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An Improved DNA Isolation Method for Metagenomic Analysis of the Microbial Flora of the Human Intestine

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The efficiency with which lysis of five strictly anaerobic and six facultatively anaerobic bacterial species, all well-known human colonic commensals, were lysed was tested using a reference method for general metagenomic analysis and an improved method that involves higher levels of lysozyme and proteinase K, as well as the addition of achromopeptidase. Ten species were lysed with an efficiency of >80% by the reference method, while the lytic efficiency for *Clostridium ramosum* JCM 1298^T was <50%. The lytic efficiency of the improved method for *C. ramosum* JCM 1298^T was 82.5%. Similarly, five samples of human feces were tested with these methods, as well as with the QIAamp DNA stool mini kit. Although the efficiency of lysis of the microbes recovered from the fecal samples fluctuated depending on the sample in the cases of the reference method (13.3–84.6%) and QIAamp DNA stool mini kit (38.8–69.2%), the improved method gave stable and high-level lysis (>90%) for all the fecal samples. Accordingly, since the DNA samples isolated by the improved method can reflect nearly true genomic information in the microbial flora, our improved method should be applicable to metagenomic analyses, not only for bacteria in the human intestine but also for bacteria in other environments.

Key words: metagenomics, DNA isolation, human feces, microbial flora

Metagenomics involves the application of modern genomic techniques to achieve a better understanding of the microbial flora in the gut environment without the need for isolation or laboratory cultivation of individual species. This approach originates from the culture-independent retrieval of ribosomal RNA genes from various environments, as pioneered by Pace and colleagues two decades ago[12]. A typical metagenomics project begins with the construction of a clone library from the DNA retrieved from an environmental sample. The recent remarkable increase in the sequencing capacity in worldwide sequencing centers has facilitated metagenomic analyses of entire clone libraries that are derived directly from various environments

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using the whole-genome shotgun sequencing method. To date, at least five shotgun sequencing projects involving prokaryotic communities in various natural environments have been completed[6,7,18–20].

Recently, the target for metagenomics of prokaryotic communities has been extended to a human and an animal, and the first report of metagenomic analysis of the human intestinal microbial flora has been published[6]. The microbial flora of humans is a particularly interesting target because it is known that gastrointestinal tract communities play important roles in health and disease. From numerous studies of enteromicrobes, prokaryotic species are estimated to be present in more than 1000 species and ~10^{13} cells in the human intestine[23]. Detailed information regarding the human intestinal microbial flora and its gene pool is likely to emerge from metagenomic studies in the near future.

The most basic and essential process in any metagenomic analysis is the isolation of DNA samples from the microbial communities and the construction of the DNA library. Unfortunately, the experimental procedures for DNA isolation have not received the attention they deserve, especially with respect to the lytic processes needed for DNA isolation. The recovery of DNA samples representative of each of the bacteria present in the microbial flora represents a major obstacle, as each flora consists of different prokaryotic species with different susceptibilities to lytic enzymes and chemicals. Gill and colleagues have pointed out that the reason why the relative levels of *Bacteroides* sequences in random assemblies and clone libraries conflict with the data obtained in other studies may be the known biases associated with fecal sample lysis and the DNA extraction method used in their study[6]. Most metagenomic analyses have employed an SDS-based extraction method in combination with freezing-thawing or grinding with a mortar and pestle in the presence of liquid nitrogen or heating, without lytic enzymes[22], although Venter and co-workers used 150 μg/ml of lysozyme in their study of microbial communities in the Sargasso Sea[20]. In addition, the concentrations of SDS added to the cell suspensions have ranged form 0.1% to 1% without any particular basis, depending on the specific metagenomic project. Since the enzymatic lysis method is not applicable to all species, especially Gram-positive bacterial and archaeal species, mechanical cell disruption techniques are often used effectively for DNA isolation. However, mechanical disruption often leads to DNA fragmentation, which makes it difficult to prepare genomic libraries with large insertions using lambda phages and fosmids.

To overcome these problems, we attempted to improve the enzymatic lysis method, using prokaryotic cells recovered from human fecal samples as the test system. The lytic efficiency and quality of the extracted DNA are compared for: (1) the improved method; (2) the reference method used for the metagenomic study of the Sargasso Sea; and (3) the QIAamp DNA stool mini kit, which is frequently used for PCR amplification with 16S rDNA primers.

