INFECTION AND IMMUNITY, July 2004, p. 3793–3802 Vol. 72, No. 7
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.7.3793–3802.2004

Use of a Continuous-Flow Anaerobic Culture To Characterize Enteric Virulence Gene Expression

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Received 16 February 2004/Returned for modification 17 March 2004/Accepted 24 March 2004

We developed an in vitro culture method to characterize the expression of bacterial genes under conditions mimicking the colonic environment. Our culture system (the intestinal simulator) comprised a continuous-flow anaerobic culture which was inoculated with fecal samples from healthy volunteers. As a test organism, we employed enteroaggregative *Escherichia coli* (EAEC), an emerging diarrheal pathogen that is thought to cause infection in both the small and large intestines. After the simulator culture achieved equilibrium conditions, we inoculated the system with prototype EAEC strain 042 and assessed the expression of three EAEC virulence-related genes. We focused particularly on expression of *aggR*, which encodes a global transcriptional regulator of EAEC virulence factors, and two AggR-regulated genes. By using real-time quantitative reverse transcription-PCR, we showed that *aggR* expression in the simulator is increased 3- to 10-fold when 042 is grown under low-pH (5.5 to 6.0) conditions, compared with results with neutral pH (7.0). Interestingly, however, this effect was seen only when the strain was grown in the presence of commensal bacteria. We also found that expression of *aggR* is 10- to 20-fold higher at low NaCl concentrations, and this effect was also observed only in the presence of commensal bacteria. Using coculture and conditioned-media experiments, we identified specific strains of *Enterococcus* and *Clostridium* that upregulated *aggR* expression; in contrast, strains of *Lactobacillus* and *Veillonella* downregulated *aggR* expression. Our data provide new insights into regulation of virulence genes in EAEC and suggest the utility of intestinal simulation cultures in characterizing enteric gene regulation.

Enteric infections continue to exact a heavy toll on the world's most indigent populations. These infections include many of the most intensively studied bacterial agents. One obstacle to more complete understanding of bacterial infections is the paucity of available whole-animal models. Moreover, a hallmark of enteric pathogens is host specificity, which limits the applicability of laboratory animal models. Thus, investigators have resorted to more-limited models of interaction with the human gastrointestinal mucosa, such as the use of xenographic transplantation and in vitro intestinal cell culture systems (2, 19).

The most important limitation of traditional in vitro models is that the bacteria cannot be cultivated under conditions mimicking those found in the lumen of the gastrointestinal tract. Signals found in the lumen include low oxygen tension, varying of pH and osmolarity by site, and the presence of specific substances rarely included in bacteriologic media, such as mucin and volatile fatty acids. Moreover, it has become increasingly clear that intra- and interspecies communication among bacteria in the gastrointestinal lumen (traditionally called quorum sensing) is a common mechanism of gene regulation in pathogenic bacteria (23–26). Thus, models that do not provide exposure of the pathogen to an environment similar to that of the human gastrointestinal ecosystem do not provide an adequate assessment of virulence gene expression characteristics.

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Continuous-flow anaerobic cultures of the human fecal microbiota have been available for decades (10). These systems have been employed to study the dynamics of bacterial populations (11, 17), the production of helpful and harmful by-products under different conditions (5, 16), the actions of probiotic bacteria, and other features of the human gastrointestinal tract (1). To our knowledge, however, these systems have not previously been used to study the response of enteric pathogens to the environmental signals they experience in the human gut.

In this study, we have developed a continuous-flow anaerobic fecal culture based on previously described systems and in which we assessed specific gene expression profiles using real-time quantitative reverse transcription-PCR (qRT-PCR). To understand the response of a bacterial pathogen in this system, we employed enteroaggregative *Escherichia coli* (EAEC), an organism with several recently described virulence genes whose expression profiles are not well characterized. We focused particularly on expression of *aggR*, which encodes a global transcriptional regulator of EAEC virulence factors that include aggregative adherence fimbriae (AAF) (18), the dispersin surface protein (21), an ABC transporter (20), and at least 16 additional genes (E. Dudley and J. P. Nataro, unpublished data). EAEC has been shown to adhere most abundantly to sections of the human colon (14) and to cause mucosal damage to colonic epithelium (19). Our findings suggest that colonic flora may exert important and unexpected influences on the expression of EAEC virulence-related genes.

TABLE 1. Medium used for intestinal simulation culture[a]

Compound	Vendor	Concn
Fermenter media		
NaCl	Sigma	30 mM
$NaHCO_3$	Sigma	5 mM
KCl	Sigma	5 mM
Glucose	Sigma	2.5 g/liter
Maltose	Sigma	3.0 g/liter
Starch	Sigma	0.6 g/liter
Hemin	Sigma	10^{-4} g/liter
Mucin	Sigma	2.5 g/liter
Bacto-peptone	Difco	5.0 g/liter
Preculture media		
Tryptone	BIO 101 Systems	10 g/liter
Yeast	BIO 101 Systems	5 g/liter
NaCl	Sigma	10 g/liter
Glucose	Sigma	5 g/liter
Maltose	Sigma	6 g/liter

[a] Modified from reference 2.

MATERIALS AND METHODS

Continuous-flow cultures. The continuous-flow intestinal simulator was essentially as described in references 2 and 17, with the modifications described below. The system comprised a continuous anaerobic culture of human feces maintained to a set pH or osmotic concentration. The basic growth medium of the culture was previously shown to provide a stable culture of colonic bacteria and is designed to mimic the conditions of the human colon (Table 1) (8). The entire fermentation apparatus was maintained inside an anaerobic chamber (Coy Laboratories Products, Inc., Grass Lake, Mich.) flooded with 90% N_2, 5% CO_2, and 5% H_2. Oxygen scavenging was accomplished by two heated copper coil units provided by the chamber manufacturer. The main fermenter culture vessel (1,000-ml Bactolift culture bottle; Lofstrand Labs Ltd., Gaithersburg, Md.) was connected to a source of fresh medium and to an exit port for collection of spent medium. Flow in the system was maintained by a peristaltic pump, which was set to assure one complete medium change (500 ml) every 24 h. A pH electrode connected to a pH meter provided continuous monitoring and servo-controlled addition of 4N NaOH to maintain the pH at the setpoint. Fresh medium in the reservoir was maintained at pH 2 to prevent retrograde contamination of the system.

