Selective growth of mucolytic bacteria including *Clostridium perfringens* in a neonatal piglet model of total parenteral nutrition[1–3]

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ABSTRACT

Background: Compromised barrier function and intestinal inflammation are common complications of total parenteral nutrition (TPN).

Objective: We tested the hypothesis that the lack of enteral nutrients in TPN might select commensal or pathogenic bacteria that use mucus as a substrate, thereby weakening the protection provided by the intestinal mucus layer.

Design: Ileal microbiota profiles of piglets fed by total enteral nutrition (TEN; $n = 6$) or TPN ($n = 5$) were compared with the use of 16S ribosomal DNA polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis and with a PCR-based method developed to specifically measure *Clostridium perfringens* concentrations. Ileal bacteria from TEN and TPN piglets were also examined for their ability to grow on mucin or sulfated monosaccharides.

Results: Bacterial community structure was equally complex in the ileum of TEN and TPN piglets, but profiles clustered according to mode of nutrition. Sixty-two percent of total mucus-associated bacteria (100 colonies tested) in TPN compared with 33% of mucus-associated bacteria (100 colonies tested) in TEN ileal samples grew on mucin. Bacteria capable of using sulfated monosaccharides were also enriched in TPN samples. *C. perfringens*, an opportunistic pathogen, was specifically enriched in the TPN ileum ($P < 0.05$). These results were corroborated by cultivation-based studies that showed rapid growth of *C. perfringens* on mucin-based substrates.

Conclusions: Mucolytic potential is widespread among intestinal bacteria. Mucolytic bacteria in general and *C. perfringens* in particular were selected when enteral nutrients were withheld in this TPN piglet model. Similar enrichment processes may occur in humans nourished by TPN and may thereby contribute to intestinal dysfunction. *Am J Clin Nutr* 2002;76:1117–25.

KEY WORDS Total parenteral nutrition, neonatal piglet model, intestinal microbiota, mucus, mucolysis, *Clostridium perfringens*, intestinal inflammation

INTRODUCTION

Total parenteral nutrition (TPN) is used extensively in the support of very-low-birth-weight infants and critical care patients with compromised gastrointestinal function (1). This mode of nutrition is associated with reductions in mucosal mass, alterations in villus-crypt morphology, lower brush border enzyme activities, and the disruption of barrier function (2–5). Although these deleterious effects may be less pronounced in adults (6), a significant portion of the morbidity associated with TPN in high-risk infants may be the result of the ingress of intestinal bacteria, with consequent bacterial sepsis and associated inflammation (7–10). The mechanisms contributing to intestinal inflammation in TPN are not well defined. Similarly, little is known about the effects of TPN on the composition of the intestinal microbiota.

We recently observed, in a neonatal piglet model, that TPN also alters the distribution of villus cells among absorptive enterocytes and goblet cells. The changes are marked by a substantial expansion (3- to 4-fold) of goblet cell number and an enrichment of acidomucins (sialo- and sulfomucins; 5). Given these changes in villus architecture and goblet-cell phenotype, we hypothesized that TPN, and thus the absence of exogenous nutrients, alter the qualitative nature of the microbial population with a selective enrichment of mucolytic bacteria, ie, bacteria that are able to grow within the mucus layer by degrading and fermenting mucins.

Accordingly, we report a study in which the mucus-associated bacteria (MAB) communities were characterized from the ileum of piglets that were nourished either with a conventional milk formula [total enteral nutrition (TEN)] or parenterally. A novel molecular ecology approach was used in combination with conventional culture techniques to specifically examine the effects of TPN on the percentage and type of mucolytic bacteria relative to the total number of MAB.

MATERIALS AND METHODS

Animals and study design

Experimental animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois (Urbana-Champaign). The study design is described in detail elsewhere (5). Briefly, 1-d-old piglets (Yorkshire-Duroc) were

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obtained from a rigorously cleaned farrowing facility and randomly assigned to the TEN (n = 6) or TPN (n = 5) group. Orally fed piglets received 100% of their energy requirements as a nutritionally complete commercial formula (Advance Baby Pig Liqui-Wean; Milk Specialties, Dundee, IL). Parenterally fed piglets received 100% of their energy requirements intravenously as a complete TPN solution that meets the nutritional requirements of neonatal piglets, as described previously (5). The solution was prepared daily as a 3:1 admixture under sterile conditions with 8.5% amino acid, 50% dextrose, and 20% lipid emulsion (Intralipid; Clintech Nutrition Company, Deerfield, IL) solutions. The solution provided 3752.3 J energy and 53.1 g protein/L with 50% nonprotein energy as fat and the remaining 50% as dextrose. Vitamins, minerals, and trace elements were added daily to the infusate before administration. The TPN solution was provided continuously via a volumetric infusion pump (IMED 980; HCI, St Louis) at a final infusion rate of 225 $mL \cdot kg^{-1} \cdot d^{-1}$. The surgical procedure for insertion of the TPN infusion line was described in detail by Park et al (11). Briefly, piglets were anesthetized (98% oxygen/2% Isoflurane; Anaquest, Liberty Corner, NJ), and TPN catheters were inserted into the umbilical artery and advanced 22 cm through the external iliac artery until its tip reached the aorta. The TPN lines were threaded through a jacket with a tether-swivel apparatus (Alice King Chatham Medical Arts, Los Angeles), which facilitated free movement of the animals within the cages while preventing catheter displacement. TEN piglets were also anesthetized and subjected to sham operations. Feeding of both treatment groups was initiated simultaneously. All piglets were injected intramuscularly (1.0 mg/kg body wt) with the antibiotic ceftiofur-cephalosporin (Naxcel; SmithKline Beecham, Philadelphia) for the first 3 d of the study. General health status, weight gain, and formula intake of each piglet were recorded daily.