Materials and Methods

Reference strains and cultivation

Five strict anaerobes and one facultative anaerobe (Table 1) were chosen as representatives of human colonic commensals for the evaluation of the reference lysis method used in the metagenomic analysis of the microbial communities in the Sargasso Sea[20]. The first three strains (*Clostridium ramosum* JCM 1298^T, *C. inoculum* JCM 1292^T, and *Bacteroides vulgatus* JCM 5826^T) were cultivated in 5 ml of GAM (Gifu anaerobic medium) liquid medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) at 37°C for 18 h. *Lactobacillus gasseri* JCM 1131^T, *Eubacterium cylindroids*

Table 1. Bacterial reference strains used in this study

Strain	Medium	Source	Reference
Absolute anaerobic			
Clostridium ramosum JCM 1298^T	GAM	Infant & adult feces	(8)
Clostridium inocuum JCM 1292^T	GAM	Appendiceal adscess	(16)
Bacteroides vulgatus JCM 5826^T	GAM	Human feces	(10)
Eubacterium cylindroides JCM 10261^T	MRS	Human feces	(2)
Bifidobacterium bifidum JCM 1255^T	MRS	Feces of breast-fed infant	(13)
Facultative anaerobic			
Lactobacillus gasseri JCM 1131^T	MRS	Human intestine	(11)

^{T} Type strain

Table 2. Bacterial strains isolated from human fecal samples

Strain	Species	Medium	Sample	Age
AE1-2	*Escherichia coli*	Schaeffer/LB	Male A	35-year-old
AE4H	*Staphylococcus pasteuri*	HA	Male B	22-month-old
AE1-3	*Corynebacterium nigricans*	Schaeffer/LB	Male A	35-year-old
AE4-3	*Bacillus subtilis*	Schaeffer/LB	Male B	22-month-old
CKY	*Enterococcus gallinarum*	MRS	Female A-1	24-year-old

Male samples, A and B were collected on November 2005. Female A-1 is the same sample listed in Table 3.

JCM 10261^T, and *Bifidobacterium bifidum* JCM 1255^T were cultivated in 10 ml of MRS broth for the cultivation of lactobacilli (Oxoid Ltd., Hampshire, UK) at 37°C for 18 h. Bacteria from each culture were harvested and washed with PBS (phosphate-buffered saline, pH 7.4) with centrifugation at 5,000×*g* at 4°C. The washed bacterial cells were resuspended in 5 ml of TE (10 mM Tris-HCl, 1 mM EDTA) buffer (pH 8.0) and used for cell counting and lysis.

Fecal samples

Two male fecal samples (Male A and B) listed in Table 2 were supplied by a Japanese volunteer family (35-year-old father and 22-month-old boy). The fecal samples of 24- and 39-year-old females (Female A-1, A-2, and B) and a male infant at 3 months and 4 months of age (Male C-1 and C-2) were purchased from Crossfield-Bio Inc. (Tokyo, Japan) (Table 3). All samples were supplied based on informed consent for the purpose of this study. The three samples, Male A, Male B, and Female A-1, were used for isolation of human colonic commensals and the other samples were used for isolation of bacterial DNA.

Recovery of bacteria from fecal samples

The collected fecal samples were placed immediately in an anaerobic bag and stored under anaerobic conditions at −80°C until used. Three grams of wet fecal sample was suspended vigorously in a 50-ml Falcon tube that contained 45 ml of PBS. The suspension was divided into three aliquots (15 ml) and 15 ml of the suspension was filtered through a 100-μm-mesh nylon filter using agitation with two plastic bars. The debris on the filter was washed twice with 10 ml of PBS with agitation from the plastic bars. The remaining two aliquots were filtered through new filters and washed in the same manner. The three filtrates were centrifuged at 5000×*g* for 10 min at 4°C, and each precipitate was washed with 35 ml of PBS and 35 ml of TE buffer (pH 8.0) under the same conditions. All the precipitates were combined in a single 50-ml Falcon tube that contained 10 ml of TE buffer and mixed vigorously. This suspension was used for the isolation of bacteria, cell counting, and lysis.

Table 3. Fecal samples used for isolation of DNA

Sample	Age	Sampling date
(Adult)		
1. Female A-1	24-year-old	February 2006
2. Female A-2	24-year-old	June 2006
3. Female B	39-year-old	July 2006
(Infant)		
4. Male C-1	3 month-year-old	April 2006
5. Male C-2	4 month-year-old	May 2006

Female A-1 and A-2 were collected from the same 24-year-old female on deferent sampling date. Male C-1 and C-2 were collected from the same infant 3 months and 4 months after birth, respectively.