To inoculate the simulator culture, 60 g of stool sample from one of three normal subjects was precultured in 500 ml of preculture medium (Table 1) anaerobically without flow. Each experiment was repeated with the stools of each subject. After overnight growth, 60 ml of the preculture was filtered through sterile gauze sponges to remove large particles and was inoculated into 440 ml of fresh fermenter medium in the continuous-flow system. The system was stabilized for at least 24 h prior to inoculation of test bacteria.

Once the simulator culture was stabilized, 50 ml of overnight Luria-Bertani (LB) broth culture of EAEC strain 042 ($\sim 10^8$ CFU/ml) was inoculated into the system. Four to eighteen hours after addition of 042, 25 ml of culture sample was withdrawn to characterize the bacterial populations and to perform RNA extraction. The sodium chloride concentration was modified by preparing medium with 0, 30, 100, 200, or 400 mM NaCl. Twenty-five milliliters of fermenter culture was sampled daily, and the bacterial populations were enumerated and characterized by using standard selective media (Anaerobic Systems and Becton Dickinson) (13) and biochemical analysis kits (Remel, Inc., Lenexa, Kans.; BioMerieux, Marcy L'Etoile, France). Strain 042 was distinguished from commensal *E. coli* by virtue of resistance to chloramphenicol and streptomycin. Those stool samples from healthy volunteers that harbored choramphenicol-resistant strains were not used for experiments.

Coculture assays. EAEC 042 was anaerobically cocultured with bacterial strains isolated from the stool of healthy subjects in an approximate proportion of 1:100. In separate experiments, EAEC 042 was cultured in preconditioned media as described previously (24). Cocultures were performed in 50-ml Falcon tubes containing 45 ml of fresh fermenter media (with 1.0 g of thioglycolate/liter) or 45 ml of preconditioned media, and cultures were incubated anaerobically at 37°C (pH 6.0) overnight. Test anaerobic and aerobic strains were propagated anaerobically on blood agar plates for 48 or 24 h, respectively. To prepare culture supernatants, a bacterial inoculum was picked from a culture dish and resuspended in fresh fermenter medium to an approximate initial bacterial density of 10^5 CFU/ml; this culture was incubated for 24 to 48 h to yield a final bacterial density of 10^7 to 10^8 CFU/ml. The supernatants from these cultures were filter sterilized using 0.45-μm-pore-size filter units (Corning Co., Corning, N.Y.), the nutrients of the filtered media were corrected to a final concentration of 0.5× LB, and the pH was adjusted to 6.0 or 7.0 using 1N NaOH.

RNA extraction and RT-PCR. At designated time points, whole-cell RNA was isolated from a 20-ml sample of the simulator culture using the Trizol method (Invitrogen, Carlsbad, Calif.) according to manufacturer's protocols. RNA was treated with RNase-free DNase I (Roche Diagnostics Corporation, Indianapolis, Ind.) to eliminate contaminating DNA. DNase I and salts were removed after treatment by using the RNeasy kit (QIAGEN, Inc., Valencia, Calif.). cDNA was synthesized from 1 to 5 μg of RNA by using random hexamer primers (Invitrogen) and the Thermoscript RT enzyme (Invitrogen) for 10 min at 25°C, 1 h at 60°C, and 5 min at 85°C; alternatively, cDNA was synthesized by using the Improm II reverse transcriptase enzyme (Promega, Madison, Wis.) for 10 min at 25°C, 1 h at 42°C, and 15 min at 75°C. PCR was performed according to standard procedures with *Taq* or *Platinum Taq* DNA polymerase (Invitrogen). Negative controls for all reactions included samples without reverse transcriptase.

Primers used included *cat* forward (5′-GCGTGTTACGGTGAAAACCT-3′) and *cat* reverse (5′-GGGCGAAGAAGTTGTCCATA-3′); *aggR* forward (5′-CCTAAAGGATGCCCTGATGA-3′) and *aggR* reverse (5′-GAATCGTCAGCATCAGCTACA-3′); *aafD* forward (5′-TCCCCCATTATTTCGACTTG-3′) and *aafD* reverse (5′-CGGGATCCCTTTTACACAAA-3′); and *aap* forward (5′-TGGAACGCAGATAATGTGGA-3′) and *aap* reverse (5′-GGGTGTCTGTCTCTGGGGTA-3′).

Real-time quantitative PCR experiments. Transcript levels were determined by using the QuantiTect SYBR Green PCR kit (QIAGEN, Inc.) after cDNA synthesis as described previously (22). RNA treated as described above was tested for DNA contamination by a standard PCR with the same primers used in the real-time assay. Reactions were performed in a 25-μl volume using the manufacturer's protocols; the reaction mix comprised 2 μl of template cDNA, 12.5 μl of 2× QuantiTect SYBR Green PCR master mix [HotStarTaq DNA polymerase, 2.5 mM $MgCl_2$, Tris-Cl, KCl, $(NH_4)_2SO_4$ (pH 8.7), 100 μM (each) dATP, dCTP, and dGTP, 200 μM dTTP-dUTP, SYBR green I and Rox dyes], a 300 μM final concentration of each primer, and diethyl pyrocarbonate-treated H_20 to 25 μl. Amplification was performed under the following conditions: 15 min at 95°C (to activate HotStarTaq DNA polymerase), followed by 40 cycles of 30 s at 95°C, 40 s at 55°C, and 15 s at 72°C. Fluorescence was detected with the Geneamp 5700 sequence detection system machine and accompanying software (version 1.1) (Applied Biosystems, Foster city, Calif.). A standard curve was plotted for the reaction with critical threshold values obtained from the amplification of known quantities of DNA from target genes. The chromosomal constitutively expressed *cat* (chloramphenicol acetyltransferase) gene of strain 042 (2) was used to normalize RNA abundance for all reactions.

