

Quantitative PCR assays for mouse enteric flora reveal strain-dependent differences in composition that are influenced by the microenvironment

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Abstract

The mammalian gastrointestinal (GI) tract is inhabited by over a hundred species of symbiotic bacteria. Differences among individuals in the composition of the GI flora may contribute to variation in *in vivo* experimental analyses and disease susceptibility. To investigate potential interindividual differences in GI flora composition, we developed real-time quantitative PCR-based assays for the detection of the eight members of the Altered Schaedler Flora (ASF) as representative members of different bacterial niches within the mammalian GI tract. Quantitative and reproducible strain-specific variations in the numbers of the ASF members were observed across 23 different barrier-housed inbred mouse strains, suggesting that the ASF assays can be used as sentinels for changes in GI flora composition. A significant cage effect was also detected. Isogenic mice that cohabited at weaning, whether from the same or different litters, showed little variation in ASF profiles. Conversely, litters split among different cages at weaning showed divergence in ASF profiles after three weeks. Individual ASF profiles, once established, were highly stable over time in the absence of environmental perturbation. Furthermore, cohabitation of different inbred strains maintained most of the interstrain variation in the GI flora, supporting a role of host genetics in determining GI flora composition.

Introduction

The number of resident bacteria associated with the mucosal surfaces of adult humans exceeds the number of somatic and germ cells by upward of an order of magnitude (Lucky 1972; Savage 1977). The gastrointestinal (GI) tract contains the largest number of microorganisms of any organ in the body (Falk 1998), where they exist as a vast ecosystem whose stability is indispensable to the health of the host. Some members of this ecosystem have been found to be important in disease progression, like several of the *Clostridium* sp. (Hopkins and Macfarlane 2002) and *Bacteroides* sp. (Foulon et al. 2003; Wilson and Limaye 2004), while others may play a role in disease prevention, like *Lactobacillus* sp. and *Bifidobacteria* sp. (Tuohy et al. 2003). However, the majority of adults have "normal" GI health because of the balance between protective and potentially harmful bacteria. When this ecosystem is disrupted, diseases like chronic and antibiotic-associated diarrhea (Gustafsson et al. 1999; Todar 2002), irritable bowel syndrome (Nobaek et al. 2000), *Helicobacter pylori*-associated gastroenteritis, inflammatory bowel disease, small bowel bacterial overgrowth (Rolfe 2000), cancer of the large intestine (Fukui et al. 2001), and disorders like sucrose maltase deficiency and lactose intolerance can develop (Fuller and Gibson 1997; Lin et al. 1998).

Although numerous reports suggest a role for members of the GI flora in pathogenesis and disease protection, investigations have been hampered largely by the problems associated with cultivation and detection (Salzman et al. 2002). Different identification methods have been used

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with variable success. However, without specialized equipment and a variety of selective culture media, the technical challenge of culturing the majority of these organisms impedes research into the role of the enteric flora in disease processes. Contributing to the difficulties are the extremely oxygen-sensitive (EOS) fusiform bacteria and unculturable segmented filamentous bacteria, comprising the bulk of the GI flora and making identification of these GI isolates to the species level difficult. However, the advent of real-time quantitative polymerase chain reaction (qPCR) technologies has made it possible to quantify levels of individual species within complex bacterial populations, without the need for specialized microbiological culture. DNA-based PCR methods also avoid the problems associated with recovering live anaerobic microorganisms and simplify the testing of environmental samples from fecal material and necropsies.

To circumvent the problems of characterizing an unknown GI flora, a standardized enteric flora that contains eight species supplied as the Altered Schaedler Flora (ASF) (Orcutt et al. 1987) is commonly given to germfree mice originating from most U.S. and foreign production facilities to provide these immuno-immature animals with a normalized flora and to provide protection from opportunistic pathogens. The ASF models more complex mixtures of the normal GI flora whose members are known to exhibit tissue tropism, occupying different niches in the mouse GI tract (Dubos et al. 1967; Savage et al. 1968; Savage 1981; Akada et al. 2003; Sarma-Rupavtarm et al. 2004). In addition, 16S rDNA sequences for the members of the ASF have been determined (Dewhirst et al. 1999), facilitating the development of quantitative assays for the ASF (Sarma-Rupavtarm et al. 2004).

Previous studies have shown that the composition of the GI flora can be modulated by genetic factors such as the major histocompatibility complex (MHC) (Toivanen et al. 2001; Zoetendal et al. 2001). Consequently, using PCR-based detection assays, the ASF can be exploited, particularly in ex-germfree mice, to evaluate the influence of genetic and environmental factors on GI colonization and to control for variations in GI flora composition during analysis of *in vivo* experiments. The availability of germfree mice, which can be populated with specific enteric strains by a number of methods including orogastric administration, enema, and association with bedding from donor animals, allows for the controlled population of mouse GI tracts and simplifies the examination of the colonic microenvironment.

We report here the development of a rapid, accurate, and sensitive PCR-based method for monitoring bacterial colonization of the mouse GI tract using the ASF as a model flora in germfree and gnotobiotic mice. We also demonstrate the utility of the ASF as a sentinel flora in barrier-housed mice for monitoring the influence of genetic and environmental factors on the composition of the GI flora.

Materials and methods

Mice. To assess the colonization patterns of the ASF in inbred strains, fresh fecal pellets were obtained from 2-4-month-old 129S6/SvEvTAC mice from Taconic (Germantown, NY) and from 129S1/SvImJ, A/J, AKR/J, ALS/LtJ, APN, BALB/cByJ, BALB/cJ, BTBR, C3H/HeJ, C58/J, CFW/J, C57BL6/J, DBA2/J, FVB/NJ, JF1/Ms, KK/HlJ, LG/J, MOLF/EiJ, SJL/J, SPRET/EiJ, SWR/J, and WSB/EiJ mice from The Jackson Laboratory (Bar Harbor, ME). All strains were housed under specific pathogen-free conditions in ventilated caging with HEPA-filtered air, given sterilized chow (Purina 3500), and had autoclaved water available *ad libitum*. Bedding was changed weekly at the same time for all cages. Germ-free BALB/cJ and 129S6/SvEvTAC mice, obtained from the UNC Center for Gastrointestinal Biology and Disease (CGIBD) Gnotobiotic Core, were colonized with the ASF microorganisms. The ASF were administered by oral gavage followed by rectal enema. Gnotobiotic mice from Taconic had been inoculated with all eight members of the ASF. Four male and four female 5-week-old C.B-17 mice were shipped from Taconic to UNC in germfree shippers.