Isolation of bacteria from fecal samples

The fecal samples suspended in 35 ml of PBS as described above were used for the isolation of bacteria. Aliquots (10–100 ml) of the fecal suspension were spread onto LB (Luria-Bertani) agar, MRS agar, HA (Haloarcula) agar (20% NaCl, 2% $MgSO_4 \cdot 7H_2O$, 0.05% $CaCl_2$, 0.0125% $MnCl_2$, 1% yeast extract, and 1.5% agar), and Schaeffer spore-forming agar [0.8% nutrient broth (Becton Dickinson Co., Franklin Lakes, NJ, USA), 0.025% $MgSO_4 \cdot 7H_2O$, 0.1% KCl, 1 μM $FeSO_4$, 1 mM $Ca(NO_3)_2$, 10 μM $MnCl_2$, and 1.5% agar]. Each plate was incubated at 37°C for 2–14 days. Colonies that appeared on the plates were picked up and streaked on new agar plates of the same type for purification. Single isolates were cultivated in 50 ml of the appropriate liquid medium, and these liquid cultures were used for the isolation of chromosomal DNA using Genomic-Tip 5000G (Qiagen, Tokyo, Japan).

16S rDNA sequencing

Polymerase chain reaction (PCR) amplification of the 16S rDNA was performed with a DNA thermal cycler (model 9600; PerkinElmer, Wellesley, MA, USA) using a

50-µl PCR mixture under the conditions recommended by the enzyme's manufacturer (Takara, Shiga, Japan) and according to a procedure reported previously[17]. Sequencing of PCR-amplified fragments was performed with the MegaBase 1000 DNA sequencer (GE Healthcare Biosciences K.K., Tokyo, Japan).

Bacterial cell counting

The total number of bacterial cells recovered from the fecal samples was determined before and after lytic treatment. A bacterium counting chamber of with a depth of 0.02 mm (Erma, Tokyo, Japan) was divided into 400 small squares. Each small square measured 5×10^{-5} mm^3. Fifty of the 400 small squares were counted at 600x power (40× objective lens, 15×eye-piece) under a phase-contrast microscope (Leitz DMR model; Leica, Tokyo, Japan). To obtain a representative sample, the cell suspension was diluted to give approximately 10 cells per square. When the cells formed chains, each cell within a chain was counted as one cell. For the cell suspensions treated with lytic enzymes or the QIAamp DNA stool mini kit (Qiagen), only intact cells were observed, with the exception of cell debris and small particles undergoing Brownian motion. The bacterial counting was performed three times for each sample. The number of bacteria in 1 ml of suspension was determined using the following equation: cells counted (50 squares)×8÷0.02×10^3 ×dilution factor.

Lytic treatment

The reference lytic treatment was essentially that used in the metagenomic study of the Sargasso Sea microbial flora[20]. To 10 ml of the cell suspension prepared as described above, 1.5 mg of lysozyme was added. After the cell suspension was incubated at 37°C for 1.5 h with gentle mixing, 2 mg of proteinase K was added and the mixture was incubated at 55°C for 5 min. Subsequently, 1.2 ml of 10% SDS was added to the cell suspension, which was further incubated at 55°C for 1 h with gentle mixing. An aliquot of the enzyme-treated cell suspension (100 µl) was used for cell counting, to assess the efficiency of the lysis.

The improved lysis procedure was basically the same as the reference method, except that the levels of added lysozyme and proteinase K were higher, and achromopeptidase was also added. Achromopeptidase is a bacteriolytic enzyme derived form *Archomobacter lyticus*[9]. Lysozyme (150 mg, to give a final concentration of 15 mg/ml) was added to a 50-ml Falcon tube that contained 10 ml of the bacterial suspension. After 1 h incubation at 37°C with gentle mixing, 30 mg of achromopeptidase was added to the suspension, which was incubated for an additional 30 min at 37°C. In the next step, a 10-fold excess of proteinase K (20 mg) was added to the cell suspension, and the remaining steps were performed as for the reference method. All enzymes used in this study were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). A 100-µl sample of the final suspension was used for cell counting. The subsequent DNA purification was performed according to the standard method used in molecular biology.

Although lysis with the QIAamp DNA stool mini kit was basically performed according to the manufacturer's recommendations, some of the steps were modified for 3-g fecal samples. Three grams of feces was divided into three samples, and one gram each was suspended vigorously in a 50-ml Falcon tube that contained 10 ml of ASL solution prepared from the kit. The fecal suspension was incubated at 95°C for 5 min and stirred vigorously for 15 sec. An aliquot (100 µl) of this suspension was used for cell counting.