RESULTS

Establishment of the intestinal simulator model. Following from previously described systems, we established a continuous-flow anaerobic culture system that mimics conditions of the human colon. Predominant bacterial flora in the fermenter vessel after 7 days of growth resembled those of the initial fecal inoculum (Fig. 1). Abundance of bacterial species was in close agreement with expected profiles from the published literature, with *Bacteroides*, *Eubacterium*, *Clostridium*, *Lactobacillus*, and *Bifidobacterium* being the most commonly isolated anaerobic species (9); *Enterococcus* spp. and *E. coli* were the predominant facultative anaerobes. Bacterial populations reached maximum densities of approximately 10^{10} CFU/ml, consistent with bacterial concentrations in the ascending colon (6, 9). There was a trend for certain species, such as *Escherichia*, *Clostridium*, *Bacteroides*, and *Bifidobacterium*, to reach slightly higher levels in the simulator culture than in the fecal inoculum; *Proteus* and *Eubacterium* were typically more easily recovered after several days of cultivation in the intestinal simulator. There was significant intersubject variation of bacterial species, and to a lesser extent, variation within the same subject

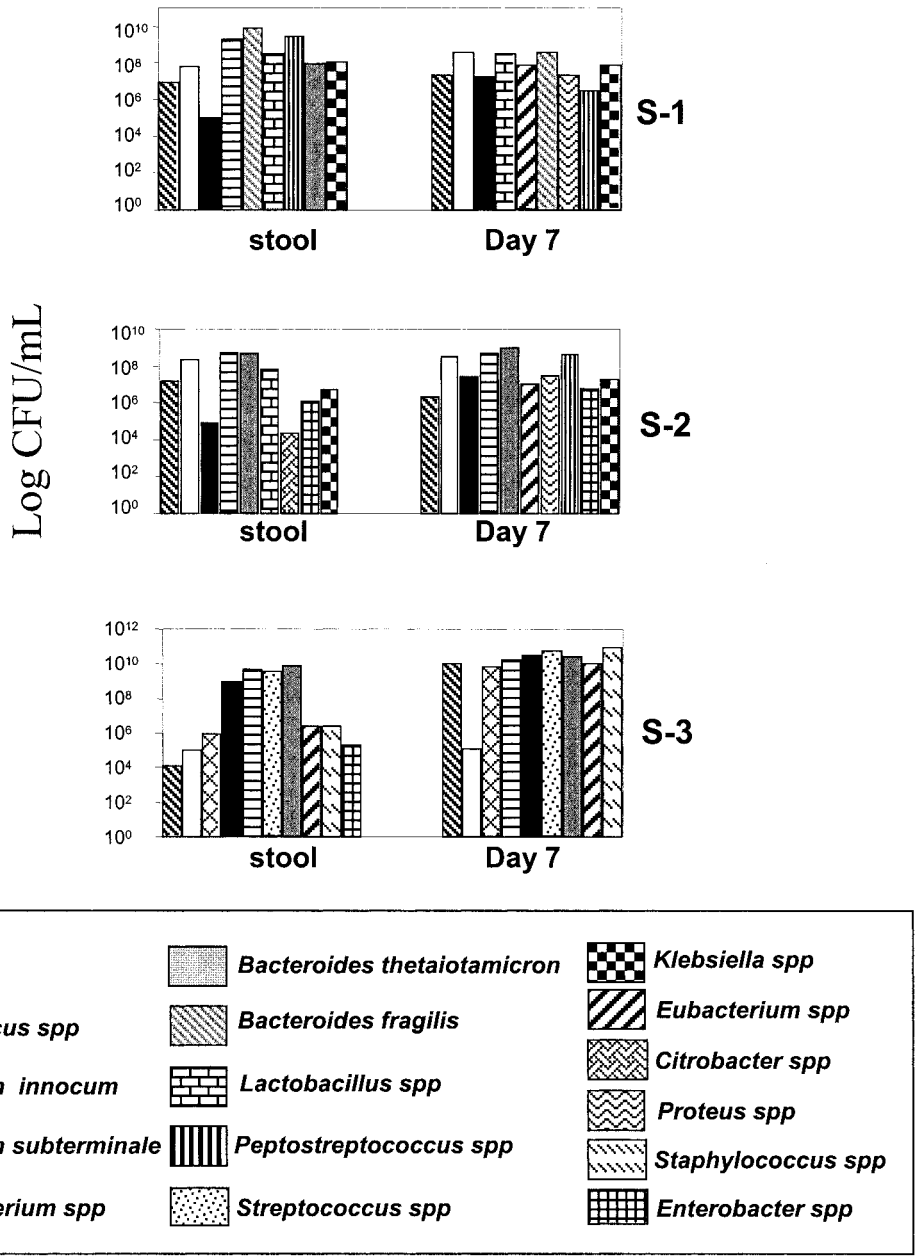

FIG. 1. Main bacterial populations present in precultures of stool samples and after 7 days' growth in the simulator. The simulator culture was inoculated with 60 ml of precultured stool from a single subject and was incubated under continuous flow for 7 days. The dominant culturable bacterial populations were determined by biochemical analysis. The results for three representative experiments (S-1, S-2, and S-3) are shown.

over time (data not shown). All experiments performed in the simulator were repeated with stool samples from at least three different normal donors.

Effect of pH manipulation on the abundance of bacterial species. One advantage of the continuous-flow anaerobic culture system is that certain characteristics of the culture can be manipulated. We sought first to assess the influence of pH on the relative abundance of bacterial flora. The culture vessel was established as above at an initial pH of 7.0. Thereafter, at daily intervals, the pH of the system was adjusted by resetting the pH controller, which responded via on-demand addition of NaOH. After equilibration, the system was inoculated with EAEC strain 042, which was distinguishable from commensal *E. coli* by virtue of its resistance to both streptomycin and chloramphenicol.

We found that most bacterial species isolated at the beginning of the experiment were recovered at all pH values. *Clostridium*, *Bacteroides*, *Lactobacillus*, and *Enterococcus* were present under all pH conditions tested. However, the relative abundance of some bacterial species changed substantially. In particular, commensal *E. coli* and *Bifidobacterium* spp. were recovered at pH 6.0 but did not tolerate pH 5.5 in most of the experiments (Fig. 2). In contrast, EAEC 042 was consistently recovered at all pH values, including pH 5.5. In separate ex-