Biological samples. Stomach, small intestine (duodenum, jejunum, and ileum), cecum, colon (proximal and distal), fecal, and liver (for negative control) samples were collected from ASF-associated male and female C.B-17 mice (Taconic) after CO₂ asphyxiation. Duplicate fecal samples were obtained from three cages each of 23 strains of inbred mice. Triplicate fecal samples were obtained from each of seven cages of individually housed A/J and FVB/NJ mice and from each of five FVB/NJ and A/J mice cohoused by strain at one-week intervals for three weeks. Duplicate samples were taken every 12 h from the same mouse over a one-week (FVB/NJ) or two-week (A/J) period.

Litters from FVB/NJ, C3H/HeJ, and 129S6/SvEvTAC mice were separated at weaning into different cages and fecal samples were collected once a week for three weeks. Weanlings from separate litters of FVB/NJ mice were combined in the same cage

to form new grouping and fecal samples were collected from these combinations once a week for three weeks. Similarly, representative adults from different strains were put in the same cage and were sampled weekly over three weeks.

DNA isolation from biological samples. Tissues were homogenized with a PRO200 homogenizer (PRO Scientific, Oxford, CT) for 1 min using a 7-mm-diameter \times 75-mm flat-bottom generator at 6 m/sec. DNA was isolated from all tissue samples by DNAzol (Invitrogen, Carlsbad, CA) before resuspending in weak base (8 mM NaOH), neutralizing in Tris-EDTA (TE), and storing at -20°C . Fresh fecal samples were taken from the rectum, placed in lysis buffer (Qiagen, Valencia, CA), and resuspended by repeated pipetting for isolation with the QIAamp DNA Stool kit. DNA from feces was resuspended in TE and stored at -20°C .

DNA isolation from mock samples. Three varieties of mock samples were prepared using mouse tissues and mixtures of the ASF. First, sections of conventional mouse colons (0.2 mg) were dissected and added to bacterial suspensions containing 10^8 bacteria from mixed ASF strains before DNA isolation. Second, fecal samples from barrier-raised 6-week-old CD-1 male mice (Charles River, Wilmington, MA) were collected and mixed with 10^8 bacteria from each ASF strain. Approximately 200 μl of the fecal suspension was used to isolate DNA. Third, 200- μl aliquots of colonic fluids were obtained from barrier-housed mice by flushing the colons with 1 ml of phosphate-buffered saline, to which ASF from pure cultures were added. DNA was isolated using DNAzol (Invitrogen) and used in real-time qPCR.

Bacterial strains and extraction of genomic DNA. The bacterial strains ASF 356, 492, 500, 502, 457, 360, 361, and 519 (Taconic) were grown in prerduced Schaedler broth or blood-enriched anaerobic agar according to established methods. Anaerobiosis was maintained using anaerobic pouches (Remel, Lenexa, KS) or anaerobic environmental chambers in a stationary 37°C incubator containing 5% carbon dioxide, 10% hydrogen, and 85% nitrogen gas. Anaerobiosis was monitored with a resazurin or methyl blue indicator (Remel). DNA was extracted with DNAzol (Invitrogen) or using a QIAamp DNA Mini Kit (Qiagen) followed by resuspension in weak base (8 mM NaOH) and neutralization in TE before storing at -20°C . The stored samples were evaluated by both traditional and real-time qPCR.

Colony counts. Gastrointestinal fluids, organs, and bacterial cultures were analyzed for bacterial content by colony counts, direct microscopy, and PCR. Traditional colony counts were performed by plating serially diluted aliquots of the samples on Schaedler blood agar plates followed by anaerobic culture for 72 h. The colonies were classified by species using morphology, Gram staining, and biochemical testing.

Primers and probes. The published 16S rDNA sequences for each bacterial strain were used to design primers and probes for real-time PCR analysis using the Primer Express program (Applied Biosystems, Foster City, CA) (Dewhirst et al. 1999). The primers, designed to result in amplicons of 100 bp, were from regions of the 16S rDNA with unique sequences and were chosen to result in PCR products with similar melting temperatures. The primers and fluorescently labeled probes were synthesized by the oligonucleotide core facilities in the Department of Pathology and the Lineberger Comprehensive Cancer Center (UNC).

Real-time qPCR. TaqMan Universal Master Mix containing Amplitaq and UNG was used in all real-time qPCR assays (Applied Biosystems). The master mix contained 0.1 $\mu\text{g}/\text{ml}$ each of forward and reverse primer and 10 μM probe. The real-time assays were performed using the conditions for the Rapid Assay Design (Applied Biosystems) and were run at 50°C for 2 min and at 94°C for 10 min before 40 cycles of 94°C for 15 sec and 60°C for 1 min. The resulting data were collected and analyzed using both the 7700 and the 7900 Sequence Detector System (Applied Biosystems). Bacterial DNA was quantified by comparing Ct values to samples of known quantity that were used to generate standard curves for each ASF member. These numbers were compared to the quantification by colony counts.

Statistics. Data were analyzed using the analysis of variance (ANOVA) module of the Statview (SAS Institute, Cary, NC). Values were expressed as the mean of at least three measurements plus standard deviation. Significance was set at $p \leq 0.05$ and highly significant values at $p \leq 0.01$. A *post hoc* test, Fisher's PSLD test, was used to determine how the groups with significant effects differed.

