Am J Physiol Gastrointest Liver Physiol 303: G1288–G1295, 2012.
First published October 11, 2012; doi:10.1152/ajpgi.00341.2012.

Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice

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Submitted 30 August 2012; accepted in final form 8 October 2012

Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K, Koga Y, Sudo N. Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. *Am J Physiol Gastrointest Liver Physiol* 303: G1288–G1295, 2012. First published October 11, 2012; doi:10.1152/ajpgi.00341.2012.—There is increasing interest in the bidirectional communication between the mammalian host and prokaryotic cells. Catecholamines (CA), candidate molecules for such communication, are presumed to play an important role in the gut lumen; however, available evidence is limited because of the lack of actual data about luminal CA. This study evaluated luminal CA levels in the gastrointestinal tract and elucidated the involvement of gut microbiota in the generation of luminal CA by comparing the findings among specific pathogen-free mice (SPF-M), germ-free mice (GF-M), and gnotobiotic mice. Substantial levels of free dopamine and norepinephrine were identified in the gut lumen of SPF-M. The free CA levels in the gut lumen were lower in GF-M than in SPF-M. The majority of CA was a biologically active, free form in SPF-M, whereas it was a biologically inactive, conjugated form in GF-M. The association of GF-M with either *Clostridium* species or SPF fecal flora, both of which have abundant β-glucuronidase activity, resulted in the drastic elevation of free CA. The inoculation of *E. coli* strain into GF-M induced a substantial amount of free CA, but the inoculation of its mutant strain deficient in the β-glucuronidase gene did not. The intraluminal administration of DA increased colonic water absorption in an in vivo ligated loop model of SPF-M, thus suggesting that luminal DA plays a role as a proabsorptive modulator of water transport in the colon. These results indicate that gut microbiota play a critical role in the generation of free CA in the gut lumen.

β-glucuronidase; catecholamines; gut microbiota; germfree

ACCUMULATING EVIDENCE HAS SHOWN that bacterial chemical molecules, such as *N*-acyl-homoserine lactones (24) and a family of autoinducers (22), signal to eukaryotic cells. Conversely, host hormones can signal to microbial cells through converging pathways to bacterial signaling molecules. This type of bidirectional communication is often called "microbial endocrinology" (27) or "interkingdom signaling" (19, 35), which thus mediates the symbiotic and pathogenic relationships between the bacteria and mammalian hosts.

Catecholamines (CA), such as norepinephrine (NE) and dopamine (DA), are utilized in the central and peripheral nervous systems, which regulate various types of body functions, such as cognitive abilities, mood, and gut motility (14). Lyte and colleagues first demonstrated that some species of pathogens could recognize exogenous CA in vitro, and such recognition increased bacterial proliferative capacity in their pioneering studies in the 1990s (27, 29–31). Sperandio and colleagues subsequently showed that enterohemorrhagic *Escherichia coli* (EHEC) virulence increases on exposure to epinephrine (E) and NE and that E binds and signals through the QseC receptor (11, 19). Work from other groups (3) also showed that E and NE increase EHEC chemotaxis, motility, adherence to epithelial cells, and virulence gene expression. A large number of researchers from different fields have become increasingly interested in this relationship, because such recognition of host hormones by pathogens is likely to provide a rational answer to a long-standing question as to why hosts become susceptible to opportunistic infections when exposed to extreme stress (19, 27, 35).

Numerous kinds of bacteria inhabit the close proximity of the gastrointestinal tract of mammals; thus such an interkingdom signaling through CA is presumed to be performed continually in the gut lumen (37) and to play an important role in the regulation of various pathophysiological functions. However, available evidence is still extremely limited because of the paucity of actual data about luminal CA. Alverdy and associates (1) showed the cecal NE level to increase following surgery in a mouse surgical stress model. These findings suggest the possible existence of gut luminal CA under some pathological conditions; however, the precise mechanism of how and to what extent CA is produced and regulated under normal or pathological conditions is largely unknown.

The present study evaluated luminal CA levels through gastrointestinal tract using a reliable and reproducible HPLC method for measuring luminal CA levels and furthermore tried to elucidate the involvement of gut microbiota in the regulation of luminal CA by comparing the findings among specific pathogen-free (SPF) mice, germ-free (GF) mice that had no bacteria, and gnotobiotic mice that were reconstituted with a mixture of *Clostridia* species or SPF fecal flora (EX-GF).

METHODS

Animals. Adult male BALB/c mice, 7–9 wk of age, were obtained from Charles River Japan (Shizuoka, Japan) and kept under SPF condition. GF BALB/c mice were originally obtained from the Central Institute for Experimental Animals (Kawasaki, Japan), as described previously (47, 48). These mice have been bred >10 generations in isolators without any bacterial contamination in this laboratory. Pairs of male and female GF mice were selected as the grandparents of mice for experiments. Their first offspring were used as parents of such mice for gnotobiotic and control GF. The parent mice were orally given stools of SPF mice to make gnotobiotic mice associated with

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gut microbiota from SPF mice, and their offspring were used as EX-GF mice (32). Gnotobiotic mice associated with a mixture of 46 related *Clostridium* species from the *coccoides* and *leptum* groups (*Clostridia* cocktail) (4), were produced by intragastrically inoculating the parents with 10^9 CFU of *Clostridia* suspended in 0.5 ml of PBS, and their offspring were used as *Clostridia*-associated mice in the experiments. The first offspring of the parents without microbiota were used as control GF mice in the experiments. Only male mice were used in the present study. GF and gnotobiotic mice were maintained in Trexler-type flexible-film plastic isolators with sterile food (CL-2, CLEA Japan, Tokyo, Japan) and water. Surveillance for bacterial contamination was thoroughly performed by a periodic bacteriological examination of mouse feces. In addition, all the GF and gnotobiotic mice were also examined after the experiments to determine whether there was any contamination with other bacteria by incubating their stool samples on agar plates for 3 days. Some GF mice were orally given 10^9 CFU of either *E. coli* mutant strain JW1609 that is deficient of the gene *uidA* encoding β-glucuronidase or its parent *E. coli* strain BW25113 (both strains from Keio *E-coli* Collection, Tokyo, Japan) (2) at 4 wk of age. Their tissue and luminal samples from the cecum were subjected to HPLC measurement 4 wk later. Some luminal samples were also used to count the number of bacteria residing in the cecum according to our previously reported method (46, 47). All experiments were approved by the Ethics Committee for Animal Experiments of both Tokai and Kyushu University.