To pellet the stool particles, the suspension was centrifuged at 7000×*g* for 5 min at room temperature. Five tablets of InhibitEX were ground using a mortar and pestle and mixed with 10 ml of the supernatant. The mixture was incubated at room temperature for 1 min, and then centrifuged at 7000×*g* for 5 min at room temperature. The supernatant was recovered in a new tube, and then the centrifugation was repeated under the same conditions. The recovered supernatant was used for DNA isolation, and the subsequent steps were performed according to the manufacturer's recommendations.

Results and Discussion

Isolation and identification of fecal isolates

We isolated facultative anaerobic bacteria from human fecal samples supplied by a Japanese family (35-year-old father and 22-month-old boy) using several types of agar media. From the colonies that grew on each agar plate, morphologically different colonies were picked randomly and purified on the same type of agar plate. The 16S rDNA sequence of each isolate was determined and those isolates that showed >99% similarity with a known species, for which the 16S rDNA sequence has been determined, were assigned to the same species. *Bacillus subtilis* was isolated from the samples obtained from the mother and two boys and grown on Schaeffer spore-forming agar medium. All of the isolates showed similarity of >99.9% with the *B. subtilis* strain in the public database, although these isolates were not derived from the Japanese fermented soybean called *natto*, which is produced by *B. subtilis*. The colony mor-

phology of our isolates was clearly different from that of the strain isolated from commercial *natto* and there were major differences in the amount of polyglutamic acid (slime) produced by *B. subtilis*, as well as the smell and taste of the two strains (data not shown). Although Japanese often eat *natto*, these results suggest that the *B. subtilis* strains isolated in this study are not transient but commensal. Indeed, it is known that some *Bacillus* species, including *B. subtilis*, thrive in the human intestine as a member of colonic commensals[1,21]. On the other hand, *Escherichia coli* and

Table 4. Comparison of lytic efficiency of fecal isolates with the two lytic methods

Strain	No treatment cell number (mean±SD)	Reference method		Improved method	
		After treatment		After treatment	
		cell number (mean±SD)	lysis %	cell number (mean±SD)	lysis %
Absolute anaerobic					
Clostridium ramosum (JCM 1298^T)	$5.77\pm1.19\times10^8$	$3.03\pm0.15\times10^8$	47.5	$1.01\pm0.15\times10^8$	82.5
Clostridium innocuum (JCM 1292^T)	$7.20\pm0.66\times10^8$	$1.12\pm0.24\times10^7$	98.4	$<10^6$	99.9<
Bacteroides vulgatus (JCM 5826^T)	$6.49\pm2.71\times10^8$	$<10^6$	99.8<	$<10^6$	99.8<
Eubacterium cylindroides (JCM 10261^T)	$7.21\pm0.66\times10^8$	$<10^6$	99.9<	$<10^6$	99.9<
Bifidobacterium bifidum (JCM 1255^T)	N.C.	$<10^6$	—	$<10^6$	—
Facultative anarobic					
Lactobacillus gasseri (JCM 1131^T)	$2.42\pm0.28\times10^8$	$2.67\pm1.22\times10^6$	98.9	$<10^6$	99.9<
Escherichia coli (AE1-2)	$1.22\pm0.16\times10^{10}$	$<10^6$	99.9<	$<10^6$	99.9<
Staphylococcus pasteuri (AE4H)	$7.41\pm1.59\times10^9$	$1.42\pm0.18\times10^9$	80.8	$7.21\pm0.82\times10^8$	90.3
Corynebacterium nigricans (AE1-3)	$6.19\pm0.71\times10^9$	$8.11\pm1.53\times10^8$	86.9	$<10^6$	99.9<
Bacillus subtilis (AE4-3)	$4.51\pm1.89\times10^9$	$<10^6$	99.9<	$<10^6$	99.9<
Enterococcus gallinarum (CKY)	$3.93\pm0.71\times10^9$	$<10^6$	99.9<	$<10^6$	99.9<

N.C.: Not countable due to multiform cells. The JCM strains were cultivated as described in the text. *Enterococcus gallinarum* CKY was cultivated in 10 ml of MRS liquid medium at 37°C for 18 h. The other strains, *Escherichia coli* AE1-2, *Staphylococcus pasteuri* AE4H, *Corynebacterium nigricans* AE1-3, and *Bacillus subtilis* AE4-3, were incubated with shaking in 10 ml of LB liquid medium at 37°C for 16 h.