Results

All eight ASF bacterial strains were cultured and verified by colony examination and Gram staining (Table 1). Using 16S rDNA sequences (Dewhirst et

Table 1. Altered Schaedler's Flora characteristics and PCR primers and probes

Organism	Identity	Morphology ^a	GenBank	Primer ^b	Sequence
ASF 360	<i>Lactobacillus</i> sp.	FA, Gram+, rod	AF157050	F P R	CGGCGGATGGGTGAGTAAC CGTGGGTAACCTGCCCTAAAGTCT GTATTAGCACCTGTTTCCAAGTGGTA
ASF 361	<i>Lactobacillus murinus</i>	FA, Gram+, rod	AF157049	F P R	GTGGCGAACGGGTGAGTAA ACGTGGGCAACCTGCCCAAAGA GCACCTGTTTCCAAGTGTATCC
ASF 519	<i>B. distasonis</i> - <i>Porphyromonas</i>	A, Gram-, coccobacillus	AF157056	F P R	ATTGCCGTTGGAAATGTTATCC AGGCGGAATGCGTGGTGTAGCG CCTTCGCAATCGGAGTTCTG
ASF 457	<i>Mucispirillum schaedleri</i>	A, Gram-, spiral-shaped bacterium	AF157055	F P R	TGCAAGAATGAAACTCAAAGGAAT CACAAGCGGTGGAGCACGTGGTT TAAGGTTCTTCGGTTAGCATCGA
ASF 356	<i>Clostridium</i> sp.	EOS fusiform, Gram+, tapered rod	AF157052	F P R	AACGCCGCGTGAAGGAA CTACGTGCGAGCAGCCGCG ACGCTTGCCCCCTACGTATT
ASF 492	<i>Eubacterium plexicaudatum</i>	EOS fusiform, Gram+, tapered rod	AF157054	F P R	TGGAGCATGTGGTTTAATTCCGA ACCTTACCAGGCCTTGACATCCCGA TGCACCACCTGTCTGCAATG
ASF 500	<i>Firmicutes</i> sp.	EOS fusiform, Gram+, tapered rod	AF157051	F P R	GCTGCTAATACCGCATGATATGTC TGTGTCGCATGGCACTGGACATC CAGACGCGAGTCCATCTCAGA
ASF 502	<i>Clostridium</i> sp.	EOS fusiform, Gram+, tapered rod	AF157053	F P R	GCTACAATGGCGTAAACAAAGAGA CGACCACGCGAGTGTGAGCGA TCCGAACTGAGACGTTATTTTTGA

^a FA = facultative anaerobe; A = anaerobe; EOS = extremely oxygen sensitive.

^b F = forward; P = probe; R = reverse.

al. 1999), specific qPCR assays were designed in a region immediately downstream of an area of common homology between all the species. These primers and probes were used in all subsequent qPCR experiments using reaction conditions specified by the Rapid Assay Development protocol (Applied Biosystems).

Specificity and sensitivity. We used a series of negative controls to test the integrity and specificity of the qPCR assays. Negative controls included PCR reaction mix with water instead of DNA, amplification of mouse DNA using bacterial primers, amplification of bacterial DNA using mouse primers, and amplification of irrelevant bacterial DNA using each bacterial primer set. While these tests resulted in amplification only at the limits of detection for nonhomologous sequences, the individual qPCR assays resulted in the specific amplification of DNA from each cognate ASF member, showing that all primer-probe combinations are highly specific with no cross-reactivities (data not shown).

Serially diluted and purified bacterial DNA was then used to assess assay sensitivity and linearity (Fig. 1). Amplification was linear over 6 logs and the y intercept, a measure of the Ct at which one copy of the organism is amplified, was near 40 cycles for each strain, demonstrating the ability of the assays

to detect a single bacterium. A comparison of bacterial numbers detected by traditional colony counting with empirical determinations using qPCR showed less than a onefold difference (Table 2). Similarly, standard curves generated from serially diluted bacterial cultures gave identical qPCR results to those from serially diluted DNA (data not shown).

Distribution along the GI tract. We next tested our assays for the ability to detect and enumerate bacterial numbers in the GI tracts of commercial, ASF-colonized, ex-germfree C.B-17 mice (Fig. 2). Only *Lactobacillus* sp. were detected throughout the GI tract, with the presence of ASF 361 more consistent compared with that of ASF 360. ASF 519 and ASF 457 were detected in all tissues except the stomach, while the EOS species ASF 356, ASF 492, ASF 500, and ASF 502 were abundant in the lower GI tract, increasing in numbers from the cecum to the distal colon. No sex-dependent differences were detected, except for ASF 500 and ASF 502, which showed greater sex-dependent organ-to-organ variation. ASF 360 was the most variably detected species, often detected only transiently; this was also observed in subsequent *in vivo* experiments and is consistent with previous observations (Sarma-Rupavtarm et al. 2004). Independently generated ex-germfree C.B-17 mice