Sample preparation for CA measurement. The mice were killed by cervical dislocation, and then segments of the ileum, cecum, and colon were removed and cut open along mesentery. Their luminal contents were weighed and collected into a 1.5-ml tube. The samples were homogenized by vigorous pipetting and vortexing in 1 ml of 0.01 M PBS. The supernatants were collected by centrifugation for 15 min at 13,000 *g* at 4°C. They were then mixed with 1 ml of 0.2 M perchloric acid (Sigma) for deproteinization. The solutions were centrifuged at 13,000 *g* for 15 min, and the deproteinized supernatant was processed for catecholamine analysis by using HPLC system.

Ileum, cecum, and colon tissue samples were weighed and homogenized in 1 ml of 0.2 M perchloric acid. The homogenates were centrifuged at 13,000 *g* for 15 min, and the supernatants were used for measuring the tissue CA contents.

Analysis of CA by HPLC. CA concentrations (pg/ml) in the luminal and tissue of the gut were determined by post column HPLC (HLC-8030, Tosoh, Tokyo, Japan) using diphenylethylenediamine as a fluorogenic reagent (21, 34). This three-column HPLC system was originally developed to evaluate plasma and urine-free CA levels; however, preliminary results showed the system to be also applicable for the measurement of luminal NE, E, and DA levels with a high recovery rate and good reproducibility (coefficient of variation of <3%).

Measurement of conjugated CA levels using enzymatic deconjugation. Glucuronide and sulfate CA conjugates in the gut lumen were analyzed by the previously described method for measuring plasma or urine samples (12, 41, 54), with some modifications. In brief, the deproteinized samples described above were divided into the three aliquots: the first aliquot was adjusted to pH 7.0 and incubated for 1 h at 37°C without any supplementation of enzymes; the second aliquot was adjusted to pH 6.8 and incubated for 1 h with β-glucuronidase (GUS, type IX from *E. coli*, 750 U/sample, Sigma, St. Louis, MO); the third aliquot was adjusted to pH 7.1 and incubated for 1 h with sulfatase (type VI from *Aerobacter aerogenes*, 0.26 U/sample, Sigma). The first, second, and third aliquots were used for the measurement of free, glucuronide- and sulfate-conjugated CA, respectively. Each incubated sample was given 1 ml of 0.2 M perchloric acid to terminate the enzymatic reactions and centrifuged again for 15 min at 13,000 *g* at 4°C. The resulting supernatants were collected and processed for the HPLC system. Glucuronide-conjugated CA was calculated as the difference between the first (free CA) and the second (free CA + glucuronide-conjugated CA) value. Likewise, sulfate-conjugated CA was calculated as the difference between the first (free CA) and the third (free CA + sulfate-conjugated CA) value. The enzyme concentrations used in this experiment were chosen because they were found to be optimal for the complete hydrolysis of conjugated CA.

Analysis of β-glucuronidase activity. Enzymatic activity of GUS from luminal or tissue contents in the cecum was measured using the previously described method (9, 36), with some modifications.

The luminal contents in the cecum were suspended in 1 ml of 0.01 M PBS and subjected to ultrasonic disintegration on ice (Bioruptor UCD 130TM, 20 kHz, Tosho, Kanagawa, Japan). The homogenates were centrifuged, and the protein concentration was quantified using a NanoDrop proteinA280 LMS spectrophotometer. A reaction mixture (pH 7.0), containing 100 μl of the supernatant fraction, 10 μl of 0.01 M phenolphthalein glucuronide (Sigma), and 50 μl of PBS, was incubated for 1 h at 37°C. The reaction was terminated by adding a glycine-NaOH solution (pH 10.2) containing 8 μl of 1 M alkaline glycine (Sigma) and 3 μl of 5% trichloroacetic acid (Wako, Osaka, Japan). Fluorescent levels of liberated phenolphthalein (PheP) were measured at 570 nm by a spectrophotometer. The concentration of GUS in the samples was calculated from a standard curve and expressed as micrograms of PheP per hour per milligram of protein.

The enzyme activity in the cecal tissue was determined by homogenizing the tissue in 1 ml of 0.01 M PBS including 0.5% Triton-X. The homogenates were sonicated three times of sonication (20 kHz) on ice and centrifuged at 13,000 *g* for 15 min at 4°C, and the supernatants were collected and analyzed for their GUS activity. The measurement was done using a reaction mixture whose pH was adjusted to 4.5, since the tissue type of GUS activity has an optimal pH of 4.5.

In vivo colon loop study. The in vivo effects of luminal CA on water absorption of the gut were examined using the method described previously, with some modification (10, 13). SPF BALB/c mice were given an intraperitoneal injection of 50 mg/kg pentobarbital sodium (Tokyo Kasei, Tokyo, Japan). The abdomen was opened by a midline incision. Approximately 3 cm of colon loop was prepared by ligating the splenic flexure and the cecal junction of colon by silk 5/0. The contents inside the loop were removed by a flushing solution (140 mM NaCl, 10 mM HEPES, pH 7.4) warmed to 37°C. Three milliliters of test solution containing 50 mM NaCl, 5 mM HEPES, 2.5 mM KCl, 20 mM glucose, 20 mM sodium ferrocyanide (Wako, Osaka, Japan), with (n = 8) or without (n = 8) 10 μM DA hydrochloride (Sigma) were injected into the loop. The loop was incubated for 30 min, and the test solution was collected for a ferrocyanide colorimetric assay at 405 nm as reported previously (7, 13). The ferrocyanide colorimetric assay was performed before (OD*before*) and after (OD*after*) incubation. Water absorption was expressed as (*Vbefore* − *Vafter*)/30 min/cm. In this equation, *Vbefore* is the initially injected volume (300 μl), and *Vafter* is calculated as *Vbefore*(OD*before*/OD*after*).