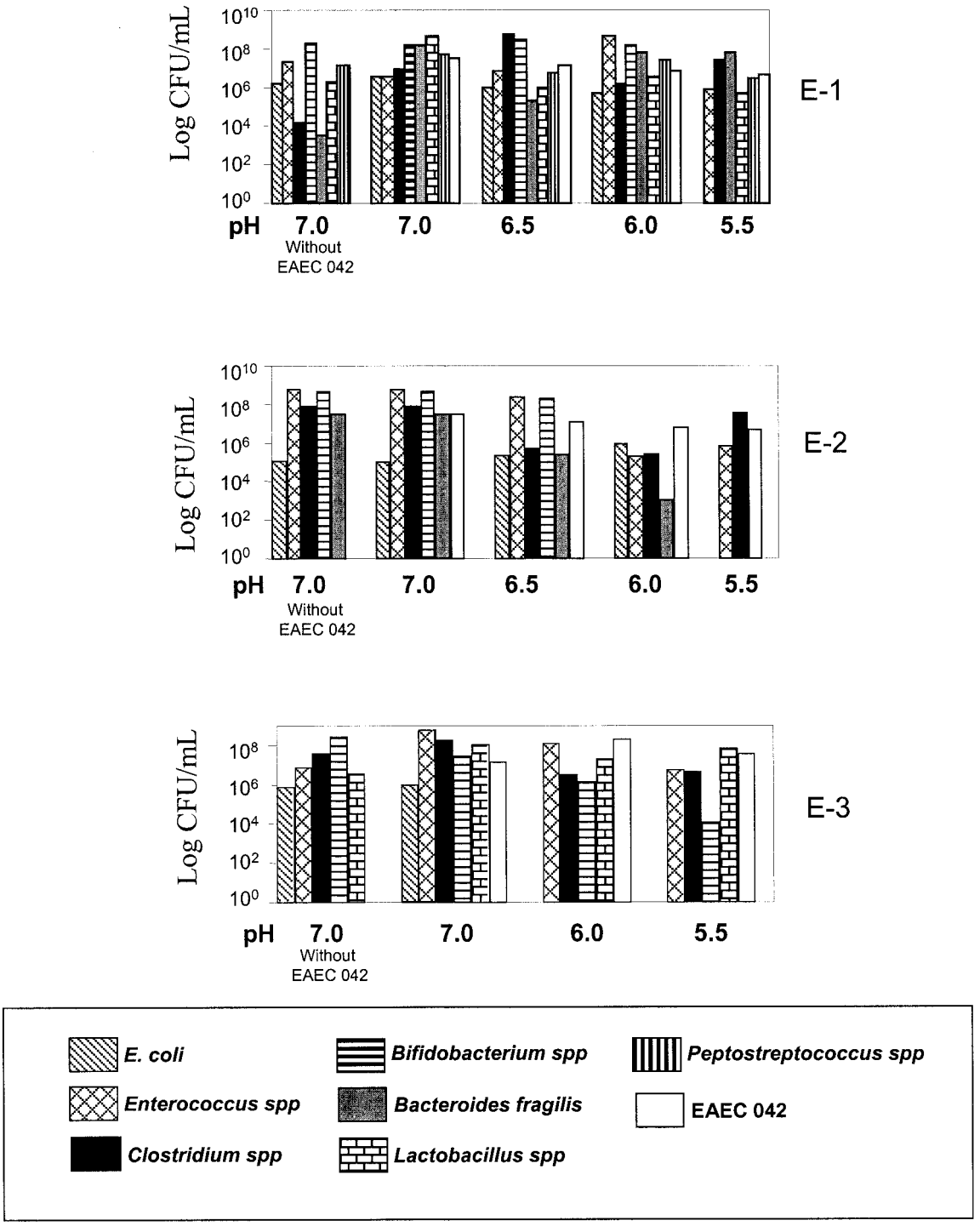

FIG. 2. Dominant culturable bacterial populations present in the simulator at different pH setpoints. EAEC 042 was inoculated in the intestinal simulator, and the system was set to maintain pH 7.0, 6.5, 6.0, or 5.5 for 7 days at each pH, at which time dominant bacterial populations were enumerated. Three representative experiments are shown, using stools from different subjects.

periments, we determined that this strain was acid tolerant, withstanding pH as low as 2.0 (data not shown).

Effect of pH on expression of EAEC genes in the intestinal simulator. We have previously reported that *aggR* expression is responsive to different environmental conditions when manipulated individually (18). We sought to extend these observations, using the continuous-flow simulator, specifically examining how the bacteria would respond when multiple signals were integrated.

Strain 042 was cultured in the simulator under different pH conditions over 7 days; each day the system was adjusted to a different setpoint. In the initial series of experiments, the pH

was first set to 7.0, followed by 6.5, 6.0, and finally 5.5. This series was reversed in a subsequent experiment. At each pH setpoint, a sample was withdrawn from the culture vessel, the bacteria were collected by centrifugation, and total RNA was extracted for quantitation of mRNA specific for three virulence-related genes: *aap*, *aafD*, and *aggR*. The RNA was reverse transcribed, and cDNA was subjected to real-time qRT-PCR with specific primers. To normalize the quantity of RNA, transcript abundance was compared with that of the constitutive 042 chromosomal *cat* gene (2) and expressed in specific transcript/*cat* transcript ratios. Stool samples which yielded *cat* mRNA prior to inoculation of strain 042 were discarded.

We found that *aggR* expression was 3- to 10-fold higher at pH 6.0 than at pH 7.0 (Fig. 3A). These results were similar whether or not fresh 042 was added to the culture vessel with each change in pH setpoint. The *aafD* and *aap* genes were also expressed more strongly at low pH (Fig. 3A), consistent with the observation that these two genes are activated by AggR. *aggR*, *aafD*, and *aap* expression was consistently maximal at low pH, regardless of the sequence of pH changes (Fig. 3B).

Our studies suggested that there was differential relative strength of expression of the different EAEC genes. When compared directly, transcription of *aap* was consistently at least 10 times more abundant than that of *aafD*, regardless of pH. Expression of *aggR* was also consistently higher than that of *aafD* (Fig. 4).

Effect of sodium chloride concentration on EAEC gene expression. Our system also permitted us to assess the effect of osmolarity (as NaCl concentration) on EAEC gene expression. These experiments were performed as described above, except that the NaCl concentration was changed in the fresh medium reservoir every 24 h, ranging from 0 to 400 mM. The pH of the culture was maintained at 6.0 for these experiments. After every medium change, the simulator was permitted to stabilize for 24 h, over which time one full volume exchange occurred. After this time, the culture was sampled for gene expression as described above.