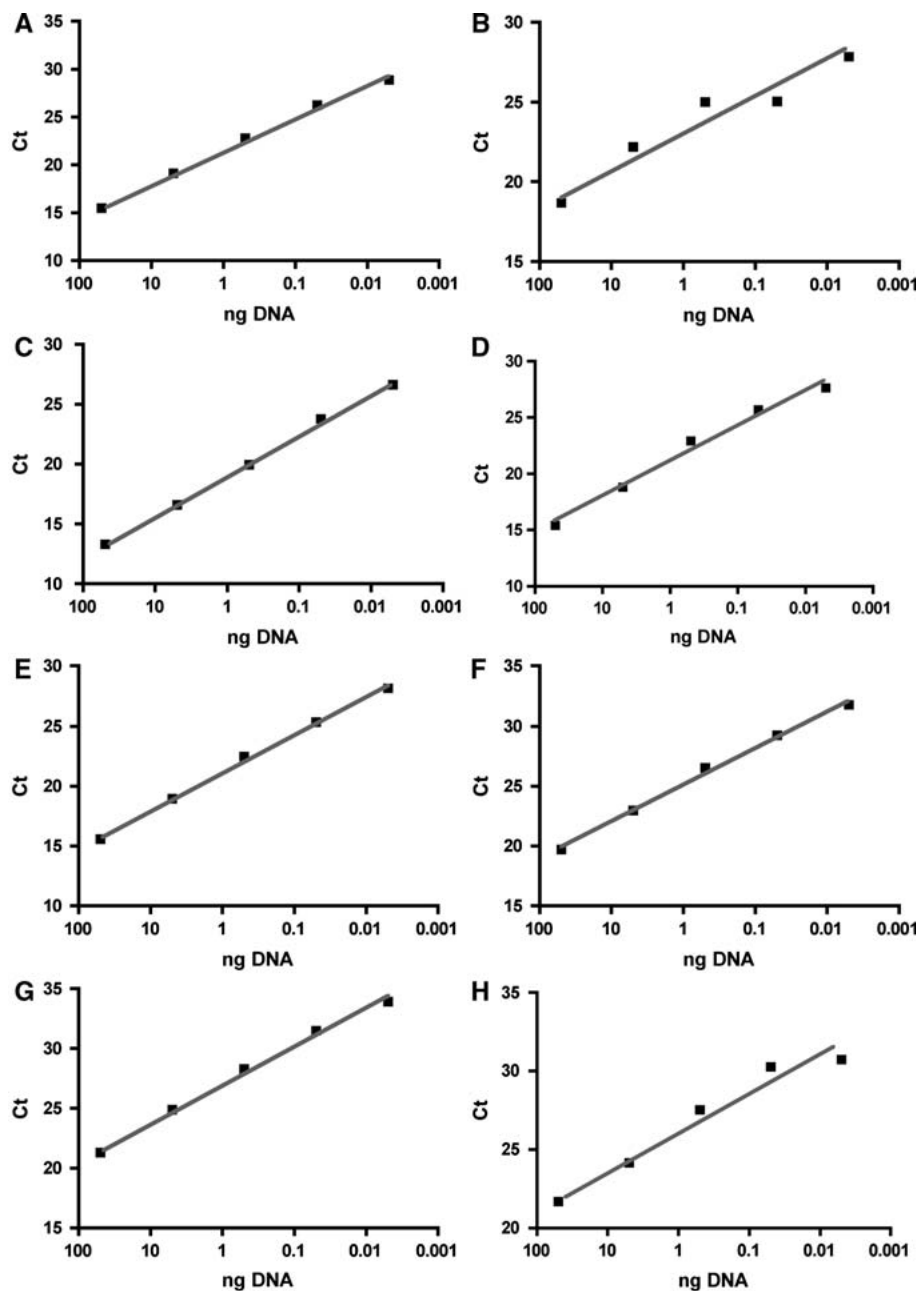


Fig. 1. Standardized qPCR curves from serially diluted ASF DNA. Ct values for reactions containing various concentrations of purified bacterial DNA diluted to range over 5 logs for (A) ASF360, (B) ASF361, (C) ASF519, (D) ASF457, (E) ASF 356, (F) ASF492, (G) ASF500, and (H) ASF502. Actual values with standard deviations are marked by squares and linear regression values by gray lines.

Table 2. Comparison of viable colony forming units (CFU) and real-time qPCR methods of quantification

Sample	Viable counts CFU/ml	Real-time qPCR deduced CFU/ml
ASF 360	1.7×10^6	2.2×10^6
ASF 361	4.6×10^6	4.6×10^6
ASF 519	2.9×10^8	2.7×10^8
ASF 457	2.0×10^7	1.17×10^7
ASF 356	1.3×10^4	1.33×10^4
ASF 492	2.4×10^6	4.14×10^6
ASF 500	5.1×10^5	4.7×10^5
ASF 502	1.1×10^5	8.07×10^4

also had extensive differences in the ASF distribution within the GI tract. This was particularly evident in the numbers of ASF 356, 492, and 519 in the cecum.

Analysis of the GI organs taken from germfree and ex-germfree, ASF-colonized 129S6/SvEvTAC and BALB/cJ mice verified trends observed in C.B-17 animals (data not shown). Measurements from the fecal samples were not representative of the distribution in colonization of the GI flora among mice or strains, but were a good surrogate for global differences between strains. In addition, the association of