Table 5. Comparison of lytic efficiency of bacteria recovered from the human fecal samples with the three methods

Fecal samples	No treatment	Reference method		Improved method		QIAamp kit method	
	cell number (mean±SD) cell/ml (cell/g-feces)	cell number cell/ml (mean±SD)	lysis %	cell number cell/ml (mean±SD)	lysis %	cell number cell/ml (mean±SD)	lysis %
(Adult)							
Sample 1 (Female A-1)	$3.31\pm0.29\times10^{10}$ ($2.21\pm0.19\times10^{11}$)	$1.07\pm0.34\times10^{10}$	67.7	$<10^6$	<99.9	N.T.	N.T.
Sample 1* (Female A-1)	$9.83\pm1.68\times10^9$ ($4.92\pm0.84\times10^{10}$)	$4.91\pm1.07\times10^9$	50.1	$8.80\pm3.56\times10^7$	99.1	$6.07\pm1.36\times10^9$	38.3
Sample 2 (Female A-2)	$1.20\pm0.10\times10^{10}$ ($4.01\pm0.32\times10^{10}$)	$1.04\pm0.02\times10^{10}$	13.3	$1.23\pm0.25\times10^9$	89.8	$4.27\pm0.16\times10^9$	64.4
Sample 3 (Female B)	$1.12\pm0.14\times10^{10}$ ($3.73\pm0.29\times10^{10}$)	$1.73\pm0.45\times10^9$	84.6	$4.45\pm0.88\times10^7$	99.6	$3.45\pm0.74\times10^9$	69.2
(Infant)							
Sample 4 (Male C-1)	$1.07\pm0.29\times10^{11}$ ($3.56\pm0.36\times10^{11}$)	$2.16\pm0.17\times10^{10}$	79.8	$3.80\pm1.23\times10^9$	96.4	$5.89\pm0.74\times10^{10}$	45.0
Sample 5 (Male C-2)	$3.90\pm0.58\times10^{10}$ ($1.30\pm0.19\times10^{11}$)	$2.81\pm0.24\times10^{10}$	27.9	$8.00\pm6.90\times10^5$	<99.9	$2.24\pm0.05\times10^{10}$	42.6

* Sample 1 is frozen for one month after first use. N.T.: not tested.

Corynebacterium nigricans were isolated from the stool sample of the father grown on the Schaeffer spore-forming agar medium (Table 2). Although first isolated from the female urogenital tract, which is a natural habitat for a multitude of commensal microbes[14], *C. nigricans* may be a commensal organism that resides not only in the female urogenital tract but also in the human intestine, since this species was isolated from the fecal sample of one of the males in the present study. The 16S rDNA sequence of strain AE4H isolated from the samples obtained from the 22-month-old boy and grown on slightly modified HA medium that contained 20% NaCl showed 99% similarity to *Staphylococcus pasteuri*, which was originally isolated from humans and animals, as well as from food specimens[3]. It took at least two weeks for strain AE4H to form a colony on HA agar, whereas this strain grew faster in medium that contained a low concentration of NaCl, such as LB medium. Thus, strain AE4H is halotolerant, as is the case for staphylococci. On the other hand, strain CKY, which showed 99% similarity to the 16S rDNA sequence of *Enterococcus gallinarum*[4], was isolated from the sample that was obtained from the 24-year-old female and grown on MRS agar.

The 16S rDNA sequences of the fecal isolates listed in Table 2 have been deposited in DDBJ with the accession number AB269763 for *E. coli* AE-1-2, AB269764 for *C. nigricans* AE1-3, AB269765 for *S. pasteuri*, AB269766 for *B. subtilis* AE4-3, and AB269767 for *E. gallinarum* CKY.

Lytic efficiency for fecal isolates

Five strict and six facultative anaerobic isolates from human feces (Table 4) were tested for lytic efficiency with the reference method used for metagenomics of the microbial communities in the Sargasso Sea and with the improved method developed in this study. The reference method, which involves the addition of 1.5 mg of lysozyme (150 µl/ml final concentration) to 10 ml of bacterial suspension, gave a lytic efficiency of >80%, although not for *C. ramosum* JCM 1298^T, as shown in Table 4. The lytic efficiency for *C. ramosum* was low at 47.5%, whereas another clostridial species, *C. innocuum*, was lysed with 98.4% efficiency. Since at least 21 *Clostridium* species are known to be human colonic commensals[1], it is expected that the lysis of *Clostridium* species in feces using the reference method should give variable results depending on the species. Whereas *B. subtilis* isolated in this study was lysed completely by the reference method, the lysozyme concentration of 150 µg/ml was too low to disrupt the well-known laboratory strain of *B. subtilis* 168 whose whole genomic sequence has been determined (http://genolist.pasteuri.fr/SubtiList). Indeed, a 10-fold higher concentration of lysozyme is required to prepare protoplasts from *B. subtilis* strain 168. This result indicates that the patterns of bacterial lysis are not necessarily predictable by species name.