Intestinal samples

On the seventh day of the experiment, the piglets were killed by electrocution and exsanguination. The small intestine from the pyloric sphincter to the ileocecal valve was excised, freed from the mesentery, and arranged on a smooth surface to form 13 segments of approximately equal length; duodenum (1–2), jejunum (3–10), and ileum (11–13) (12). Mucosa samples were collected by scraping the luminal surface from the ileum (segment 12) with a sterile glass slide after draining the luminal fluid. Thus, each mucosal sample contained the mucus itself with entrapped bacteria (MAB) as well as epithelial and lamina propria cells and, in the case of the TEN piglets, a residual amount of luminal contents. Mucosa samples were snap-frozen in liquid nitrogen and stored at −80 °C.

Polymerase chain reaction amplification of 16S ribosomal DNA and denaturing gradient gel electrophoresis

Genomic DNA was extracted from pure cultures, enrichment plates, and ileal samples with the use of a modified Tsai and Olson protocol (13), described in detail by Simpson et al (14). These genomic DNA preparations were used as templates for polymerase chain reaction (PCR) amplification of 16S ribosomal DNA (rDNA) and subjected to denaturing gradient gel electrophoresis (DGGE) analysis as described (14). A DGGE ladder was created by mixing a collection of equal concentrations of species-specific 220–base pair (bp) fragments obtained by PCR amplification of the 16S rDNA V3 region with the primers 341F and 534R (15). PCR template DNA was extracted from anaerobically grown pure cultures of *Bacteroides thetaiotaomicron* (VPI 5482), *B. fragilis* (VPI 2553), *Ruminococcus albus* strains 7 and 8 (laboratory collection), *Streptococcus bovis* (laboratory collection), *Desulfovibrio vulgaris* (ATCC 29579), *D. desulfuricans* (NCIB 8307), *Bilophila wadsworthia* (ATCC 49260), *Escherichia coli* K-12 NM522 (ATCC 47000), *C. parvum* (laboratory collection), and *C. perfringens* (laboratory collection).

To analyze the resulting DGGE gel, we first used the DIVERSITY DATABASE version 2.1 of The Discovery Series (Bio-Rad, Hercules, CA) to determine the migration distances and intensity of individual bands within each gel. This yielded a linear plot of selected gel lanes and allowed the detection of bands that were common among different samples. The resulting data were further recorded in binary format (0 for absence and 1 for presence of band) and then subjected to cluster analysis with the use of the polymorphism parsimony criteria of the program DOLLOP in the PHYLIP software package 3.57c to generate dendrograms (16).

Sequence analysis of polymerase chain reaction products

PCR products were excised from DGGE gels, reamplified, and sequenced directly with an automated sequencing system (Applied Biosystems, Foster City, CA) at the WM Keck Center for Comparative and Functional Genomics, University of Illinois Biotechnology Center (Urbana). Sequences were analyzed with the BLAST (Basic Local Alignment Search Tool) family of programs to search and align nucleotide sequences with similar sequences in GenBank (National Library of Medicine, Bethesda, MD; Internet: www.ncbi.nlm.nih.gov/BLAST/).

Cultivation of mucolytic bacteria

To assess the relative diversity of bacterial species able to grow exclusively on mucus (ie, mucolytic bacteria), mucosal samples (n = 3) from both TEN and TPN animals were serially diluted over 6 orders of magnitude in anaerobic diluent and spread on petri plates containing the basal habitat-simulating medium 10 modified to contain mucin as the carbon source (17). The medium contained (final volume per liter) K_2HPO_4, 0.23 g; KH_2PO_4, 0.23 g; $(NH_4)_2SO_4$, 0.23 g; NaCl, 0.46 g; $MgS0_4 \cdot 7H_2O$, 0.09 g; $CaCl_2 \cdot {}^2H_2O$, 0.04 g; yeast extract (Difco, Sparks, MD), 0.5 g; a pancreatic digest of casein (Trypticase; Difco), 2 g; resazurin, 0.001 g; and hemin, 1×10^{-5} g, pH 6.5. Pig gastric mucin (Sigma, St Louis) was added at 1% final volume and agar at 1.5% final volume. The medium was boiled to eliminate oxygen. Finally, 4 g Na_2CO_3/L and 0.5 g cysteine-HCl/L were added under carbon dioxide incubation. Cultures were incubated at 37 °C under anaerobic conditions. After 5 d of incubation, colonies of mucolytic bacteria were swiped from the plates. Total bacterial DNA was extracted and subjected to PCR amplification and DGGE analysis.

To assess the acidomucolytic activity (the potential to specifically degrade acidomucin moieties), ileal bacterial samples were cultivated anaerobically at 37 °C in liquid medium 10, containing pig gastric mucin (0.1%, type II; Sigma) as the sole carbohydrate source. Sialic acid constitutes the predominant source of acidic moieties in pig gastric mucin (0.5%) (18), whereas sulfated oligosaccharides represent a minor portion of acidomucin moieties. Immediately after inoculation and every day for 4 d, 1 mL of each culture was collected anaerobically; 0.2 mL was used to assess bacterial growth by measurement of the optical density at 600 nm. The remainder of the sample was centrifuged at room temperature for 4 min at 12 000 × g, and 0.1 mL of the supernatant

fluid was used to determine the extent of acidomucin disappearance with the use of an assay described by Stanley et al (19). Briefly, mucin present in the 0.1-mL sample was precipitated in a 1.5-mL tube by adding in the following order: 0.1 mL aqueous alcian blue (pH 2.5), 0.7 mL H_2O, and 0.1 mL $MgCl_2$ (0.25%), followed by a 30-s vortex and overnight precipitation at room temperature. Tubes were then centrifuged at room temperature for 4 min at 12 000 × *g*, the supernatant was discarded, and the precipitated pellet was washed twice in 40% ethanol · 0.1 mol HCl/L to eliminate non-acidomucin-associated alcian blue. The remaining pellet was then resuspended in 1 mL 10% sodium dodecyl sulfate, and 0.2 mL of this suspension was used to assess the acidomucin concentration by measurement of the optical density at 620 nm. A noninoculated medium was used as a negative control.