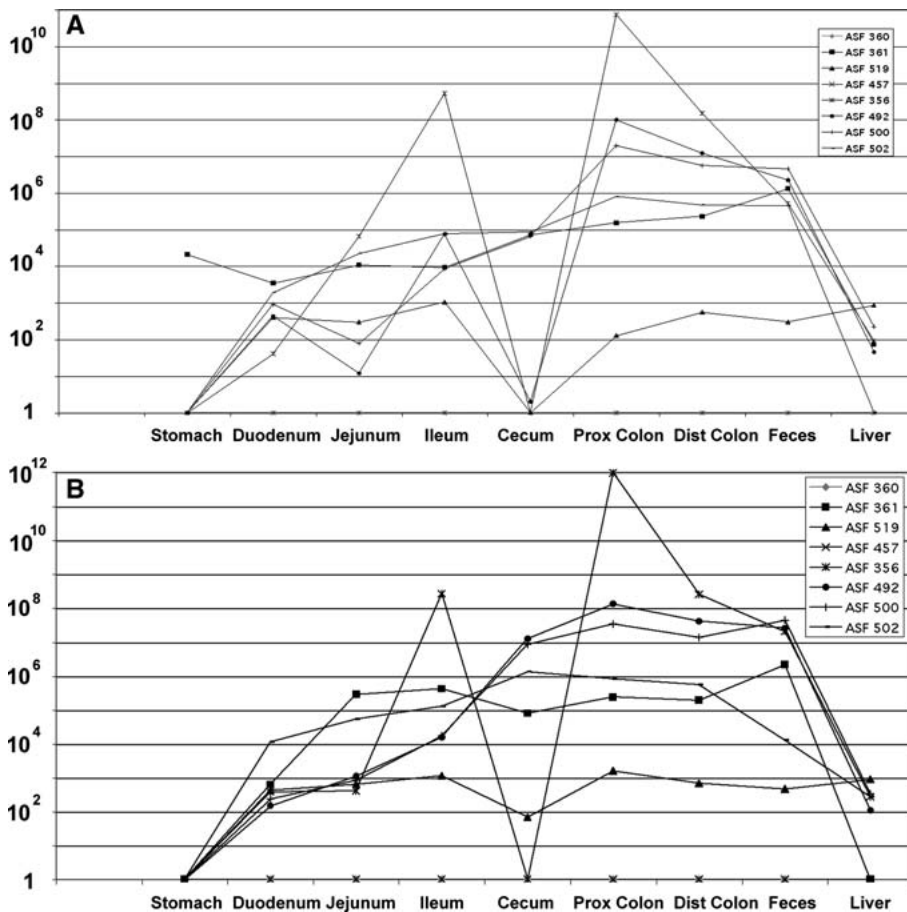


Fig. 2. Detection of ASF members in samples taken from defined flora C.B17 mice. Average values for (A) female and (B) male mice. Quantities have been converted to CFU using standard curves from Figure 1. S1, duodenum; S2, jejunum; S3, ileum; Colon 1, proximal colon; Colon 2, distal colon.

the germfree mice with the ASF restored the cecal morphology to a size comparable with cecal sizes in conventional or barrier-housed animals, confirming the presence and functionality of the ASF. These results are similar to a previous study that reported the distribution of the ASF in the mouse GI tract (Sarma-Rupavtarm et al. 2004).

Interstrain variation. Because the ASF were originally isolated from Swiss outbred, barrier-reared mice, we tested for the presence and population variability of the ASF members in fecal samples from 23 inbred, barrier-reared mouse strains to evaluate their usefulness as a sentinel flora. In agreement with data from the colonization of germfree mice, ASF 361 numbers were consistently higher than ASF 360 in all strains tested (Fig. 3). ASF 360 was found to be abundant only in BTBR mice, although JF1/MsJ, LG/J, and WSB/EiJ strains also had detectable levels. ASF 519 was found in highest abundance in CFW/J mice, while ASF 457 was highest in BALB/cJ and 129S6/SvEvTAC and ASF 492 in JF1/MsJ mice. Of the EOS species, ASF 356 and ASF 500 were high in many strains, while ASF 502 was highest in SJL/J

mice. Only BTBR mice were positive for all eight ASF species, and the only ASF members detected in all 23 strains were ASF 519 and ASF 356.

Significant variation in the numbers of all ASF was found when comparing across inbred strains ($p < 0.0001$). Interstrain variation accounted for approximately 40% of the total variation, while the number of significant ($p \leq 0.05$) pairwise strain differences among the 23 strains was greater than 100. Interestingly, there was general agreement in the ASF numbers between strains with a recent common origin; the strain pairs 129S1/SvImJ and 129S6/SvEvTAC ($p = 0.98$), despite originating from different production facilities, and BALB/cJ and BALB/ByJ ($p = 0.1$) were not significantly different in ASF profiles, suggesting a strong host genetic component to the levels of ASF.

Intrastrain variation. To obtain a better estimate of the amount of ASF variation within strains, the level of temporal variability of the ASF in samples taken from the same A/J mice over time was determined (Fig. 4a). The levels of the ASF appear to be stable once established because samples taken