The improved method, which uses a 100-fold higher con-

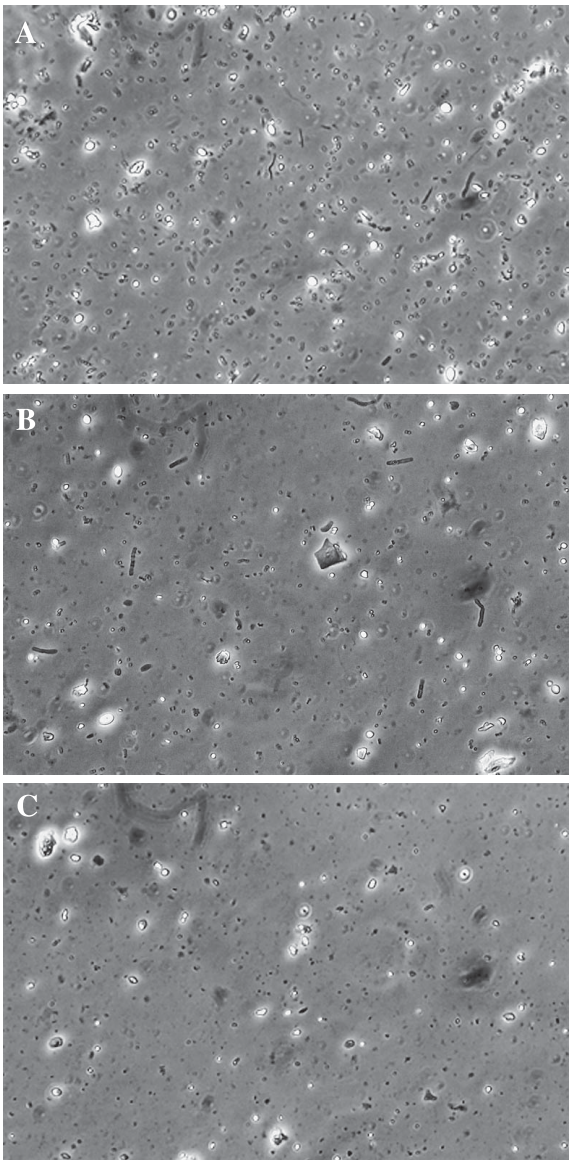

Fig. 1. Microscopic observations of fecal samples before and after enzymatic treatment. The female A-2 sampled on June 2006 was used for this study (Table 2). (A) Before enzymatic treatment, many cocci are evident. (B) After enzymatic treatment using the reference method, some intact rod-shaped cells still remain. (C) After enzymatic treatment using the improved method, no intact cells are observed, except for cell debris and the particles derived from the feces. Cell counting was performed with a counting chamber and a phase-contrast microscope at 600×power.

centration of lysozyme (15 mg/ml) and involves the addition of achromopeptidase (3 mg/ml), increased the efficiency of *C. ramosum* lysis to 82.5% (Table 4). Achromopeptidase, which is a bacterolytic enzyme derived from *Achromobacter lyticus*[9], has been reported to lyse a relatively wide spectrum of bacteria, including *Micrococcus*, *Bacillus*, *Sarcina*, *Staphylococcus*, *Streptococcus*, and *Clostridium* species, as well as some Gram-negative bacteria[5,15]. Although this enzyme is usually used on its own, a stronger effect is seen when it is used in combination with lysozyme, based on our experiences with *Bacillus* species. The high lytic efficiency observed in the present study is presumably brought about by the synergistic effect of an increased lysozyme concentration and addition of achromopeptidase.

Lytic efficiency for prokaryotes recovered from feces

Five fecal samples from three individuals (female adults A-1, A-2, and B, and male infant C-1 and C-2; Table 3) were tested for lytic efficiency with the QIAamp DNA stool mini kit method, as well as with the two methods described above. The lytic efficiency of the reference method for microbes recovered from the fecal samples ranged from 13.3% to 84.6% depending on the fecal sample; when the lytic efficiency is low, lysozyme-resistant Gram-positive species are considered to predominate in the feces (Table 5). In the case of sample 2, which showed only 13.3% lysis, most of the cells observed microscopically were Gram-positive cocci, in contrast to other fecal samples (Fig. 1). Gram-positive anaerobic cocci, such as the genera *Peptostreptococcus*, *Coprococcus*, and *Ruminococcus*, are known to be lysozyme-resistant human colonic commensals, while many of the strains belonging to these genera are susceptible to achromopeptidase[1,5]. On the other hand, the lytic efficiency of the improved method for sample 2 was dramatically higher at 89.8% (Table 5 and Fig. 1), and most of the cocci observed in the cell suspension disappeared after treatment with 3 mg/ml of achromopeptidase. This demonstrates that achromopeptidase is effective for the lysis of human commensal Gram-positive cocci.