To determine the ratio of mucolytic to total MAB, serial dilutions of pooled mucosal samples ($n = 3$) from TPN or TEN piglets were also spread on petri plates containing habitat-simulating medium 10 with mucin (1% final) and simple carbohydrates (glucose and galactose, 2 g/L each) as carbon sources. After 5 d of anaerobic growth, a total of 200 colonies were isolated from the plates (100 colonies each for the TPN and TEN mucosa samples) and transferred to 24-well plates containing 2 mL media/well containing either no carbohydrate or mucin, glucose-6-sulfate, or galactose-6-sulfate as the single source of carbohydrate for growth. Each type of medium was prepared as described above, but with 0.1 g yeast extract /L and 0.4 g pancreatic digest of casein/L. To assess the relative growth in these media, 100 μL medium from each 24-well was transferred to a 96-well plate after 2 d of anaerobic growth, and the optical density was measured at 650 nm in a Vmax Plate Reader (Molecular Devices, Sunnyvale, CA). Growth on the sulfated monosaccharides provides evidence for *O*-sulfatase activity and thus an ability to degrade sulfated mucins (20, 21). In addition, the mucolytic potential of the intestinal isolate *C. perfringens* was examined separately in pure culture, also with these media.

Analysis of *Clostridium perfringens* colonization by real-time quantitative polymerase chain reaction

Genomic DNA preparations from ileal mucosal samples were used as templates for PCR amplification in a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Amplification was performed in a 25-mL final volume containing 2X SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μmol/L (each) primer, and 5 mL DNA template. The oligonucleotide primers used to detect *C. perfringens* were CP.1 (5′-AAAGATGGCATCATCATTCAAC-3′) and CP.2 (5′-TACCGTCATTATCTTCCCCAAA-3′) as designed by Wang et al (22), which target a 279-bp-specific region of the 16S rDNA sequence of *C. perfringens*. To assess the total concentration of eubacterial DNA, the variable V3 regions of 16S rDNA genes were amplified with the primers 341F, without the additional 5′ 40-nucleotide GC-rich sequence (GC-clamp) as described above, and 534R (15). PCR protocols for both *C. perfringens*–specific and eubacterial primer sets were as described previously (14, 22), with modifications to obtain a linear regression curve when log DNA concentrations were plotted against C_T values. The C_T value represents the threshold cycle, or the PCR cycle at which an increase in fluorescence from SYBR Green, generated via the binding of SYBR Green to double-stranded DNA, above a baseline signal (derived from samples without DNA template) can first be detected. The standard amplification protocol included 1 cycle of 2 min at 50 °C and 10 min at 95 °C to activate the AmpliTaq Gold DNA polymerase (Applied Biosystems) in the SYBR Green PCR Master Mix and 35 cycles of 3 steps each, composed of 15 s at 95° C, 10 s at 65° C, and 1 min at 76 °C. For the amplification of the 279-bp 16S rDNA fragment of *C. perfringens*, the annealing step was 15 s at 60 °C instead of 10 s at 65 °C. Fluorescent products were detected at the last extension step of each cycle. For both primer sets, a standard curve was generated with DNA from a pure *C. perfringens* culture. The DNA concentrations used were respectively 10, 100, and 1000 pg/mL, and these concentrations were plotted against the C_T value. The GeneAmp 5700 Sequence Detection System then generated a standard curve comparison with the log DNA concentration for all standards and determined the DNA concentration of unknowns by interpolation. All standards and unknowns from ileal mucosal samples were analyzed in triplicate. The concentration of *C. perfringens* in the ileal mucosal samples is expressed as the average percentage of *C. perfringens* DNA to total eubacterial DNA.

Statistical analysis

For DGGE band number and the quantification of *C. perfringens*, means and SEMs were calculated for each piglet from each diet, and treatment effects were compared by Student's *t* tests with the DATA ANALYSIS package from Microsoft EXCEL (Redmond, WA). Statistical analyses of bacterial growth and acidomucolytic activity were performed with the general linear model procedure in SAS (version 6.09; SAS Institute, Cary, NC). A 2 (treatment: TEN compared with TPN) by 4 (days 0, 1, 2, and 3) factorial analysis of variance with interaction was used to compare differences due to diet (TEN compared with TPN) between and within all days. Diet-dependent differences were determined by the Tukey test with an assigned P value of < 0.05.

RESULTS

PCR-DGGE analysis of ileal MAB diversity

To investigate the effect of mode of nutrition on mucosal microbiota profiles, total genomic DNA was isolated from individual ileal samples from 7-d-old TEN and TPN piglets and used as a template for PCR amplification of the V3 regions of bacterial 16S rDNA followed by DGGE analysis. Representative DGGE banding patterns are presented in **Figure 1**A. Ileal microbiota profiles were relatively complex in both TEN and TPN samples. The number of DGGE bands in any individual lane ranged from 3 to 18. A greater number of bands was detected in TPN ($\bar{x} \pm$ SD: 14 ± 6) than in TEN ileal samples (10 ± 4) (Figure 1A), although this difference was not significant ($P < 0.23$). Sample TEN3 was not included for further analysis of the DGGE gel and subsequent molecular analyses because PCR of DNA from this sample did not yield visible bands.