We found that *aggR*, *aafD*, and *aap* were more abundantly expressed under low NaCl concentrations. *aggR* transcript in 0 or 30 mM NaCl was 10- to 20-fold more abundant than at an NaCl concentration of 200 or 400 mM (Fig. 5A). *aafD* and *aap* genes yielded the same expression profiles as *aggR* (Fig. 5A). *aafD* was expressed 5- to 10-fold more abundantly at 0 or 30 mM than at higher concentrations, whereas the *aap* gene was expressed at 10 times higher levels at low NaCl concentrations than at high ones. In one experiment only (E-2), expression of *aap* demonstrated an unusual second peak at 400 mM.

Since our observed pH effects could have been influenced by the NaOH concentration, we performed experiments in which the pH of the fresh medium was adjusted to 6.0 or 7.0 by using phosphate buffers at a uniform final concentration of 100 mM. *aggR* expression revealed the same response to pH as in the NaOH-controlled system (data not shown).

Effect of pH and sodium chloride concentration in the absence of commensal flora. The continuous-flow system also permitted us to assess EAEC gene expression in the absence of commensal flora. We first cultivated 042 aerobically in LB media buffered to pH 6.0, 6.5, or 7.0 and measured expression of *aggR*, *aafD*, and *aap* by using real-time qRT-PCR. These experiments revealed relatively modest effects of pH on gene expression: abundance of *aggR* and *aap* transcripts was only approximately twofold higher at pH 7.0 than at pH 6.0, and *aafD* expression was threefold higher at pH 7.0 than at pH 6.0 (data not shown). We then cultivated strain 042 in simulator medium under conditions of anaerobic continuous flow but without the addition of fecal bacteria. Under these conditions, *aggR*, *aap*, and *aafD* were more strongly expressed at neutral pH than at pH 6.0, similar to growth in L broth (Fig. 6). Strikingly, expression of the *aggR* and *aap* genes at pH 7.0 was approximately five- to sevenfold higher than at pH 5.5 (Fig. 6).

We also tested the response of strain 042 to modulation of the NaCl concentration in the absence of commensal flora. Surprisingly, we found that the *aggR*, *aafD*, and *aap* genes were expressed better at high NaCl concentrations, contrary to what we saw when 042 was grown in the fermenter in the presence of the intestinal flora. Although a clear difference in the gene expression level could be seen at high NaCl concentrations compared with low NaCl concentrations, there were only small differences between expression levels at each increment (Fig. 5B).

Effect of specific commensal strains on 042 gene expression. Our data suggested that EAEC genes may respond differently in the presence or absence of commensal bacteria. To further characterize this phenomenon, we assessed the effects of individual enteric strains on expression of *aggR*. Cocultures of 042 and, individually, strains of *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Enterococcus*, and *Veillonella* were established. The relative proportion of EAEC 042 to the specific intestinal strains was approximately 1:100. After overnight anaerobic growth at pH 6.0 in fermenter media, RNA from cocultures was extracted and analyzed for *aggR* expression by qRT-PCR. We found that *aggR* expression varied substantially depending on the species cocultured. The level of *aggR* expression increased two to fivefold when 042 was cultured with strains of *Enterococcus* or *Clostridium* (Fig. 7). In contrast, *Lactobacillus* and *Veillonella* species were found to downregulate *aggR* expression nearly sixfold (Fig. 7). Strains of *Bacteroides*, *Bifidobacterium*, and commensal *E. coli* did not exert any demonstrable effect on *aggR* expression.

In order to verify the observations of coculture, we repeated these experiments using preconditioned media. Expression of an *aggR-lacZ* fusion was assessed in media previously conditioned by growth with EAEC 042 or with EPEC or EHEC strains. In contrast to the EHEC *lee1-lacZ* promoter fusion control, we did not observe any effect of *E. coli*-preconditioned media on *aggR* expression (data not shown). However, media conditioned with other enteric strains exerted pronounced effects on *aggR* expression. The *aggR* promoter was upregulated two- to threefold in media conditioned by growth of *Enterococcus* or *Clostridium* spp., similar to the results of coculture experiments (Fig. 7). Media preconditioned by growth of *Lactobacillus* or *Veillonella* downregulated expression of the *aggR* gene to a similar degree (Fig. 7).

DISCUSSION

Few whole-animal models are available for the study of diarrheal pathogens. For some organisms, such as EAEC, no animal model carrying a conventional enteric flora has yet been described. At the same time, it has become increasingly