Statistical analysis. All the continuous data are presented as means ± SE. Comparison of changes among two groups was performed using paired or unpaired *t*-test. The comparison of the changes among three or more groups was performed by Dunnett's post hoc test after the ANOVA. All analyses were performed using the JMP 9 software package for Windows (SAS Institute Japan). A P value of <0.05 was considered to be significant.

RESULTS

Presence of considerable amounts of catecholamines in the lumen of the cecum. Preliminary experiments showed that an ordinary HPLC method optimized to measure brain CA levels was not applicable to the determination of luminal CA contents of the cecum, because luminal samples contained substantial artifact peaks and contaminants that inhibited the precise evaluation of luminal CA levels. Therefore, an HPLC method with

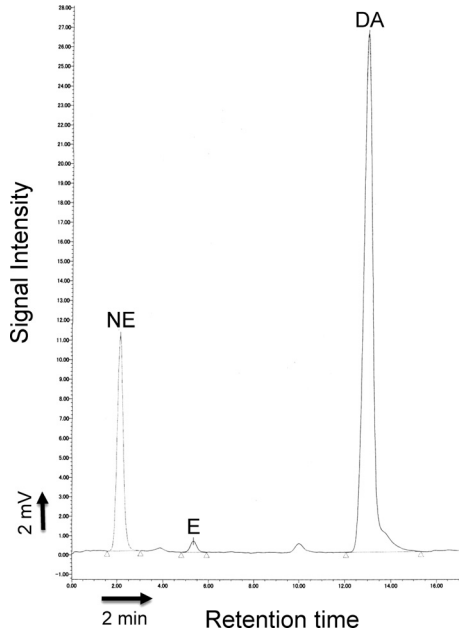

Fig. 1. Luminal catecholamine (CA) concentrations in the cecum of specific pathogen-free (SPF) mice. A representative of chromatogram data of luminal contents of the cecum is shown. Peaks NE, E and DA indicate norepinephrine, epinephrine, and dopamine, respectively.

diphenylethylenediamine as a precolumn fluorescence derivatization reagent (21, 34) was used to accurately measure the luminal CA levels.

NE, E, and DA were each clearly identified as a unique peak (Fig. 1). Considerable amounts of biologically active free NE and DA were present in the lumen of the cecum. The DA levels were the highest among the three CA, whereas the E levels were only marginally detectable (NE, 22 ± 3 ng/g; E, 1.4 ± 0.2 ng/g; DA, 93 ± 8 ng/g).

Distribution of NE and DA in the lumen and tissue of the gastrointestinal tract. Luminal CA levels of SPF mice were measured in the ileum and colon, as well as in the cecum.

Figure 2*A* shows that free NE and DA were also present in the lumen of the ileum and colon. The NE or DA level in the lumen was the highest in the colon among the three parts of the gut [NE: ileum, 10 ± 2 ng/g; cecum, 22 ± 3 ng/g; colon, 48 ± 7 ng/g ($P < 0.001$ vs. ileum value); DA: ileum, 16 ± 3 ng/g; cecum, 93 ± 8 ng/g ($P < 0.001$ vs. ileum value); colon, 132 ± 19 ng/g ($P < 0.001$ vs. ileum value)]. In contrast, there were no significant differences in tissue NE or DA levels among the ileum, cecum, and colon (Fig. 2*B*; NE: ileum, 218 ± 29 ng/g; cecum, 310 ± 24 ng/g; colon, 275 ± 16 ng/g; DA: ileum, 14 ± 4 ng/g; cecum, 25 ± 4 ng/g; colon, 15 ± 2 ng/g). In addition, tissue NE and DA levels showed no significant correlation with luminal NE and DA levels in any parts of the digestive tract examined.

The majority of CA exists in a biologically active, free form in the gut lumen of SPF mice. Previous reports showed that a large proportion of peripheral CA in blood or urine exists as a conjugated form that is biologically inactive (15, 54). Two forms of CA conjugates, glucuronide and sulfate, are found in mammals; however, glucuronidation is thought to be a more important metabolic pathway in rodents (12, 15, 52). Therefore, the study investigated free, glucuronide- and sulfate-conjugated forms of CA in the lumen of the ileum, cecum, and colon.

Figure 3 and Table 1 show that almost all NE and DA were in the free form in the cecum and colon, although substantial amounts of glucuronide-conjugated NE and DA were present in the ileum.

The majority of CA exists in a biologically inactive, conjugated form in the gut lumen of GF mice. Yoneda and coworkers (54) reported that conjugated CA in peripheral blood could be changed into free CA by in vitro incubation with bacterial GUS. This study, therefore, hypothesized that free CA present in the gut lumen might be generated via deconjugation by bacterial GUS derived from gut microbiota. Both the free and conjugated forms of luminal CA levels were measured in the gut of GF mice that were completely deficient of gut bacteria and then compared with those in the SPF mice.

Figure 4*A* shows that free NE and DA levels in the cecal lumen were lower in the GF mice than in the SPF mice. In contrast, there were no significant differences in cecal tissue free NE and DA levels between the GF and SPF mice (Fig. 4*B*). Figure 5 and Table 2 demonstrate that >90% of DA were in the glucuronide-conjugated form in all parts of digestive tracts examined of GF mice (Fig. 5*A*), whereas ~40% of NE were in the glucuronide-conjugated form (Fig. 5*B*). These results indicate that gut microbes are responsible for generating free NE and DA in the gut lumen.