Because it is difficult to interpret bacterial community profiles with respect to both the number and relative diversity of distinct assemblages by visual inspection of the gel, DGGE patterns were first displayed as linear plots (**Figure 2**) and then subjected to cluster analysis with the use of the PHYLIP software, as described above. Visual analysis of the linear plots indicated that MAB profiles among TEN piglets clustered more tightly than among their TPN counterparts. This observation is further illustrated by the tight clustering pattern within TEN groups (spheres) after cluster analysis of the TEN-TPN MAB profiles (Figure 1B), compared

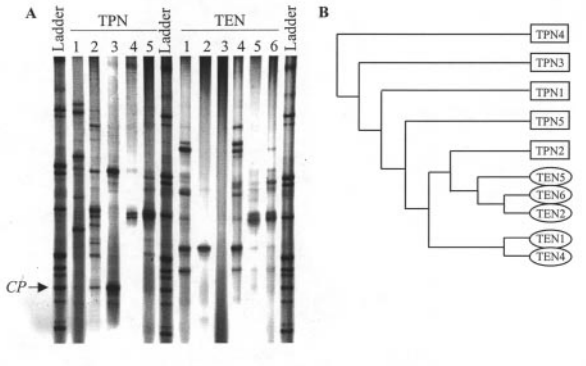

FIGURE 1. A: Denaturing gradient gel electrophoresis (DGGE) profiles generated from polymerase chain reaction (PCR)–amplified V3-16S ribosomal DNA (rDNA). DNA fragments were obtained by PCR amplification of genomic DNA extracted from mucosal samples collected from the ileum of 5 piglets nourished by total parenteral nutrition (TPN1–5) and of 6 nourished by total enteral nutrition (TEN1–6). The ladder was constructed as described in Materials and Methods and contains, from top to bottom, the following: *Bacteroides fragilis, B. thetaiotaomicron, Desulfovibrio vulgaris, Streptococcus bovis, Escherichia coli* K12*, Ruminococcus albus* 7, *D. desulfuricans*, *Clostridium perfringens* (*CP*) as indicated by the arrow, *R. albus* 8, *C. parvum*, *Fibrobacter succinogenes*, and *Bilophila wadsworthia*. B: Dendrogram representing the degree of relatedness among V3-16S rDNA banding patterns of individual animals. The dendrogram was constructed with PHYLIP analysis software (16) and is based on the migrational distances of the V3-16S rDNA PCR amplicons within the DGGE gel.

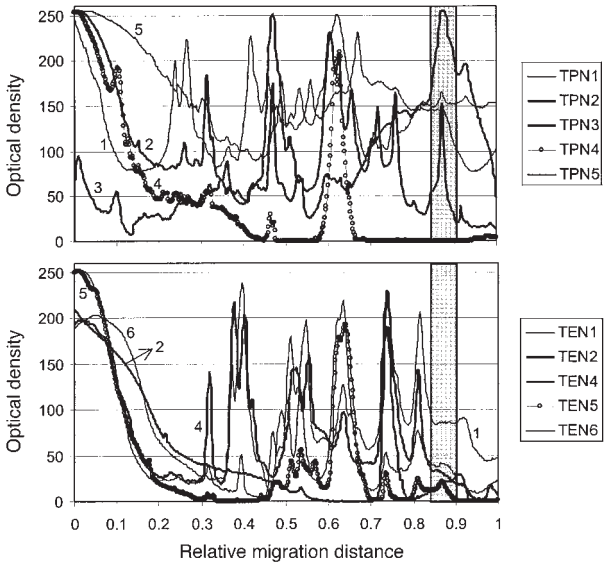

FIGURE 2. Linear plots of total parenteral nutrition (TPN; top) and total enteral nutrition (TEN; bottom) V3-16S ribosomal DNA denaturing gradient gel electrophoresis (DGGE) bacterial profiles generated by the DIVERSITY DATABASE version 2.1 of The Discovery Series (Bio-Rad, Hercules, CA). Numbers on the *x* axis reflect the relative migration distance of each polymerase chain reaction (PCR)–DGGE band, and the *y* axis is the peak height, indicating the intensity (optical density) of each band. Peaks within the box in the TPN panel are DGGE bands identified via sequence analysis as *Clostridium perfringens*. The absence of peaks in the box in the TEN panel indicates that the concentration of *C. perfringens* in the TEN samples fell below the detection limit of PCR-DGGE analysis.

with the distinct branching pattern within TPN groups (squares). Moreover, TPN and TEN DGGE banding patterns clustered separately and grouped according to mode of nutrition (Figure 1B). This result shows that the presence of exogenous nutrients in the ileal lumen superimposed a level of bacterial species variation on animal-to-animal differences, which must vary according to the nature of host-derived substrates.

Analysis of DGGE gels further showed the TPN-specific presence of *C. perfringens* in 4 of 5 TPN samples, as shown by comparison of the migration distance of the *C. perfringens* band (Figures 1 and 2) in the DGGE ladder. Further sequence analysis of the "CP" bands in the TPN ileal samples confirmed their identification as *C. perfringens*.

PCR-DGGE analysis of bacterial diversity after mucin enrichment

The relative diversity of potentially mucolytic bacterial assemblages from TPN and TEN ileal samples was compared by enrichment culture on mucin-limited medium, followed by PCR-DGGE analysis of resultant bacterial communities. For both TEN and TPN samples, petri plates (1×10^{-2} dilution) contained 20–200 colonies after 5 d of cultivation. The colonies differed substantially in morphology and size, which may reflect a differential efficacy of mucin utilization by different bacterial species or, in the case of small colonies, nonspecific growth on other medium constituents. Bacterial community profiles after growth on mucin-limited medium are shown in **Figure 3**A.