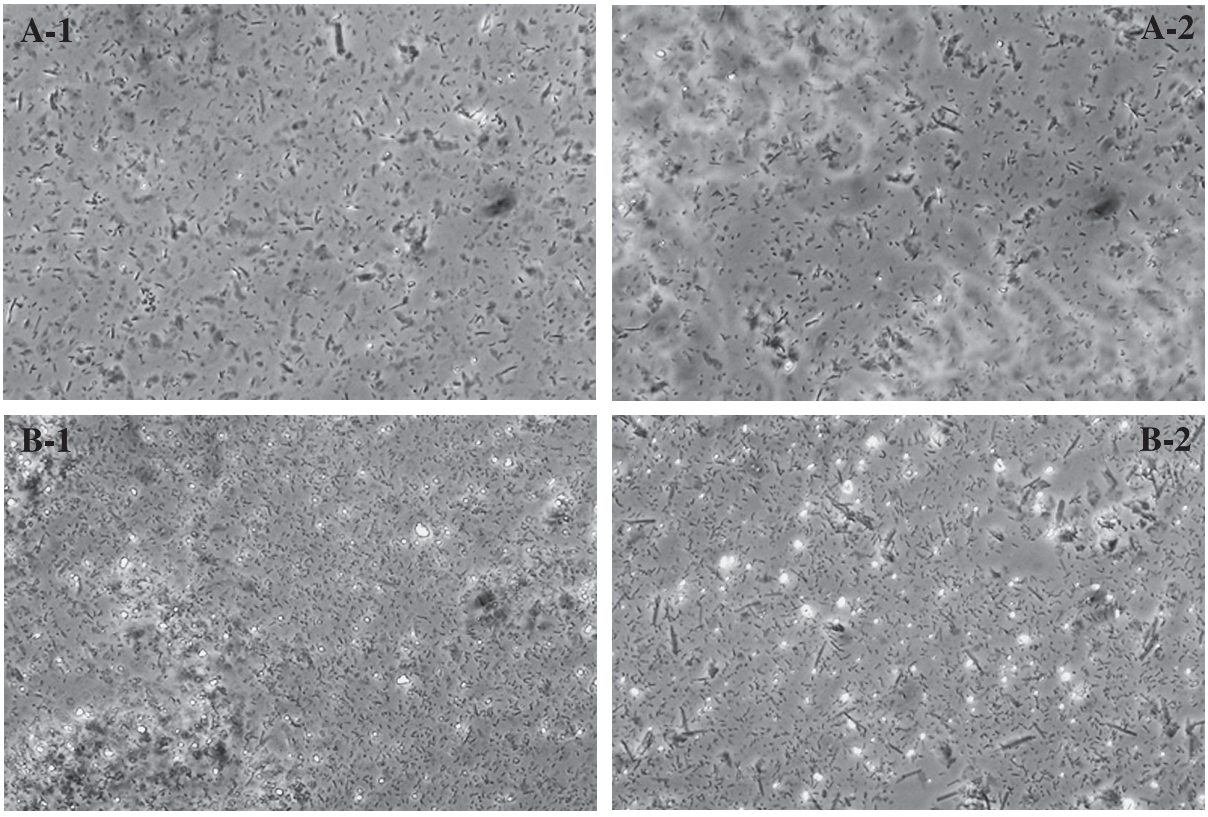

Fig. 2. Comparison of lysis after enzymatic treatment using the reference method between two fecal samples obtained from the same male infant. (A) Microscopy of the fecal sample from the infant at 3 months of age. A-1, Before enzymatic treatment; A-2, lytic efficiency is 79.8% (B) Microscopy of the fecal sample from the infant at 4 months of age. B-1, Before enzymatic treatment; B-2, lytic efficiency is 27.9% after enzymatic treatment. Cell counting was performed as described in the legend to Fig. 1.

There was a significant difference in the efficiency of lysis of recovered microbes between the two samples collected at an interval of one month from the same male infant, as shown in Table 5 and Fig. 2. Since this probably reflects a difference in the fraction of lysozyme-resistant microbes in the total fecal microbe population, it appears that the intestinal microbial flora changes in the 3–4 months after birth, although no significant changes were noted by microscopic observation (Fig. 2). As observed for the previous case, almost complete lysis of the samples from the infant was achieved with the improved method. Moreover, the improved method showed an overall stable and high lytic efficiency (>90%) for the other fecal samples (Table 5).