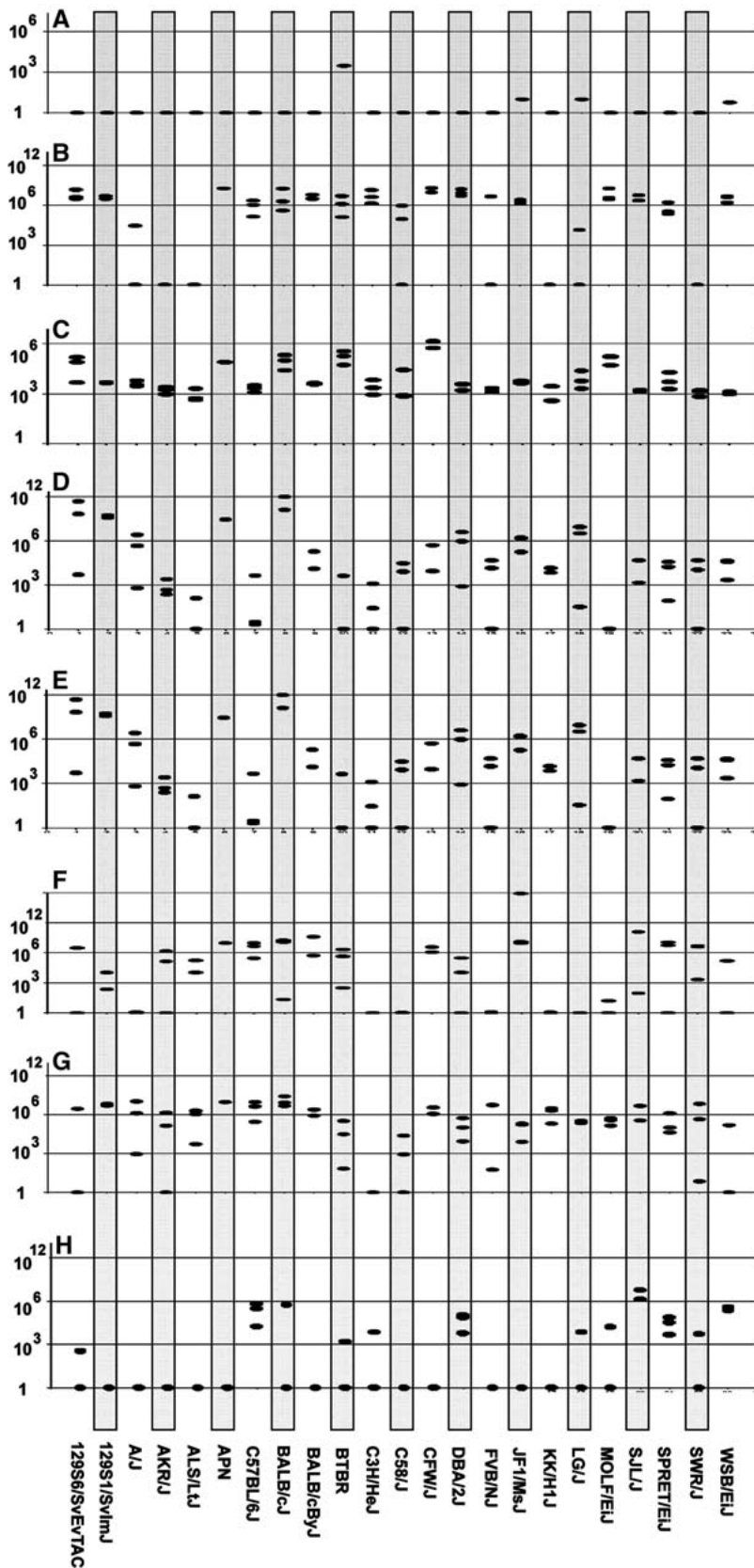


Fig. 3. Variation in quantity of ASF members among different barrier-housed mouse strains. Each oval represents the mean numbers of ASF measured in triplicate samples taken from a single cage. Three independent cages, housing mice from different litters, were measured for each strain. (A) ASF360, (B) ASF361, (C) ASF519, (D) ASF457, (E) ASF356, (F) ASF492, (G) ASF500, and (H) ASF502. Quantities have been converted to CFU using standard curves from Figure 1.

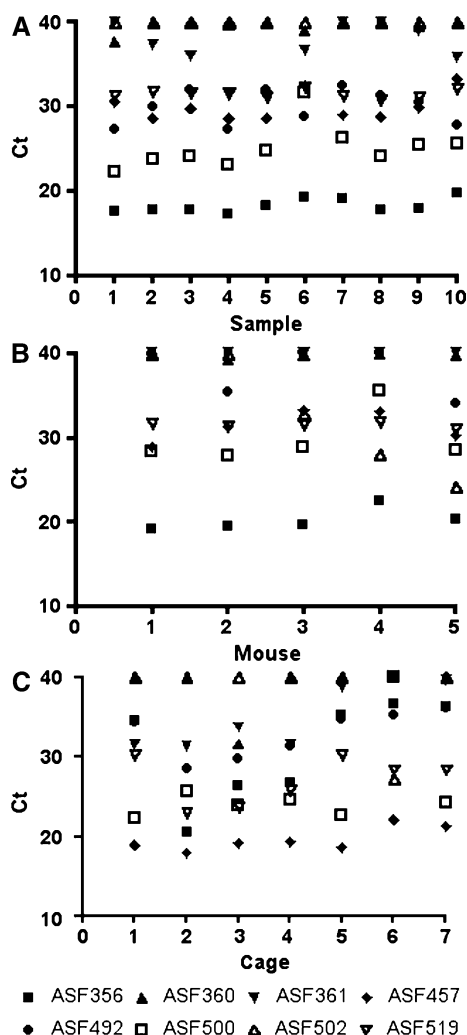


Fig. 4. Intrastrain variation in quantity of ASF members with different microenvironments. The mean value of multiple measurements for each ASF member is marked by a unique symbol and the qPCR Ct values plotted for each condition. (A) Samples taken from the same A/J mouse over a one-week window (odd samples were collected midmorning and even samples taken late evening). (B) Samples from individual A/J mice that cohobated after weaning from different litters. (C) Samples from cages of independently housed A/J mice from different litters.

over time and at different times of day from the same mouse do not vary significantly ($p = 0.99$). To further characterize the variability that exists within a strain, we examined the variability in ASF profiles among A/J mice housed in the same cage (Fig. 4b). While one mouse was significantly different from all of the other cagemates (mouse 5, $p < 0.05$), the overall effect of cohobating was that ASF profiles were similar among individual mice within the same cage ($p = 0.90$).

Because genetically identical mice with identical microenvironments were remarkably similar, we also measured ASF variability from seven indepen-

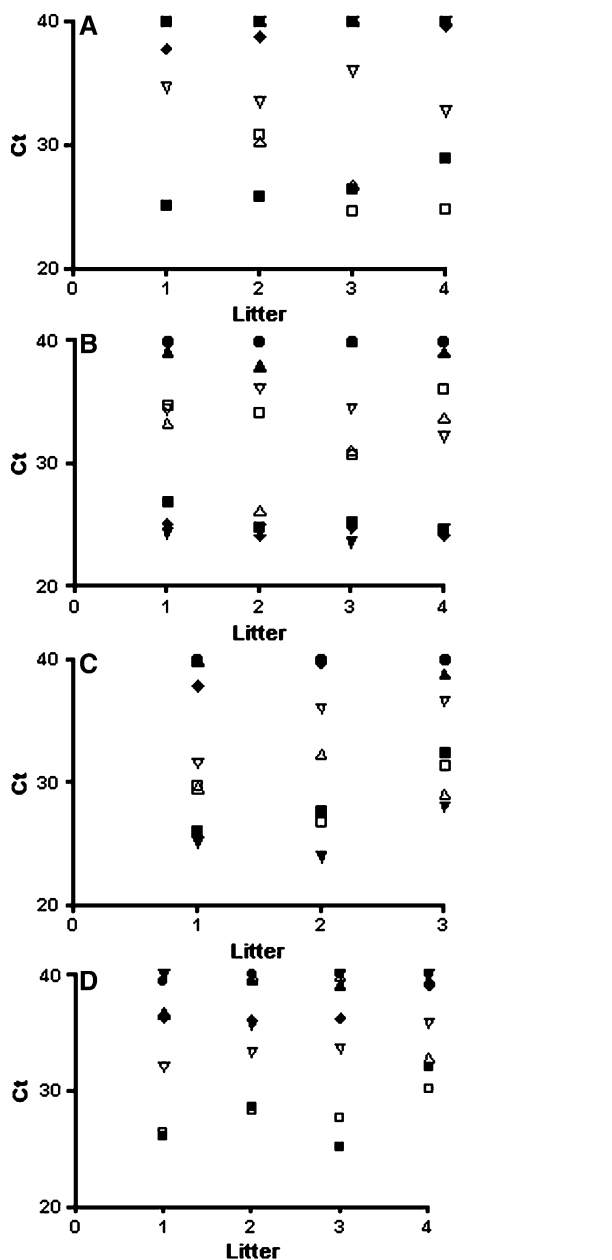
dent cages of A/J mice (Fig. 4c). The variation in the numbers of each ASF member in different A/J cages was similar to the range observed in the strain profile (Fig. 3), including absence of ASF 360 and ASF 502 from all cages except one. The level of variation between cages was not as high as the level of variability observed between strains, further suggesting that variation in the level of GI flora is determined by both host and microenvironment factors.