Community profiles of potential mucolytic bacteria from TEN ileal samples were more complex than the MAB community profiles (19 ± 3 compared with 10 ± 4 MAB 16S rDNA bands, respectively; $P < 0.05$), whereas community profiles of potential mucolytic bacteria from TPN ileal samples did not increase in complexity compared with profiles from MAB (14 ± 2 compared with 14 ± 6 MAB 16S rDNA bands, respectively). The additional and novel 16S rDNA bands detected after cultivation on mucin-limited medium apparently belonged to ileal bacteria that were present in numbers below the detection limit of direct DGGE analysis. That result indicates that the ileum of TEN piglets harbored a greater variety of bacteria than did the ileum of TPN piglets. Enrichment on mucin also promoted the growth of *C. perfringens*, as shown by the appearance of a *C. perfringens* band in all TPN samples and 5 of 6 TEN samples (Figure 3A). One of the TEN piglets exhibited a uniquely low number of 16S rDNA bands (piglet TEN3, 7 bands) relative to the remainder of the TEN group and thus was omitted for statistical and cluster analyses.

Cluster analysis of the DGGE banding patterns showed that TPN (squares) and TEN (spheres) mucolytic bacterial assemblages clustered separately and grouped according to mode of nutrition (Figure 3B), as also observed for the comparison of ileal MAB communities. Thus, potentially mucolytic bacterial communities were different in the ileum of TPN than of TEN piglets.

Assessment of acidomucolytic activity

The effects of enteral compared with parenteral nutrition on bacterial acidomucolytic activity (ie, the capacity to degrade acidomucins) were assessed, given the observed increase in acidomucin-containing goblet cells in TPN compared with TEN animals (5). Assay results are presented in **Figure 4**. Bacterial growth

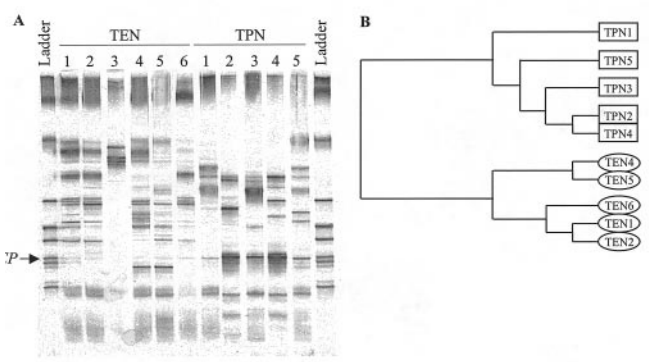

FIGURE 3. A: Denaturing gradient gel electrophoresis (DGGE) profiles generated from polymerase chain reaction (PCR)–amplified V3-16S ribosomal DNA (rDNA). DNA fragments were obtained by PCR amplification of genomic DNA extracted from mucus-associated bacteria from ileal total parenteral nutrition (TPN) and total enteral nutrition (TEN) samples after enrichment on medium 10 containing mucin (10 g/L) as the sole carbon and energy source. The ladder was constructed as described in Materials and Methods and contains, from top to bottom, the following: *Bacteroides fragilis, B. thetaiotaomicron, Streptococcus bovis, Escherichia coli* K12, *Ruminococcus albus* 7, *Clostridium perfringens* (*CP*) as indicated by the arrow, *R. albus* 8, and *C. parvum.* B: Dendrogram representing the degree of relatedness among V3-16S rDNA banding patterns of mucin-cultivated bacteria isolated from ileal mucosal samples of individual animals. The dendrogram was constructed with PHYLIP analysis software (16) and is based on the migrational distances of the rDNA PCR product within the DGGE gel (shown in panel A).

(curves in Figure 4) was observed and began to plateau by day 2 for each ileal sample tested. Acidomucin concentrations decreased during the first 3 d of culture from 1 g medium/L to concentrations of 0.39–0.68 g/L, showing that bacterial growth was associated with acidomucin degradation (bars in Figure 4). Acidomucolytic activity did not vary significantly between the TPN and the TEN ileal samples ($P < 0.1677$) but differed significantly between

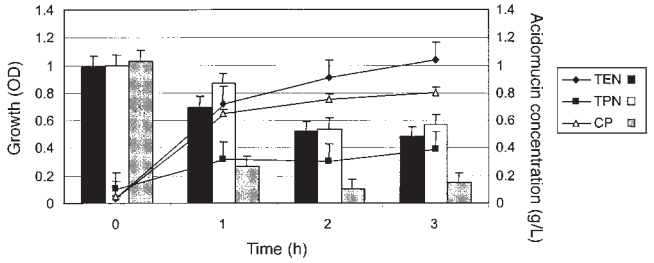

FIGURE 4. Mean (±SEM) acidomucolytic activity (shaded bars) and growth (curves) displayed by 3 total parenteral nutrition (TPN 1, 4, and 5) and 3 total enteral nutrition (TEN 1, 2, and 5) mucosal samples and *Clostridium perfringens* (CP) in pure culture. Cultures were grown anaerobically in medium 10 modified to contain mucin (10 g/L) as the sole carbon and energy source. Disappearance of acidomucin as a function of time was calculated by alcian blue precipitation of the total acidomucin contained in the medium. Bacterial growth was monitored by measurement of the optical density (OD) at 600 nm. Acidomucolytic activity did not vary significantly between the TPN and the TEN ileal samples ($P < 0.1677$) but differed significantly between days ($P < 0.0001$). Bacterial growth rate was significantly lower in the TPN than in the TEN mucosa samples ($P < 0.0004$). There was also a significant interaction ($P < 0.0407$) between time and the mode of nutrition for bacterial growth of ileal samples but not for acidomucolytic activity ($P < 0.6905$).