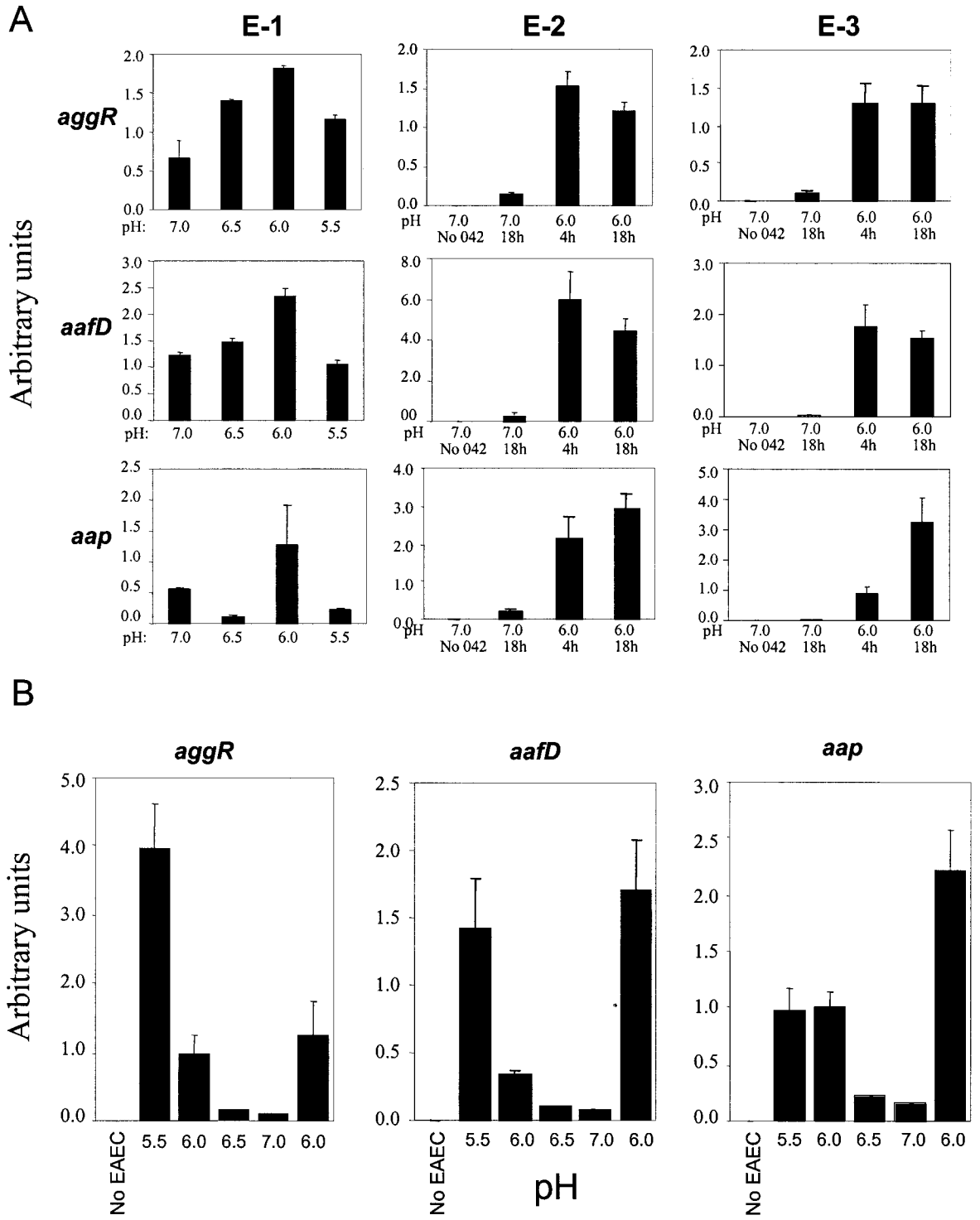

FIG. 3. Expression of *aggR*, *aafD*, and *aap* from EAEC 042 grown at different pH setpoints in the simulator culture. (A) After equilibration of the system for 72 h, EAEC 042 was inoculated at a final concentration 10^7 CFU/ml, and incubation was continued for 4 to 18 h. Real-time qRT-PCR was performed for EAEC virulence genes as described in Materials and Methods. Data are reported in arbitrary units of transcript abundance normalized against *cat* transcript abundance (quantity of target gene/quantity of *cat* gene). Three representative experiments (E-1, E-2, and E-3) are shown (A). For E-1, samples were only withdrawn 18 h after 042 inoculation. For E-2 and E-3, fermenter samples before EAEC 042 inoculation were included as negative controls. (B) Effect of increasing and decreasing pH on *cat*, *aggR*, *aafD*, and *aap* expression in the simulator system. The pH setpoint was increased from 5.5 to a maximum of 7.0 and then reduced to 6.0. The system spent at least 24 h at each setpoint. Real-Time qRT-PCR was performed as for Fig. 3A. Data are expressed in arbitrary units of transcript abundance normalized against *cat* transcript abundance.

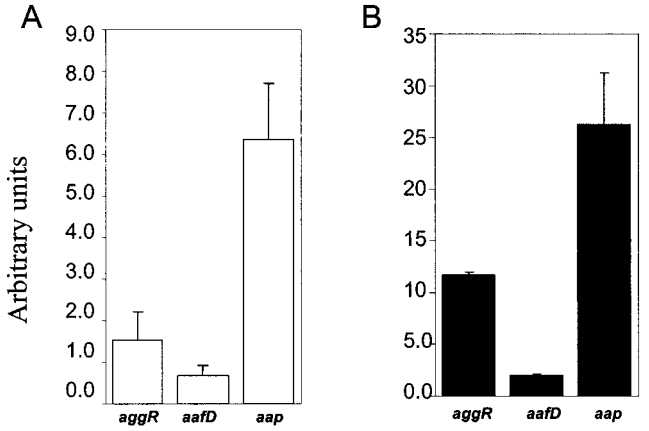

FIG. 4. Levels of *aggR*, *aafD*, and *aap* expression in EAEC 042 grown at pH 7.0 (A) or 6.0 (B) in the intestinal simulator. EAEC 042 was grown in the intestinal simulator at the respective pH setpoint, and the expression levels of the three virulence genes were compared directly by real-time qRT-PCR. Data are expressed in arbitrary units of transcript abundance normalized against *cat*.

apparent that not only are virulence genes subject to complex regulatory mechanisms, but these circuits can be impacted by signals released from the commensal flora. To understand the contributions of quorum sensing and the complexities of multiply integrated environmental signals, it is helpful to have a model whose parameters can be individually manipulated, which is not possible with whole-animal systems. To address this need, we developed a continuous-flow anaerobic culture system, based on previously published reports (2, 8, 17). Our system permitted us to address independently the contributions of pH, sodium chloride concentration/osmolarity, and commensal flora to the regulation of virulence-related loci in an enteric pathogen. In a separate report, we have demonstrated the presence of quorum sensing signals in our culture system as sensed by enterohemorrhagic *E. coli* (23).

We found that our system permitted long-term stable growth of the dominant culturable flora of the human gastrointestinal tract. Intestinal anaerobic species, such as *Bacteroides*, *Eubacterium*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*, and *Veillonella*, were maintained at high concentrations; these organisms are found in high numbers in the stools of normal adults and children outside of infancy (4). Our system also permitted the propagation of aerobic species, such as *E. coli*, *Enterococcus*, *Klebsiella*, *Proteus*, *Peptostreptococcus*, *Streptococcus*, and *Staphylococcus*. We were able to achieve stable bacterial densities of 10^8 to 10^{10}CFU/ml, similar to densities reported for the ascending and transverse colon (4).

As a test organism, we employed the enteric pathogen EAEC. EAEC strain 042 adheres abundantly to the colonic mucosa in vitro by virtue of aggregative adherence fimbriae II (AAF/II). We have partially characterized the regulation of AAF/II and have shown that expression of the fimbrial biogenesis genes requires the AraC homolog AggR (18). We have recently found that AggR is a global regulator of virulence genes in EAEC, controlling expression of a protein capsule (*aap*) (21), a secretion system (*aat*) (20), and at least one chromosomal gene cluster (*aai*) (E. Dudley and J. P. Nataro, unpublished data). Given its central role in EAEC pathogenesis, we sought to better understand control of the AggR regulon, and we hypothesized that in the absence of a suitable animal model, a continuous-flow simulator culture would permit us to begin to characterize the behavior of the bacterium under in vivo-like conditions and to dissect the contributions of various individual signals.