The QIAamp DNA stool mini kit is often used for the isolation of DNA that is used to amplify 16S rDNA by PCR. Although it is difficult to know the principle of the lysis used in this procedure, as the kit components are not revealed, the actual lytic efficiency fluctuated in the range of 38.3–69.2%. The variability in efficiency observed for this kit does not necessarily correspond to that observed for the reference method.

DNA isolation from bacteria recovered from feces

Following lysis by the three different methods described above, the bacterial DNA samples were purified by the standard phenol/chloroform protocol. To evaluate the quantity and quality of the DNA, samples 3 (Female B) and 5 (Male C-2) were selected as representatives of the adult and infant samples, respectively. The DNA isolated from samples 3 and 5 by the three different methods was electrophoresed on a 1% agarose gel in TAE buffer, while the DNA concentration after polyethylene glycol (PEG) precipitation was measured in a spectrophotometer at 260 nm. The total amount of DNA isolated from feces depends on the initial number of bacteria in the fecal sample and the efficiency of lysis, and may be affected by enzymatic degradation if microbes that possess strong DNase activities are present in the fecal sample. The amounts of DNA isolated by the improved method from 3 g each of samples number 3 and 5 were 87.6 μg and 166.5 μg, respectively. These DNA concentrations are 2.3- to 2.4-times higher than those obtained using the reference method for both samples and are sufficient for the preparation of genomic libraries. In addition, since there was no significant difference in the ratio of optical density of the DNA at 260 nm to 280 nm over 1.9 between the reference and the improved methods, it is thought that the DNA isolated by both methods is purified enough for genomic manipulation.

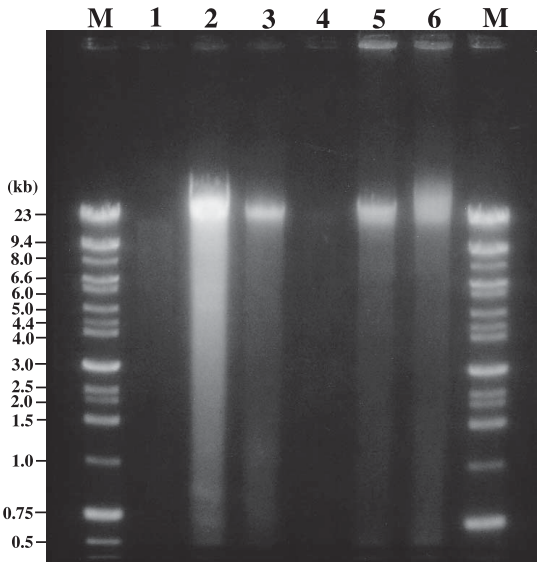

Fig. 3. Comparison of three different methods used for the extraction of DNA from prokaryotic cells recovered from human feces. DNA samples (10 μl) were loaded onto a 1% agarose gel and electrophoresis was performed in TAE buffer for 45 min. M, Molecular weight marker (λ *Hin*dIII digest+1-kb ladder); Lanes 1–3, male infant (sample 5); Lanes 4–6, female adult (sample 3); Lanes 1 and 4, QIAamp DNA stool mini kit; Lanes 2 and 5, improved method; Lanes 3 and 6, reference method.

The initial condition of the isolated DNA is very important for the preparation of genomic libraries that contain both long and short inserts. As shown in Fig. 3, the DNA samples isolated using the improved and reference methods were of sufficiently high molecular weight for the preparation of a fosmid library with 40-kb inserts, although a small amount of lower molecular weight DNA was observed in the gel. On the other hand, the recovery of DNA using the QIAamp DNA stool mini kit was very low, as evidenced by the appearance of a weak band on the gel (Fig. 3), despite high lytic efficiency (69.2%), and also most of the DNA was fragmented into small pieces, causing a long smear to appear in the gel. Thus, the QIAamp system is not useful for the isolation from fecal samples of DNA to be used in the preparation of genomic libraries.

Conclusions

Since metagenomics entails a comprehensive study of the microbial communities in various environments, it is essential to construct genomic libraries that represent all of the species present without bias. In this study, we present an enzymatic lysis method that has high lytic efficiency and that is much milder, giving more intact DNA, than mechanical methods. Indeed, using the improved method, DNA of

high molecular weight was obtained in sufficient quantities to allow the construction of genomic libraries with large inserts. We found that the combination of lysozyme and achromopeptidase conferred a lytic efficiency of >90%. We believe that this method is applicable to global metagenomic studies of other environmental samples.

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