TABLE 1
Effect of mode of nutrition on the number of mucolytic and sulfatase-positive bacteria, expressed as a percentage of total mucus-associated bacteria[1]

Medium	Mode of nutrition	
	TPN	TEN
	%	
Mucolytic	62	33
Glucose-6-sulfatase positive	37	30
Galactose-6-sulfatase positive	15	8

[1] TPN, total parenteral nutrition; TEN, total enteral nutrition. Aliquots (100 μL) of pooled ileal mucosal samples from 3 TPN or 3 TEN piglets were inoculated on nutrient agar plates containing medium 10 with mucin (10 g/L) and a mix of simple carbohydrates (glucose and galactose, 2 g/L each) as carbon sources. Isolated bacterial colonies (n = 100/mode of nutrition) were then transferred to medium containing mucin, glucose-6-sulfate, or galactose-6-sulfate, after which bacterial growth was assessed by measurement of the optical density at 650 nm after 2 d of anaerobic cultivation.

days ($P < 0.0001$). However, bacterial growth rate was significantly lower in the TPN than in the TEN mucosa samples ($P < 0.0004$; Figure 4), possibly reflecting the less dense bacterial population in TPN than in TEN samples (*see* below). There was also a significant interaction ($P < 0.0407$) between time and the mode of nutrition for bacterial growth of ileal samples but not for acidomucolytic activity ($P < 0.6905$).

For both TPN and TEN samples, bacterial growth rates were most rapid during the first 24 h of culture. That period did not correlate consistently with the rate of acidomucin disappearance, in particular for TPN samples, which may indicate initial selective utilization of neutral mucin moieties.

Quantification of mucolytic and sulfatase-harboring bacteria

To better resolve the differences in mucolytic potential of MAB from TPN compared with TEN ileal samples, the ratio of mucolytic bacteria to total MAB was assessed. After 5 d of anaerobic growth on habitat-simulating medium 10, plates for both diet groups were compared to determine the dilution that yielded at least 100 clearly visible and distinguishable bacterial colonies, which were chosen for analysis. For TPN mucosal samples, this dilution was 10^{-2}; the serial dilution that yielded ≈100 colonies for TEN mucosal samples was 10^{-3}. These observations indicate that bacterial density was greater in TEN than in TPN animals. Each of the 100 colonies from each diet group was then tested for growth on medium containing mucin as a selective substrate. The results of the assays are provided in **Table 1**. Colonies able to grow on mucin represented 62% of total cultivable MAB from TPN ileal samples, whereas 33% of total cultivable MAB from TEN ileal samples were mucolytic, indicating that a greater percentage of bacteria were able to grow on mucin in TPN samples than in TEN samples.

Bacterial growth on galactose-6-sulfate and glucose-6-sulfate was also analyzed as an initial comparison of the ability of TPN compared with TEN ileal bacteria to degrade sulfomucin. Thirty-seven percent of total cultivable MAB from TPN samples grew on medium containing glucose-6-sulfate as the sole carbohydrate source, compared with 30% of total cultivable MAB from the TEN ileum. Fifteen percent of total cultivable MAB in TPN samples grew on galactose-6-sulfate–containing medium, and 8% of total cultivable MAB from the TEN ileum were able to use this sulfated

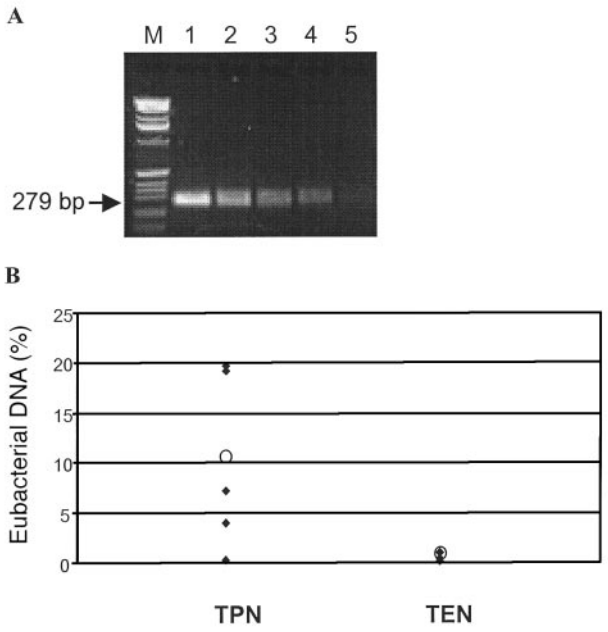

FIGURE 5. A: Gel electrophoresis of 279–base pair (bp) quantitative real-time polymerase chain reaction (PCR) amplicons generated from genomic DNA derived from a pure culture of *Clostridium perfringens*. PCR amplicons in lanes 1–3 are generated from, respectively, 10-fold serial dilutions of genomic *C. perfringens* DNA, and amplicons derived from genomic DNA from representative total parenteral nutrition (TPN) and total enteral nutrition (TEN) ileal mucosal samples are present in lanes 4 and 5, respectively. PCR amplification of genomic DNA from 2 negative controls (*Bacteroides fragilis* and *Lactobacillus oris*) did not yield PCR amplicons (data not shown). Specificity of the 279-bp PCR amplicons was also verified by sequence analysis. M corresponds to a 1-kb ladder (Gibco BRL, Rockville, MD). B: Quantitative real-time PCR analysis of the concentration of *C. perfringens* in TPN and TEN ileal mucosal samples expressed as a percentage of total eubacterial DNA. Each symbol represents one piglet. The average concentration of *C. perfringens* in both TPN and TEN samples is indicated by ○.

monosaccharide. The higher percentage of bacterial growth on glucose-6-sulfate than on galactose-6-sulfate likely reflects preferential use of glucose after cleavage of the 6-*O*-sulfate group (17). Bacterial growth was, however, generally less substantial than growth on mucin-limited medium but was not observed on medium devoid of carbohydrate substrate. Thus, although the absolute number of mucolytic and acidomucolytic bacteria may have been less in TPN than in TEN piglets, MAB in TPN samples were enriched in bacteria possessing both mucolytic and sulfatase activities.