Substantial elevation of free NE and DA in the cecal lumen upon exposure to enteric bacteria. Further experiments examined whether the association with bacteria, which have a high GUS activity, leads to an elevation of free CA in the cecal

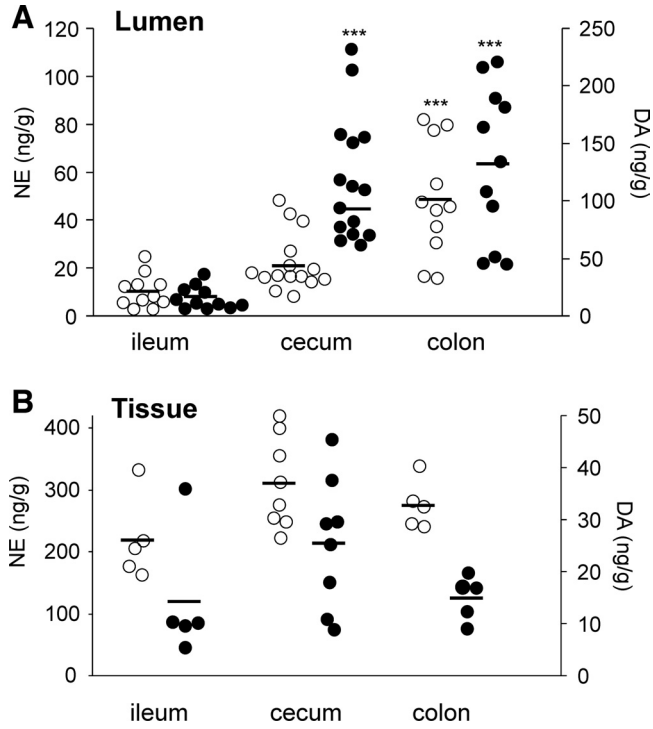

Fig. 2. Luminal and tissue CA concentration in the gastrointestinal tract of SPF mice. Luminal (n = 11–15; *A*) and tissue (n = 5–8; *B*) CA levels were measured by the method described in METHODS. Open and closed circles show NE (left vertical axis) and DA (right vertical axis) levels, respectively. ***Significantly higher than the corresponding ileum value ($P < 0.001$).

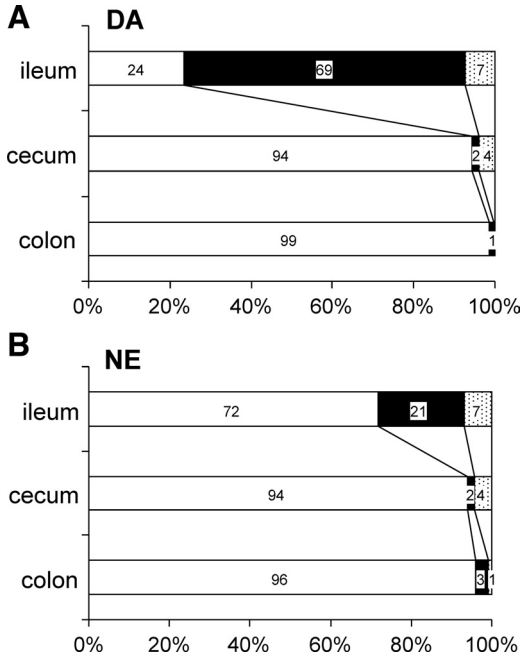

Fig. 3. Free, glucuronide, and sulfate-conjugated CA in the gut lumen of SPF mice. Luminal glucuronide- and sulfate-conjugated DA ($n = 6$; *A*) or NE ($n = 6$; *B*) levels in the ileum, cecum, and colon were analyzed by the methods described in METHODS. The mean value of each form of CA is expressed as a percentage of the total (free CA + conjugated CA). The open, closed, and dotted bars show free, glucuronide-, and sulfate-conjugated CA, respectively.

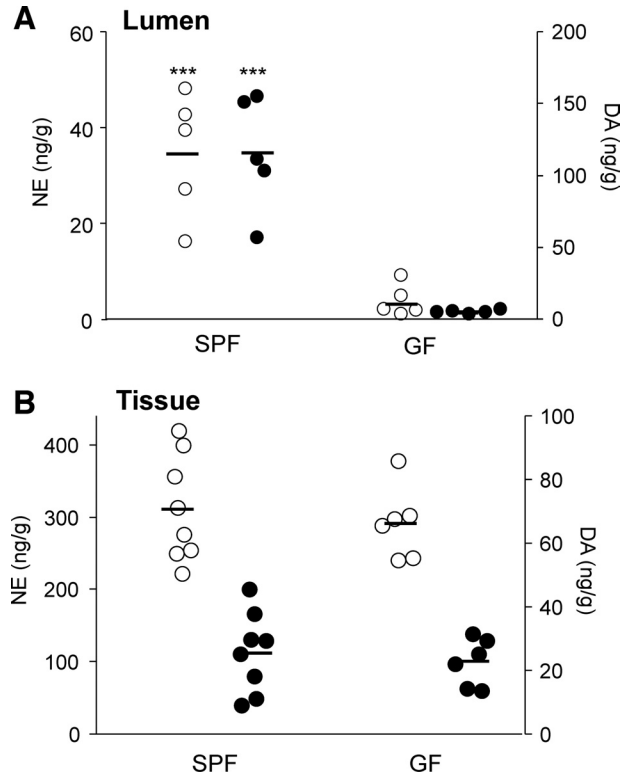

Fig. 4. Cecal free CA levels in the lumen and tissue of SPF and germ-free (GF) mice. Luminal ($n = 5$; *A*) and tissue ($n = 6–8$; *B*) free CA levels in the SPF mice and GF mice were measured as described in METHODS. Luminal NE levels (left vertical axis) in the SPF and GF mice were 35 ± 5*** ng/g and 3.8 ± 1.3 ng/g, respectively; luminal DA levels (right vertical axis) in the SPF and GF mice were 115 ± 14*** ng/g and 5.0 ± 0.5 ng/g, respectively. ***Significantly higher than the corresponding GF value ($P < 0.001$). Tissue NE levels (left vertical axis) in the SPF and GF mice were 310 ± 24 ng/g and 290 ± 19 ng/g, respectively; tissue DA levels (right vertical axis) in the SPF and GF mice were 25 ± 4 ng/g and 22 ± 3 ng/g, respectively.

lumen of the GF mice to verify the involvement of gut bacteria in the production of free CA.