It has been well established that the pH of the human colon rises gradually from 5.5 to 6.0 in the ascending colon to close to 7.0 in the rectum (12). We were particularly interested in studying the events which might occur upon passage through the ileo-cecal valve, at which time a colonic pathogen experiences a rapid diminution of pH and the presence of enormous numbers of competing bacterial species. For diarrheagenic *E. coli* in particular, an incoming pathogen will presumably need to compete effectively with the incumbent commensal *E. coli*. Presumably, *E. coli* pathogens require specific virulence genes, some conferring metabolic competitiveness, to dominate this new niche. Unexpectedly, we found that in low-pH conditions similar to those of the ascending colon (at least in some subjects), our EAEC strain outcompeted wild-type commensal *E. coli* strains and achieved high growth levels. In separate experiments, we were able to demonstrate acid tolerance by EAEC strain 042 (not previously described). Acid tolerance is a well-known property of other colonic pathogens, such as *Shigella* and Shiga toxin-producing *E. coli* (3, 15). It is likely that such acid tolerance facilitates passage through the stomach, but our data raise the intriguing possibility that this trait may also assist the colonic newcomer in competing successfully with the established flora of the ascending colon. Further experiments to address this hypothesis are under way.

Studies with the simulator culture demonstrated that the *aggR*, *aaf*, and *aap* genes are coregulated, as was expected. Although there may be subtle differences in timing of expression of these genes in vivo, we have not yet observed this phenomenon in our system. In the simulator system, we found that the *aggR* regulon appears to be maximally expressed under conditions of low pH and relatively low osmolarity. Given that pH and osmolarity rise during passage through the colon, these observations are consistent with maximal expression during initial entry into the ascending segment. These experiments did not specifically address the effect of passage through the small intestine on expression of EAEC virulence-related loci, but interestingly, we found that gene expression profiles were significantly different in the absence of commensal flora (as would prevail in most of the small bowel). In the absence of commensal bacteria, *aggR* was better expressed at pH 6.5 to 7.0, notably, the pH found in most of the small intestine. The *aggR*-regulated genes *aafD* and *aap* showed the same expression pattern. This suggests the possibility of two separate regulation circuits for *aggR*: a small bowel quorum sensing-independent system and a large bowel quorum sensing-dependent system. This will require characterization in further studies.

We noted that the effect of commensal flora was not uniform from subject to subject and hypothesized that these differences were due to heterogeneity of fecal populations. To address this hypothesis, we performed coculture and conditioned medium experiments using a collection of commensal isolates. Interestingly, we found that expression of *aggR* varied dramatically in response to the presence of different enteric strains. This effect was to some extent strain specific, since response to entero-

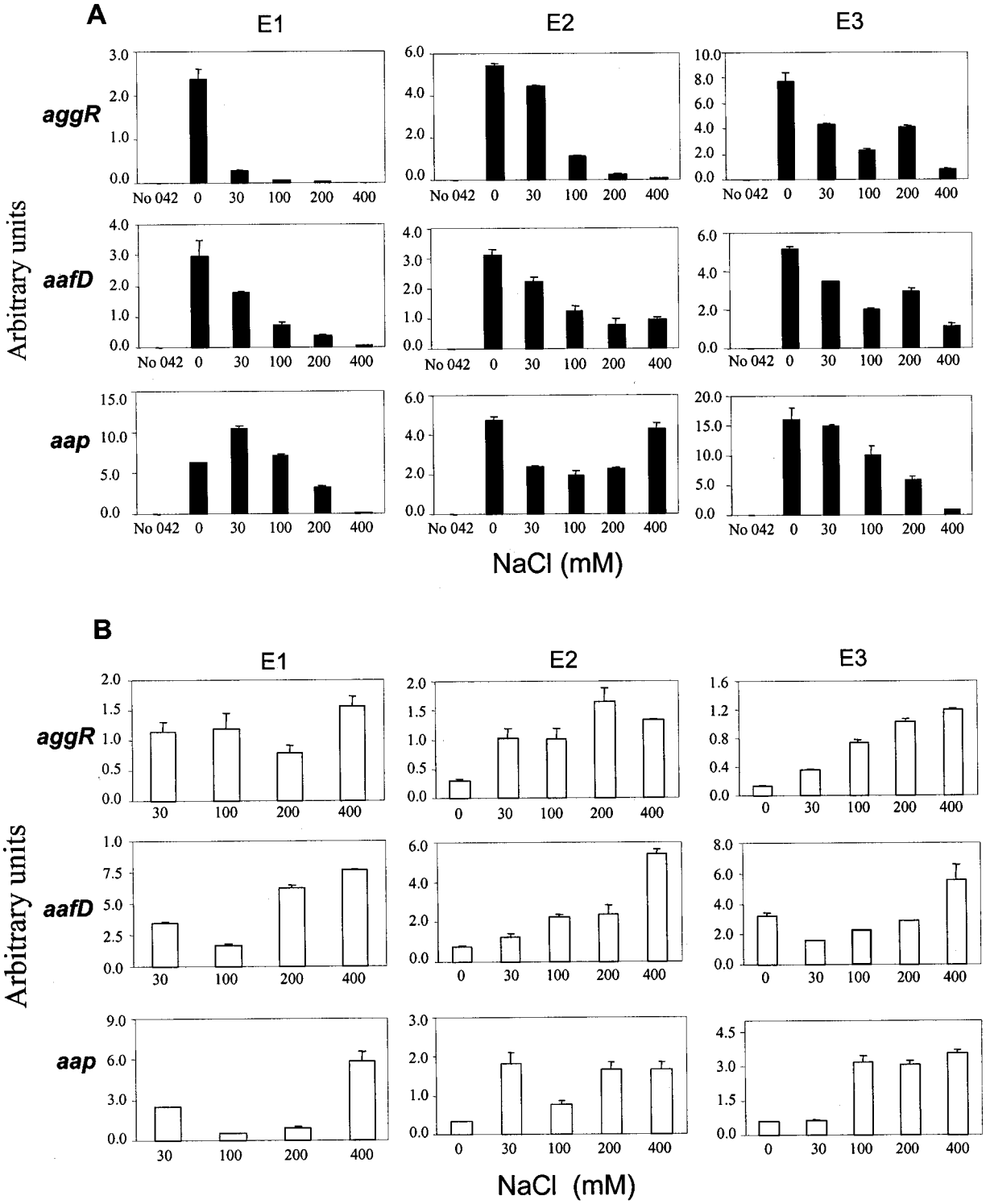

FIG. 5. Effect of the NaCl concentration on EAEC virulence gene expression in the intestinal simulator. (A) EAEC 042 was inoculated once at the beginning of the experiment and grown at different NaCl concentrations for 7 days in the simulator system including fecal flora. (B) EAEC 042 was inoculated anaerobically in simulator medium under continuous flow at various NaCl concentrations, in the absence of fecal commensals. After overnight growth at the osmotic setpoint, total RNA was isolated and cDNA was synthesized and analyzed for *cat*, *aggR*, *aafD*, and *aap* expression by real-time qRT-PCR. Data represent transcript abundance from three separate experiments normalized against *cat* transcript.

