**311–318.
47. **Swanink, C. M., J. F. Meis, A. J. Rijs, J. P. Donnelly, and P. E. Verweij.** 1997. Specificity of a sandwich enzyme-linked immunosorbent assay for detecting *Aspergillus* galactomannan. J. Clin. Microbiol. **35:**257–260.
48. **Tone-Shimokawa, Y., T. Toida, and T. Kawashima.** 1996. Isolation and structural analysis of polysaccharide containing galactofuranose from the cell walls of *Bifidobacterium infantis*. J. Bacteriol. **178:**317–320.
49. **van Langevelde, P., J. T. van Dissel, E. Ravensbergen, B. J. Appelmelk, I. A. Schrijver, and P. H. Groeneveld.** 1998. Antibiotic-induced release of lipoteichoic acid and peptidoglycan from *Staphylococcus aureus*: quantitative measurements and biological reactivities. Antimicrob. Agents Chemother. **42:**3073–3078.
50. **Ventura, M., and R. Zink.** 2002. Rapid identification, differentiation, and proposed new taxonomic classification of *Bifidobacterium lactis*. Appl. Environ. Microbiol. **68:**6429–6434.
51. **Viscoli, C., M. Machetti, P. Cappellano, B. Bucci, P. Bruzzi, M. T. Van Lint, and A. Bacigalupo.** 2004. False-positive galactomannan Platelia *Aspergillus* test results for patients receiving piperacillin-tazobactam. Clin. Infect. Dis. **38:**913–916.
52. **Walsh, T. J., S. Shoham, R. Petraitiene, T. Sein, R. Schaufele, A. Kelaher, H. Murray, C. Mya-San, J. Bacher, and V. Petraitis.** 2004. Detection of galactomannan antigenemia in patients receiving piperacillin-tazobactam and correlations between in vitro, in vivo, and clinical properties of the drug-antigen interaction. J. Clin. Microbiol. **42:**4744–4748.
53. **Wicken, A. J., J. D. Evans, and K. W. Knox.** 1986. Critical micelle concentrations of lipoteichoic acids. J. Bacteriol. **166:**72–77.
54. **Wicken, A. J., and K. W. Knox.** 1980. Bacterial cell surface amphiphiles. Biochim. Biophys. Acta **604:**1–26.