Gnotobiotic mice associated with either a mixture of 46 *Clostridia* species (*Clostridia*) or fecal flora from SPF mouse (EX-GF) showed a drastic elevation of free NE and DA levels (Fig. 6*A*). Likewise, cecal contents from such gnotobiotic mice also exhibited a higher GUS activity compared with those from GF mice (Fig. 6*B*).

Bacteria-derived GUS is necessary for full production of free CA. The change in luminal CA levels were examined in the cecum after GF mice were colonized with either *E. coli* mutant strain lack of GUS-encoded gene *uidA* (JW1609: ΔGUS) or its parent *E. coli* strain (BW25113) to demonstrate the critical role of bacteria-derived GUS in the production of luminal CA. There was no difference in the number of bacteria residing in the cecum between the BW25113- and ΔGUS-inoculated mice (BW25113, 10.4 ± 0.04 $\log_{10}$CFU/g cecal content; ΔGUS, 10.3 ± 0.04 $\log_{10}$CFU/g cecal content).

Figure 7*A* and Table 3 show that the 70% of DA was still in the glucuronide-conjugated form even after the association with ΔGUS, although the 25% of the total DA were in the free form. In contrast, the two-thirds of DA were converted into the free form, while the 29% of the total DA remained conjugated after the association with BW25113. The conjugated form of NE was 29% of the total after inoculation with ΔGUS, while representing 15% of the total after inoculation with BW25113 (Fig. 7*B*). The GUS activity in the cecal lumen of ΔGUS-gnotobiotic mice was only marginally detectable, which was almost identical with the GF value ($n = 5$ per each group; ΔGUS, 6.7 ± 0.4 μg PheP·h^{-1}·mg protein^{-1}; GF, 6.4 ± 0.3 μg PheP·h^{-1}·mg protein^{-1}). On the other hand, the BW25113 *E. coli*-gnotobiotic mice exhibited a small but significant increase in GUS activity in the cecal lumen compared with the ΔGUS-gnotobiotic mice ($n = 4$; 11.6 ± 1.3 μg PheP·h^{-1}·mg protein^{-1}; $P < 0.01$ vs. ΔGUS value). Interestingly, the GUS activity in the cecal wall was significantly increased upon exposure to ΔGUS to the comparable level found upon expo-

Table 1. *Free, glucuronide-conjugated, and sulfate-conjugated CA in gut lumen of SPF mice*

	Free CA, ng/g	Glucuronide-Conjugated CA, ng/g	Sulfate-Conjugated CA, ng/g
Ileum			
NE	9.0 ± 2.1	3.8 ± 1.9	1.7 ± 1.2*
DA	15.7 ± 4.1	44.9 ± 5.1‡	4.7 ± 1.4
Cecum			
NE	34.6 ± 4.6	0.5 ± 0.2†	1.5 ± 0.4†
DA	115.4 ± 14.4	1.6 ± 1.0†	4.1 ± 1.5†
Colon			
NE	60.5 ± 6.0	2.0 ± 0.5†	0.6 ± 0.2†
DA	177.0 ± 10.9	2.5 ± 0.7†	0.3 ± 0.2†

Values are means ± SE. Luminal glucuronide- and sulfate-conjugated norepinephrine (NE) and dopamine (DA) levels in the gut lumen of specific pathogen-free (SPF) mice ($n = 6$) were measured according to the methods described in METHODS. CA, catecholamines. Significant difference from the corresponding free NE or DA value: *$P < 0.05$; †$P < 0.001$; ‡$P < 0.001$ were significantly different from the corresponding free NE or DA value.

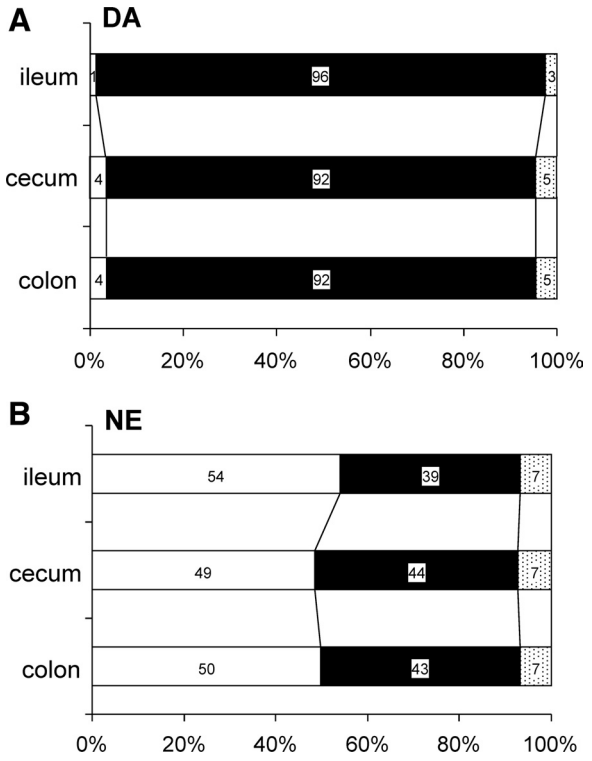

Fig. 5. Free glucuronide- and sulfate-conjugated CA in the gut lumen of GF mice. Luminal glucuronide- and sulfate-conjugated DA (*A*) or NE (*B*) levels in the ileum, cecum, and colon were analyzed according to the methods described in METHODS. The mean value of each form of CA is expressed as a percentage of the total (free CA + conjugated CA). The open, closed, and dotted bars show free, glucuronide-conjugated, and sulfate-conjugated CA, respectively.