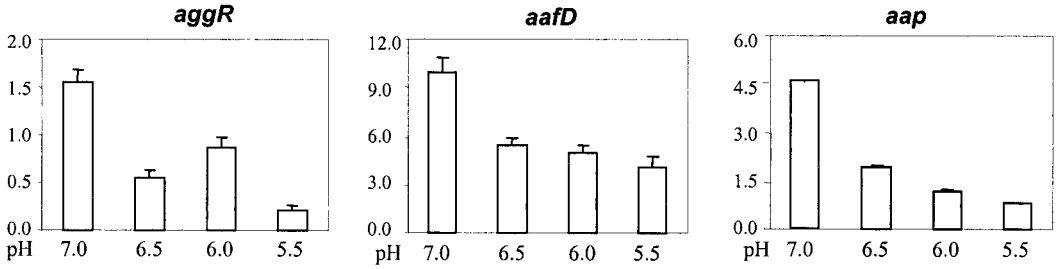

FIG. 6. Expression of EAEC virulence genes at various pH setpoints in the absence of commensal flora. Experiments were performed as for Fig. 3, except that no fecal bacteria were added to the system.

cocci was not uniform within the same species. We were also surprised to find that *aggR* responded to coculture with commensal species but not to media conditioned with the parent EAEC, suggesting that the dominant mode of interbacterial signaling is heterologous. In addition to upregulation of *aggR* by *Enterococcus* and *Clostridium*, organisms often considered harmful, we observed downregulation by *Lactobacillus*, considered to be a marker of healthy enteric ecology and a probiotic species. A specific effect of these enteric species on expression of virulence genes is another mechanism whereby the bacteria could exert their respective contributions to intestinal health. The precise mechanism whereby these interspecies effects are exerted is not yet clear. Possibilities include established autoinducer quorum-sensing systems or the effects of metabolic products not implicated in known signaling circuits. We believe that the activation of *aggR* by the intestinal flora is not due to the LuxS system, since supernatants of EPEC, EHEC, and EAEC which contain AI-2 activated *lee1* in EPEC and EHEC but not *aggR*. It has been reported that AI-2 produced by the commensal flora is important in virulence gene activation in *Pseudomonas aeruginosa*: at least 30 genes of this pathogen were activated by media preconditioned with commensal strains (7). Interestingly, these authors also reported both down- and upregulation of virulence genes by the commensal flora.

The continuous-flow anaerobic system has long been used as a tool for characterizing the activities of enteric bacteria. Here we show that coupling of the continuous-flow system with real-time qRT-PCR allows the investigator to study the effects of the intestinal environment on expression of specific bacterial genes. The results we have generated thus far have provided interesting hypotheses for further testing, including analyses of the behavior of other enteric pathogens and extension of these observations to whole-animal models.

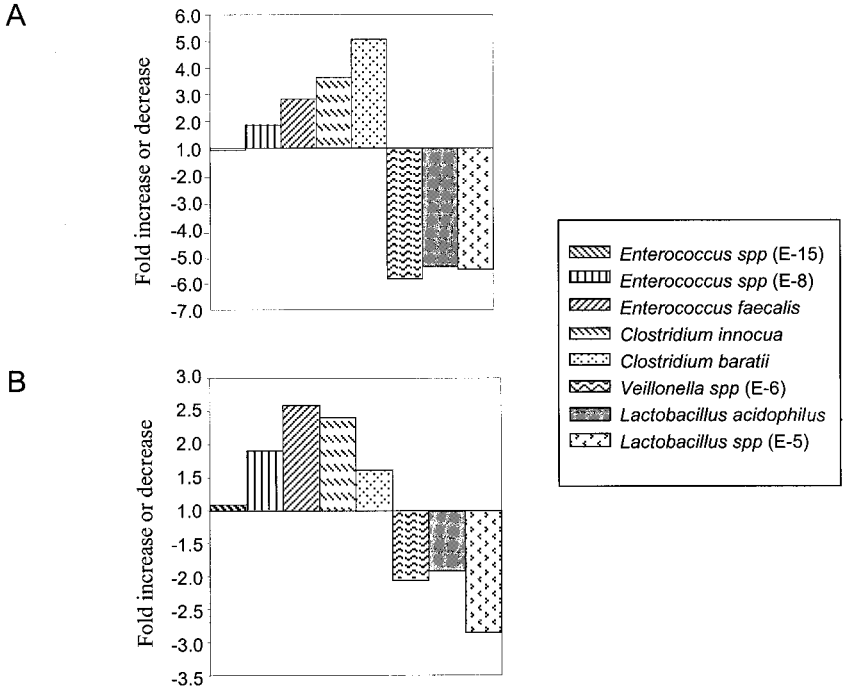

FIG. 7. Effects of commensal flora on *aggR* expression. EAEC 042 was anaerobically cocultured (A) with specific strains of intestinal bacteria in fermenter medium at pH 6.0 or cultured in media preconditioned (B) by growth of the same bacterial strains. After 18 h of growth in either system, the level of *aggR* expression was determined by real-time qRT-PCR. Data are expressed as fold increases or decreases of *aggR* transcript in coculture or preconditioned medium, normalized against *aggR* abundance in pure cultures of 042 or medium preconditioned by growth of 042.

ACKNOWLEDGMENTS

This work was supported by a grant from the Crohn's and Colitis Foundation of America to E.B. and U.S. Public Health Service grant AI33096 to J.P.N.

We are grateful to Jim Kaper for reviewing the manuscript.

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Editor: A. D. O'Brien