Quantification of *Clostridium perfringens* in ileal mucosa

A quantitative real-time PCR-based method was developed to specifically measure *C. perfringens* concentrations in TPN and TEN ileal samples, as described in Materials and Methods. Both *C. perfringens*–specific and eubacterial primer sets yielded a single PCR product of the correct size, respectively 279 bp for the *C. perfringens*-specific primer set **(Figure 5A)** and 220 bp for the eubacteria-specific primer set when visualized on an agarose gel (data not shown). Regression values for the *C. perfringens*–specific primer set and the eubacterial primer set when log DNA concentrations were plotted against C_T values were respectively 0.98 and 0.99 as determined by serial dilution of *C. perfringens* DNA in the DNA concentration range of 10–1000 pg/mL.

The percentage of *C. perfringens* relative to total eubacterial DNA was significantly higher in TPN (10.0 ± 8.9%) than in TEN ileal samples (excluding sample TEN3; 0.4 ± 0.4%; $P < 0.02$; Figure 5B). The relative percentage of *C. perfringens* ranged from 0.1–0.9% for the TEN ileal samples, compared with 0.3–19.9% for the TPN ileal samples (Figure 5B). These results are consistent with the results from the DGGE analysis (Figure 1), despite the semiquantitative property of DGGE analysis. The densest *C. perfringens* DGGE bands were found in the TPN1, TPN2, and TPN3 lanes (Figures 1 and 2), and the respective *C. perfringens* concentrations determined by real-time PCR were 19.9%, 7.1%, and 19.2%. The *C. perfringens* concentrations in the TPN4 and TPN5 ileal samples were respectively 0.3% and 4.0%, consistent with the absence of a *C. perfringens* band in the TPN4 lane and the presence of a weakly visible *C. perfringens* band in the TPN5 lane. In TEN samples, *C. perfringens* concentrations contributed, at most, 0.9% of total eubacterial DNA, which was at or below the detection limit for DGGE analysis. This finding further illustrates the sensitivity of the real-time PCR assay, because detection and quantification of *C. perfringens* was possible at concentrations below 0.1% of total eubacterial DNA.

Assessment of mucolysis by *Clostridium perfringens*

C. perfringens was able to grow on medium with mucin as a substrate, indicating that this organism possesses mucolytic activity. *C. perfringens* showed substantial acidomucolytic activity, with 72% of acidomucin in the medium disappearing after 1 d of culture, corresponding to an ≈4-fold increase in optical density of culture supernatant (Figure 4). *C. perfringens* growth in pure culture was therefore coupled more closely to acidomucin disappearance than was observed for any of the mixed ileal samples. Limited *C. perfringens* growth was further observed on both glucose-6-sulfate and galactose-6-sulfate, whereas growth on medium devoid of carbohydrate substrate was not observed.

DISCUSSION

The data presented indicate that, in a piglet model, a complex bacterial population is maintained in the TPN ileum, despite the lack of enteral nutrients. In fact, ileal bacterial community structure was equally complex in the ileum of TEN and TPN piglets. Bacterial population profiles, however, clearly clustered according to the mode of nutrition of the host. Thus, we tested the hypothesis that mucolytic potential may vary among the bacteria resident in the TEN compared with the TPN ileum. Approximately twice as many MAB from TPN samples as from TEN samples were able to grow on mucin as a sole carbon and energy source, indicating that mucolytic bacteria were initially selected in the ileum of TPN piglets. Moreover, upon enrichment of MAB on mucin-limited medium, the number of bacterial species in TEN samples, as represented by the number of PCR-DGGE bands, was increased in comparison to the original TEN ileal samples, whereas the number of PCR-DGGE bands was approximately the same before and after mucin-based cultivation for the TPN ileum. Together, these data indicate that the complex microbiota found in the TPN ileum was enriched with mucolytic bacteria.

It was of importance also that one-third of MAB from the normal microbiota of the TEN ileum were mucolytic, suggesting that mucolysis may be widespread among gut bacteria. This potentially

important clinical issue has received limited attention. In healthy adults, ≈1% of total cultivable fecal bacteria possessed mucolytic potential and were identified as strains of *Ruminococcus torques*, *R. gnavus*, and *Bifidobacterium* species, ie, Gram-positive obligately anaerobic, nonsporulating, and nonpathogenic fecal bacteria (18, 23, 24). Although pig gastric mucin was the source of mucin substrate in both the present work and in the earlier work with human feces, the sources of inocula differed substantially in these studies. Bacterial populations in the ileum, particularly of a neonatal animal, may have been more adapted to degrade gastric mucin than bacteria from adult human feces, given the general similarities in gastric and ileal compared with colonic mucins (25). This possibility is further supported by evidence that in the pig ileum, Gram-positive anaerobes, including representatives of the phylotypes identified previously as being mucolytic, are prominent (26).

In addition, the relatively low percentage of mucolysis in human feces might simply reflect the relative abundance of the Gram-negative *Bacteroides-Prevotella* group in the large intestine (27), because an extensive survey of *Bacteroides* strains isolated from human colonic contents did not identify strains capable of degrading intestinal mucin (28). However, mucolysis has been detected in both the Gram-negative *Bacteroides-Prevotella* group (*Prevotella* strain RS2, *B. fragilis*, *B. thetaiotaomicron*; 28–31) and in spore-forming Gram-positive bacteria (*C. septicum*; 32). In any case, the molecular ecology data show diversity among mucolytic intestinal bacteria. PCR-DGGE separates 16S rDNA amplicons based on their GC content, and thus the wide range of bands from mucin-selected bacteria indicates that mucolytic potential is not restricted to one particular group of bacteria. The present evidence that the ileum of the neonatal piglet is colonized by a diverse population of mucolytic bacteria provides impetus for additional investigation of the genetic and biochemical basis of bacterial mucolysis in the human gastrointestinal tract both in space and time.