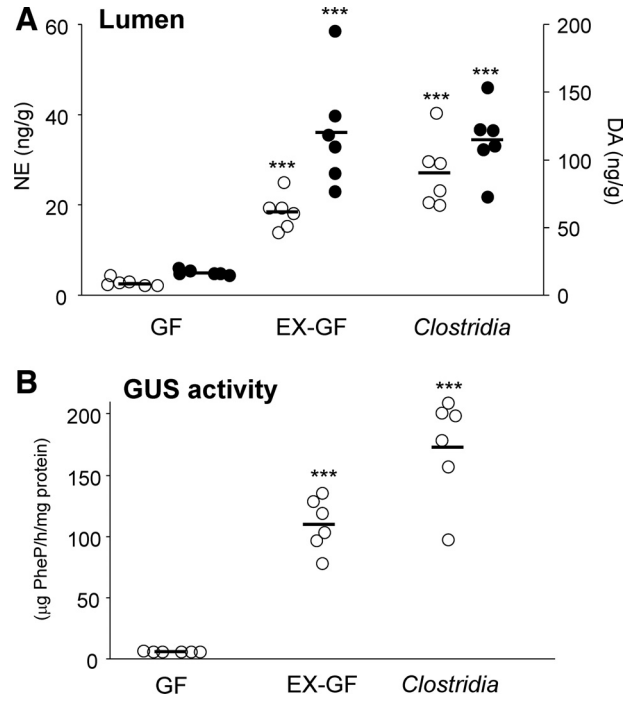

Fig. 6. Luminal free CA levels and β-glucuronidase (GUS) activity in the cecum of gnotobiotic mice. *A*: cecal luminal contents from EX-GF ($n = 6$) and *Clostridia* ($n = 6$)-associated mice were processed for free NE and DA measurement, as described in METHODS. Free NE concentrations in the GF, EX-GF, and *Clostridia*-mice were 2.6 ± 0.3 ng/g, 18 ± 1 ng/g***, and 27 ± 3 ng/g***; free DA concentrations were 16.3 ± 0.7 ng/g, 120 ± 16 ng/g***, and 114 ± 10 ng/g***. ***$P < 0.001$ was significantly higher than the corresponding GF value. *B*: cecal luminal contents of GF, EX-GF, and *Clostridia*-associated mice were subjected to the measurement of GUS activity, as described in METHODS. GUS activity in the GF, EX-GF, and *Clostridia*-mice were 6 ± 0.3 μg PheP·h^{-1}·mg protein^{-1}, 110 ± 8 μg PheP·h^{-1}·mg protein^{-1}***, and 173 ± 16 μg PheP·h^{-1}·mg protein^{-1}***. ***$P < 0.001$ was significantly higher than the corresponding GF value.

sure to BW25113 strain [$n = 5$ per each group; ΔGUS, 3.00 ± 0.25 μg PheP·h^{-1}·mg protein^{-1} ($P < 0.01$ vs. GF value); BW25113, 2.72 ± 0.18 μg PheP·h^{-1}·mg protein^{-1} ($P < 0.05$ vs. GF value); GF, 1.93 ± 0.06 μg PheP·h^{-1}·mg protein^{-1}]. Collectively, these results indicate that gut microbiota play a critical role in the generation of luminal free CA via their GUS. The bacteria-induced increase in tissue GUS activity may be involved in this generation.

Luminal DA exerts pro-absorptive effects on water transport in the colon. Luminal administration of NE or DA stimulates active ion and water absorption via adrenergic or dopaminergic receptor activation in the intestine (5, 6), thus demonstrating the role of luminal DA as a pro-absorptive modulator of ion and water transport. Therefore, the effects of DA on water absorption were examined using an in vivo colon loop model (8, 13) of SPF mice.

The injection of 10 micromoles of DA into the loop were found to induce a 30% increase in water absorption out of the gut lumen compared with the vehicle injection without DA ($n = 8$ per each group, vehicle 55 ± 5 μl·30 min^{-1}·cm^{-1}; DA 72 ± 4 μl·30 min^{-1}·cm^{-1}; $P < 0.05$).

Therefore, these results support the previous findings that luminal DA could have a pro-absorptive effect on colonic epithelial cells.

Table 2. *Free, glucuronide-conjugated, and sulfate-conjugated CA in gut lumen of GF mice*

	Free CA, ng/g	Glucuronide-Conjugated CA, ng/g	Sulfate-Conjugated CA, ng/g
Ileum			
NE	7.5 ± 3.7	1.9 ± 0.3	0.4 ± 0.1
DA	1.5 ± 0.3	130.1 ± 16.3†	3.6 ± 0.7
Cecum			
NE	3.8 ± 1.3	2.8 ± 0.6	0.4 ± 0.1*
DA	5.0 ± 0.5	136.9 ± 29.7†	6.6 ± 1.6
Colon			
NE	3.2 ± 0.6	2.8 ± 0.6	0.4 ± 0.1*
DA	4.8 ± 0.3	138.0 ± 20.1†	7.0 ± 1.1

Luminal glucuronide- and sulfate-conjugated NE and DA levels in the gut lumen of germ-free (GF) mice ($n = 6$) were measured according to the methods described in METHODS. Signficantly different from the corresponding free NE or DA value: *$P < 0.05$; †$P < 0.001$.

DISCUSSION

The present study identified substantial levels of free DA and NE in the gut lumen of the mice raised under SPF condition. The free DA and NE levels in the lumen of the cecum were particularly lower in the GF mice than in the SPF mice. Interestingly, >90% of DA and NE was a biologically active, free form in the SPF mice; on the contrary, >90% of DA and 40–50% of NE were present as a biologically inactive, conjugated form in the GF mice. Moreover, association with either a mixture of *Clostridium* cocktail or fecal microbiota