To determine whether this was a general phenomenon, we conducted identical experiments using FVB/NJ mice. Similar to results using A/J mice, variation was higher among FVB/NJ mice housed in different cages than those that cohobated (data not shown); there was no significant variation in replicate samples taken from the same mice over time ($p = 0.20$). These results show that significant host and cage effects exist that influence ASF levels.

The finding that samples taken from individual mice over time did not vary as much as samples from littermates, recaged mice, or mice from different mouse strains is consistent with studies demonstrating that GI flora composition is stable after birth and is characteristic of a particular individual (Shanahan 2004).

Cage and litter effects. Because most cages in the previous study represented different litters of mice, we sought to differentiate cage effects from possible litter effects. Individual litters of FVB/NJ, C3H/HeJ, or 129S1/SvImJ mice were split among separate cages at weaning. The parental cages and the weanlings were analyzed repeatedly over three weeks after separation. Concurrently, FVB/NJ mice from different cages, with measurable differences in ASF levels, cohobated, and ASF patterns were characterized after three weeks. Although some differences were significant between individual cages of mice from the same strain, the degree to which splitting litters among different cages changed the ASF profile after three weeks was dependent on the strain (Fig. 5a-c). Separated FVB/NJ mice did not show significant differences when analyzed by cage ($p = 0.34$). However, C3H/HeJ and 129S1/SvImJ were significantly different after three weeks ($p = 0.03$ and $p = 0.008$, respectively).

These results suggest that mouse strains are differentially susceptible to environmental changes that modulate the GI flora. As might be predicted, ASF profiles from independently reared FVB/NJ mice in "new" cohobated cages converged to have similar ASF profiles (Fig. 5d), as similar as those detected from cohobating littermates. This is likely achieved through coprophagy, the eating of feces which is important for nutrition, which can mediate GI flora



■ ASF 356 ▲ ASF 360 ▼ ASF 361 ◆ ASF 457
● ASF 492 □ ASF 500 △ ASF 502 ▽ ASF 519

Fig. 5. Effect of recaging on quantities of ASF members. The mean value of multiple measurements for each ASF member is marked by a unique symbol and the qPCR Ct values plotted for each condition. Samples taken from (A) FVB/NJ, (B) C3H/HeJ, and (C) 129S1/SvImJ littermates split into different cages. Sample 1 was the parental cages and other samples are from split daughter cages. (D) Independent cages of FVB/NJ mice (1-3) combined into a new cohabitation cage (4).

exchange between littermates. Previous studies have shown that there is a strain dependence on defecation in mice, which could translate into differences in the time and efficiency with which different

strains of recaged mice begin to homogenize their GI flora (Tang et al. 2002).

To further evaluate the influence of caging versus genetic background on GI flora population dynamics, C58/J, C57BL/6J, 129S1/SvImJ, FVB/NJ, and BTBR female mice cohabited for three weeks starting at weaning. Individual fecal samples were collected before cohabitation and weekly during three weeks of cohabitation. Cohabiting across strains removed the overall cage effects ($p = 0.49$), resulting in no intrasrain differences. However, similar to the strain profile data above, all strains were found to retain highly significant differences in ASF profiles when the data were analyzed by strain ($p < 0.001$). Importantly, when the data were grouped and analyzed by cage and then further separated by strain, there were no significant differences between mice from the same strain housed in different cages.

This experiment did reveal several significant differences between strains that cohabited. C57BL/6J mice were significantly different from FVB/NJ mice within the same cage ($p < 0.02$), and also were different from DBA/2J ($p < 0.0001$) and C58/J ($p = 0.026$) mice. Similarly, BTBR differed from FVB/NJ ($p = 0.027$) and DBA/2J ($p = 0.0007$). All other significant differences were between mice of different strains in different cages. Taken as a whole, the data indicate that while caging has an effect on flora composition, host genotype exerts a stronger effect. Strains cohabiting so that no cage had more than one mouse from the same strain seemed to control for environmental effects with the host genetic effects limiting the degree to which the microenvironment could influence the composition of the GI flora.

Discussion

Using assays designed to be specific for each ASF bacterial species, we were able to detect and quantify each member of the ASF in a variety of biological samples, including pure cultures, fecal samples, colonic flushings, and homogenized tissues from ex-germfree and barrier-housed mice. This proves the utility of these assays for ASF quantification, even in samples from complex mixtures or ecosystems. We used these assays to demonstrate ecologic differences in GI flora across and within different mouse strains. The data resulting from the use of these assays suggest that significant variation exists among strains of inbred mice and that an additional significant source of variation is the microenvironment.