The constant availability of intestinal mucus relative to the variability of enteral nutrients associated with oral feeding makes mucin an ideal substrate for supporting bacterial growth. In this regard, mucolytic bacteria would have a competitive advantage during times of nutrient deprivation such as during malnutrition or TPN (18, 33–35). The degree to which bacterial mucolysis is constitutively active in the presence of enteral nutrients is not clear, although it is certain that mucolytic bacteria also use more readily available nutrients (28). The provision of more readily soluble nutrients would likely enable growth of mucolytic bacteria at reduced costs relative to growth on the more complex mucin substrate. Consistent with that idea, mucolytic enzyme expression is suppressed by high glucose concentrations but induced in the presence of mucin and low glucose concentrations (31, 32).

The stability of a bacterial ecosystem is directly related to its diversity index, with a decrease in diversity resulting in a less stable ecosystem (36). Cluster analysis showed that TEN bacterial profiles grouped more tightly than did their TPN counterparts. In addition, the greater number of 16S rDNA bands in TEN ileal samples after mucin enrichment shows that the TEN ileum indeed contained a more diverse bacterial population than did the TPN ileum. These results support the idea that oral feeding allows for a stable association between the host and its endogenous microbiota, which contrasts with the circumstances of TPN. Destabilization of the bacterial ecosystem with TPN may then promote the proliferation of one or more bacterial species able to respond to changes in substrate availability and chemotype. We investigated this possibility by examining the possible enrichment of acidomucolytic bacteria and the opportunistic pathogen *C. perfringens*.

Acidomucolytic potential was examined because Ganessunker et al (5) observed an increase in acidomucins (sialo- and sulfomucins) in the TPN ileum compared with the TEN ileum. Increased mucin sulfation by the host may be an adaptive response to compromised integrity of the mucosal barrier because sulfated mucins are more resistant to bacterial breakdown than are their nonsulfated counterparts (21, 37, 38). Similarly, sialic acid is thought to strengthen the mucus gel by maintaining the viscoelastic properties of mucins (39). Both TEN and TPN ileal samples exhibited acidomucolytic activity when cultivated on mucin-limited medium. Critically, the percentage of total cultivable MAB able to grow on sulfated monosaccharides was consistently higher in the TPN ileum than in the TEN ileum, presumably reflecting the selection of acidomucolytic bacteria in the TPN ileum. The selective enrichment of bacteria able to degrade acidomucins would thereby counteract efforts by the host to strengthen the mucus layer through increased acidomucin synthesis (5, 37, 38), further compromising the epithelial barrier (2–5, 40, 41). The enhanced bacterial mucolysis in conjunction with local inflammation and goblet cell expansion in response to TPN suggests that the mucus layer may be maintained by the host at a homeostatic set point. To address this intriguing hypothesis, the extent to which intestinal bacteria or host immune cells (either or both) may contribute bioactive factors affecting goblet cell dynamics must be determined (42).

The potential for the proliferation of a single bacterial species fully adapted for growth on mucus in the TPN ileum is reflected by the TPN-specific detection of *C. perfringens* by PCR-DGGE. On the other hand, the detection of *C. perfringens* in TEN only after enrichment on mucin indicates that with enteral feeding, *C. perfringens* growth is minimal, but its apparent mucolytic potential renders this organism more fit in a mucin-selective environment such as the TPN intestine. To determine whether *C. perfringens* was indeed selected in the TPN ileum, we developed a real-time PCR-based method, which enables the rapid and sensitive quantification of specific bacterial species expressed as a percentage of the total bacterial population in a particular ecosystem. Results from this assay clearly showed that *C. perfringens* growth was significantly greater in the TPN than in the TEN ileum. In additiona, *C. perfringens* grew rapidly on medium with mucin as a selective substrate, accompanied by an almost complete degradation of alcian blue–stained mucin constituents after 1 d of culture, indicating significant acidomucolytic potential, consistent with earlier reports (43, 44). Although only limited growth was observed on individual sulfated monosaccharides, preliminary evidence indicates that *C. perfringens* growth on sulfated sugars plus mucin is greater than on mucin alone (B Deplancke and HR Gaskins, unpublished data, 2001). This observation is consistent with findings by Macfarlane et al (32), which suggest that mucin upregulates sulfatase expression and in this way renders sulfated sugars more accessible for degradation, resulting in increased bacterial growth.

Because of its production of toxins such as β-toxin and enterotoxin, *C. perfringens* has been recognized as an opportunistic pathogen possibly contributing to necrotizing enterocolitis in low-birth-weight infants, to infectious diarrhea, and to enteritis necroticans (45–49). Normally, β-toxin is rapidly inactivated by trypsin in the small intestine (50). Small-intestinal trypsin concentrations are, however, significantly decreased in the intestine of

low-birth-weight infants (51) and with TPN (52), suggesting that *C. perfringens* production of β-toxin in the TPN ileum may lead to local inflammation. Also, enterotoxin production is directly associated with sporulation in *C. perfringens* (48). Mechanisms leading to sporulation and thus enterotoxin production remain mostly undefined. Sporulation in some bacteria (eg, *Bacillus subtilis*) is dependent on a critical cell density and hence proliferation (53). The present data indicate that *C. perfringens* enjoys a growth advantage when exogenous nutrients are withheld because of its strong mucolytic potential, thus representing a crucial step in the pathogenesis of this organism. This finding may be particularly relevant to the practice of using TPN both to prevent necrotizing enterocolitis and to treat infants who develop the disease (1). In fact, the finding that incremental enteral feeding of dilute infant formula or breast-feeding decreased the overall incidence of necrotizing enterocolitis in parenterally nourished newborns (54) is consistent with the present data and the hypothesis that exogenous nutrients may suppress the mucolytic activity of *C. perfringens* as well as other mucolytic commensals. In contrast, other studies indicate a significant association between the development of necrotizing enterocolitis and enteral feeding (55), and the issue requires further study. The importance, however, of determining the extent to which bacterial mucolysis contributes to TPN-associated complications in newborns and other patient populations is clear.

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