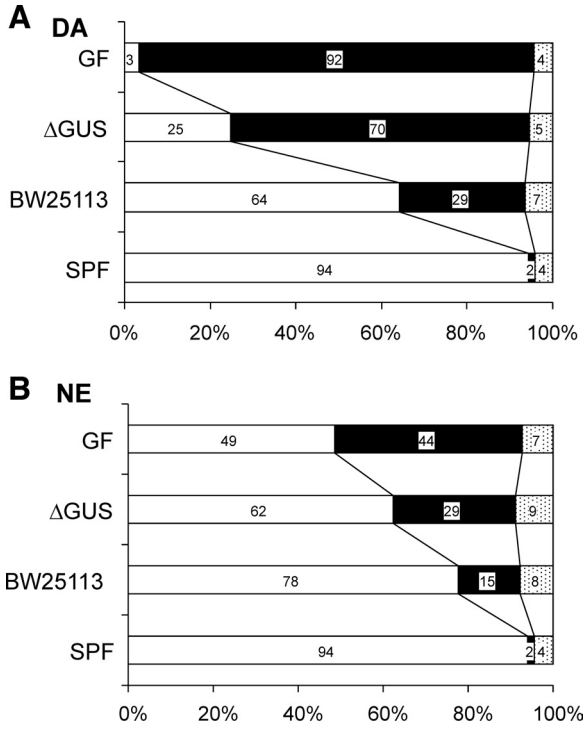

Fig. 7. Free, glucuronide- and sulfate-conjugated CA in the cecal lumen of the mice colonized with *E. coli* mutant strain JW1609 or its parent strain BW25113. The cecal contents were subjected to the measurement of free, glucuronide-conjugated, and sulfate-conjugated NE (*A*) and DA (*B*) levels 4 wk after exposure to *E. coli* mutant strain devoid of GUS ($n = 5$, JW1609: ΔGUS) or its parent strain ($n = 5$, BW25113). The GF and SPF data shown in this figure are identical with ones shown in Tables 1 and 2. The mean value of each form of CA is expressed as a percentage of the total (free CA + conjugated CA). The open, closed, and dotted bars show free, glucuronide-conjugated, and sulfate-conjugated CA, respectively.

from SPF mice, both of which had abundant GUS activity, resulted in a drastic elevation of free DA and NE. However, the 70% of DA were still in the glucuronide-conjugated form in the cecum of GF mice colonized with a mutant strain of *E. coli* that is deficient of GUS gene. Finally, the injection of 10 micromoles of DA into a colon loop significantly increased water absorption out of the gut lumen compared with the control injection without DA. This is the first report indicating that gut microbiota play a critical role in the generation of free CA in the gut lumen.

Previous findings suggest that CA originated from the host may make resident bacteria increase bacterial virulence by acting through a specific receptor on bacterial cells. Indeed, Lyte and Bailey (28) reported that release of CA following the destruction of noradrenergic terminals by the neurotoxin 6-hydroxydopamine increased the number of *E. coli* in the cecum of mice. This in vivo work is very important because it is the first report showing the possible involvement of host CA in bacterial pathogenicity; however, no actual increase in the luminal CA levels was demonstrated in that paper due to the technical difficulties encountered with the use of luminal contents. The current methods are thus expected to unravel some important unanswered questions and thus contribute to further advances in this research field.

Many exogenous and endogenous compounds are conjugated in the liver or the gut and excreted by the bile (20). Some hormones are also conjugated by glucuronidation, sulfation, and methylation (16, 17). For example, glucuronide and sulfate conjugation are reported to be important pathways in the peripheral metabolism of thyroid hormone (17). The thyroid hormone is mainly conjugated by the enzymes such as UDP-glucuronosyltransferase in the liver, and the conjugated form is excreted into the bile. The excreted iodothyronine sulfates and glucuronides are hydrolyzed by gut microbiota. Thereafter, they are presumed to be absorbed from the gastrointestinal tract as a biologically active hormone. Together with the present results that free CA was produced by the hydrolysis of conjugated CA by microorganism-derived GUS, these findings suggest the following scenario about CA absorption and metabolism: CA contained in the diet are digested and absorbed from the gut. They are conjugated in the liver and then released into the gut via the bile duct. These CA are deconjugated by bacterial GUS, and the resulting free CA might be reabsorbed from the gut, thus making up the "enterohepatic circulation." A research project using surgical technique is now in progress to clarify whether gut-liver circulation does exist as a pathway of CA metabolism.

The GUS from *E. coli* is a 290-kDa tetrameric protein and essentially free of sulfatase activity (23). Its optimal pH is 6.8, whereas that of the tissue-type GUS is 4.5 (38). The mean pH in the intestinal lumen ranges from 6.5 to ~7.9 (upper segment of the small intestine) to 6.8 to ~8.0 (colon) in rodents (20, 42, 53); thus these findings indicate that the conversion from conjugated CA to free CA in the gut lumen is largely dependent on bacterial GUS. However, the present result that gnotobiotic mice associated with ΔGUS still had the capability to generate significant amounts of free CA in the cecal contents suggests the significant contribution of tissue type GUS to the generation of free CA in the gut lumen. The colonization with ΔGUS significantly elevated cecal tissue GUS activity to the same levels as the colonization with the parent strain *E. coli* BW25113. These findings suggest a potentially important contribution of tissue-derived GUS to the de-glucuronidation process of conjugated CA, although precisely how and to what extent tissue-derived GUS is involved in the conversion of the luminal conjugated form into the free form in the gut remains to be elucidated.

The gastrointestinal tract is densely innervated by noradrenergic and dopaminergic nerves, whose fibers are found in the mucosa of the intestines (26). Therefore, it is possible that CA present in the enteric nervous system might be released into the

Table 3. *Free, glucuronide-conjugated, and sulfate-conjugated CA in cecal lumen of BW25113- and JW1609-inoculated mice*

	Free CA, ng/g	Glucuronide-Conjugated CA, ng/g	Sulfate-Conjugated CA, ng/g
BW25113			
NE	2.8 ± 0.4	0.6 ± 0.2	0.3 ± 0.1
DA	72.3 ± 8.8	31.5 ± 2.5	7.3 ± 0.8
JW1609 (ΔGUS)			
NE	2.2 ± 0.3	1.1 ± 0.3	0.3 ± 0.1
DA	31.9 ± 4.3*	87.9 ± 3.4†	6.9 ± 1.0

Luminal glucuronide- and sulfate-conjugated NE and DA levels in the cecal lumen of BW25113- ($n = 5$) and JW1609 (ΔGUS; $n = 5$)-inoculated mice were measured according to the methods described in METHODS. Significantly different from the corresponding BW25113 value: *$P < 0.01$; †$P < 0.001$.

gut lumen, thus contributing to the luminal CA levels. However, this possibility seems unlikely, because the dominance of NE levels over DA levels in gut tissue was in sharp contrast with the dominance of the latter over the former in the gut lumen. Moreover, there was no significant association between the tissue and the luminal CA levels. Nonetheless, it should be noted that the CA released from the enteric nervous system might affect luminal CA levels under some pathological conditions, such as severe stress and enteric infection.