Variation in GI flora may be important for many biological traits. For example, differential housing (group vs. individual) is a cage effect that has been found to affect mouse immunologic response (Salvin

et al. 1990), aggression (Nyberg et al. 2004), glucocorticoid resistance (Avitsur et al. 2003), and testis weight (Sayegh et al. 1990). The social position of the mice within a cage (dominant vs. inferior) can lead to variation in stress levels and psychological stress is known to inhibit small intestine transit in mice (Cao et al. 2005). Reduction in small intestine transit time, like that caused by stress, results in increased numbers of aerobes in the proximal small intestine and a decrease in the proportion of *Lactobacillus* sp. to *E. coli* (Wang and Wu 2005).

Recaging of mice has been reported to cause intercage variation (Zhou et al. 1997; Avitsur et al. 2003; Marashi et al. 2004), especially as it relates to factors associated with aggression and stress as new social positions are established. Changes in caging and population density can also modulate inflammation in mice (Scislowska-Czarnecka et al. 2004). These studies suggest a link between stress, dysbiosis, and GI function, which might be relevant to GI diseases like irritable bowel syndrome, and imply that caging differences can translate to changes in GI population dynamics.

In studies in which mice were tested for behavioral responses to ethanol using a multisite strategy, significant differences in the performance of genetically identical mice were observed, showing that nongenetic components affect ethanol preference (Crabbe et al. 1999; Crabbe and Wahlsten 2003; Wahlsten et al. 2003a, 2003b). This observation was most likely a result of environmental differences between test sites and not differences in protocols or handling (Crabbe et al. 2005), because food, bedding, light/dark cycles, experimental method, cage density, cage changing frequency, and numerous other variables were standardized. Variation in GI flora after establishment at the different test sites was not measured and could have been a major confounder contributing to cross-site, intrastain variation underlying phenotypic differences attributed to the "idiosyncratic nature" of each lab.

Similar to the behavior analyses, *in vivo* factors distinct from genetic background effects can influence obesity phenotypes (Burcelin et al. 2002). Obesity is strongly correlated with adipocyte hormone production, insulin levels, and glucose uptake, factors that have genetic underpinnings, but which may also be influenced by environmental factors. When genetically identical C57BL/6J mice are put on a high-fat diet, most mice develop insulin resistance after treatment. However, obesity differs among isogenic C57BL/6J mice, with only 50% becoming obese and diabetic and 10% remaining lean and nondiabetic after nine months. The intermouse variation in obesity has been attributed to "differ-

ential metabolic adaptation" to the high-fat diet (Burcelin et al. 2002). Changes in body weight and diabetes occurred slowly and caused the body weight and diabetic indices to start to diverge three months after diet initiation. Intriguingly, although the lean mice had significantly higher rates of glucose clearance *in vivo*, no differences were observed when tissue utilization of glucose was measured *in vitro*. This suggests that the glucose utilization differences may not be due to intrinsic host factors but to some other *in vivo* factor like GI flora; differences in the composition of the GI flora can modulate food transit time and metabolic factors regulating food intake and energy expenditure, resulting in variable obesogenic and diabetic phenotypes (Chow 2002).

Supporting a major role of the GI flora in physiology, it was reported that flora alone can affect body fat and insulin resistance (Backhed et al. 2004). Conventionalization of germfree C57BL/6J mice with a normal flora was followed by a 60% increase in body fat and the induction of insulin resistance within two weeks. Circulating leptin, insulin, and glucose levels were all significantly increased in conventionalized mice compared with their germfree counterparts. The effects were independent of the presence of mature lymphocytes and were mediated by the GI flora-regulated suppression of fasting-induced adipocyte factor. Suppression of this factor led to increases in monosaccharide absorption from the gut lumen and stimulated host hepatic lipogenesis, suggesting a link between the GI flora and energy usage and storage. Consequently, a diet-induced change in the GI flora is an environmental factor that can effect obesity studies.

In summary, our experimental data confirm that bacterial numbers derived from analyzing fecal samples alone are not a true indication of the variety in colonization that exists in specific niches (Sarmarupavtarm et al. 2004). Our data also show that there are no significant sex-specific differences in ASF colonization, except for ASF 500 and ASF 502, which show large sex-specific differences in the small intestine, cecum, and colon in ex-germfree C.B-17 mice. However, there are significant differences in the ASF colonization patterns between mouse strains that can be globally detected in fecal samples, indicative of significant host-dependent differences in GI flora across genetically distinct individuals. These differences might be exploited to evaluate intervention therapies for GI pathologies or may provide clues to the biological processes that underlie strain differences in experimental models of GI and other diseases.

We also found that interstrain differences are confounded by significant intercage variation.

Within A/J and FVB/NJ strains, cage variation is most likely due to cage effects and not litter effects because ASF profiles within a cage were not significantly different, yet litters split at weaning showed divergence of ASF profiles. Furthermore, cohabitation of mice from cages previously shown to exhibit differences in ASF profiles yielded mice whose ASF profiles, though still significantly different from each other, were more similar than when mice were housed separately, indicating that the microenvironment can modulate the composition of the GI flora. Nonetheless, replicate samples taken from the same mouse over time were not significantly different, suggesting that colonization patterns of enteric GI flora are stable over time without external perturbations. Finally, the finding that cohabitation of genetically distinct mice removed the cage effect confirms that microenvironment is a key factor in GI population dynamics, but the fact that strains remained significantly different in spite of cohabitation demonstrated the strength of genetic influence on GI flora composition.

The ASF was considered the ideal enteric model flora for these studies because it provides many of the benefits ascribed to conventional flora, like barrier protection from pathogenic organisms and the "normalization" of gastrointestinal organs in germ-free animals. Furthermore, the ASF are also members of the normal mouse GI flora, which is present in most mouse facilities, and, because they represent the major niches of the GI tract, can be used as a sentinel flora to monitor differences between mice in response to recaging, relocation, and other environmental effects as well as changes related to diet, stress, and mouse behavior. These assays may also be helpful for understanding extragenetic variations between experimental animals, allowing for more meaningful data interpretation and analysis.

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