Russian researchers (39, 49) have reported that some species of microorganisms can produce CA in an in vitro culture system. In fact, transcripts that have some similarity with mammalian tyrosine hydroxylase, a rate-limiting enzyme, are found in some species of bacteria (18, 44). The present study found no significant difference in the total DA levels (free + conjugated types) of the cecal contents among the GF, SPF, and gnotobiotic mice; however, the total NE levels of the cecal contents were still lower in the GF mice than in the SPF or gnotobiotic mice. These results suggest gut microbes are a likely source for gut luminal NE. In addition, gut bacteria enriched from mouse feces did contain substantial amounts of NE and a lesser amount of DA (unpublished observations). Therefore, it is reasonable to speculate that gut microbes may be important as a source of luminal CA, especially NE. However, some species of bacteria, such as *E. coli*, have a functional transporter for CA, such as the bacterial neurotransmitter sodium symporter family member Leu T (43). Therefore, there is insufficient evidence to determine whether the NE and DA found in gut microbes originate from bacterial production by tyrosine hydroxylase-like enzyme or if they are just taken from the gut lumen via a Leu T-like transporter.

DA 1A receptors are identified in the cells at the base of the intestinal crypts of rat small intestine (50). Moreover, α2-adrenergic receptors are also reported to be present on gut epithelial cells (25). These findings suggest an important interaction between the luminal CA and gut epithelial cells. In fact, the luminal administration of DA stimulates active ileal ion absorption via α2-adrenergic or dopaminergic receptor activation, demonstrating the role of luminal DA as a proabsorptive modulator of ion and water transport (5, 6). These results were also confirmed by the present results, in which the injection of 10 μM DA exerted pro-absorptive effects on gut epithelial cells. More importantly, Vlisidou et al. demonstrated that the injection of CA augments *E. coli* O157:H7 pathogenicity in a bovine ligated ileal loop model (51). Collectively, it is reasonable to speculate that such luminal CA might be involved in not only pro-absorptive functions and alteration in bacterial pathogenicity but also in a variety of physiological and pathological functions, such as gut motility (45) and modulation of immune reactions (33, 40). Clarifying such unidentified functions should further support the hypothesis that CA are important molecules signaling between gut microorganisms and intestinal epithelial cells under physiological conditions.

In conclusion, the present results indicate that gut microbiota play a crucial role in generating the biologically active, free form of CA in the lumen of the gut. Further studies are called for to elucidate luminal CA-related functions and their precise mechanisms, but the present data offer a firm foundation for this rapidly developing field of research and help to clarify the complex interactions and pathways involved in the bidirectional communication between gut microbes and the host.

ACKNOWLEDGMENTS

We appreciate the technical support from the Research Support Center, Graduate School of Medical Sciences, Kyushu University.

GRANTS

This work was partially supported by Grants-in-Aid for General Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology in Japan (no. 19390192, no. 22659144, and no. 24659350, N. Sudo), and a research grant from the Yakult Bioscience Research Foundation (N. Sudo).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Y. Asano and N.S. conception and design of research; Y. Asano, T.H., R.N., Y. Aiba, and T.K. performed experiments; Y. Asano, T.H., and K.Y. analyzed data; Y. Asano, T.H., K.Y., Y.K., and N.S. interpreted results of experiments; Y. Asano prepared figures; Y. Asano and N.S. drafted manuscript; Y. Asano, T.H., R.N., Y. Aiba, T.K., K.Y., Y.K., and N.S. approved final version of manuscript; K.Y., Y.K., and N.S. edited and revised manuscript.

REFERENCES

1. **Alverdy J, Holbrook C, Rocha F, Seiden L, Wu RL, Musch M, Chang E, Ohman D, Suh S.** Gut-derived sepsis occurs when the right pathogen with the right virulence genes meets the right host: evidence for in vivo virulence expression in *Pseudomonas aeruginosa*. *Ann Surg* 232: 480–489, 2000.
2. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2: 2006.0008, 2006.
3. **Bansal T, Englert D, Lee J, Hegde M, Wood TK, Jayaraman A.** Differential effects of epinephrine, norepinephrine, and indole on *Escherichia coli* O157:H7 chemotaxis, colonization, and gene expression. *Infect Immun* 75: 4597–4607, 2007.
4. **Barnes MJ, Powrie Immunology F.** The gut's Clostridium cocktail. *Science* 331: 289–290, 2011.
5. **Barry MK, Aloisi JD, Pickering SP, Yeo CJ.** Luminal adrenergic agents modulate ileal transport: discrimination between alpha 1 and alpha 2 receptors. *Am J Surg* 167: 156–162, 1994.
6. **Barry MK, Maher MM, Gontarek JD, Jimenez RE, Yeo CJ.** Luminal dopamine modulates canine ileal water and electrolyte transport. *Dig Dis Sci* 40: 1738–1743, 1995.
7. **Berliner R, Kennedy And Hilton J T.** Renal clearance of ferrocyanide in the dog. *Am J Physiol* 160: 325–329, 1950.
8. **Caballero-Franco C, Keller K, De Simone C, Chadee K.** The VSL#3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 292: G315–G322, 2007.
9. **Cardona MME.** β-Glucuronidase activity in germ-free, monoassociated and conventional mice. *Microb Ecol Health Dis* 18: 38–41, 2006.
10. **Charoenphandhu N., Laohapitakworn S., Kraidith K., Nakkrasae L.I, Jongwattanapisan P, Tharabenjasin P, Krishnamra N.** Electrogenic Na^+/HCO_3^- co-transporter-1 is essential for the parathyroid hormone-stimulated intestinal HCO_3^- secretion. *Biochem Biophys Res Commun* 409: 775–779, 2011.
11. **Clarke MB, Hughes DT, Zhu C, Boedeker EC, Sperandio V.** The QseC sensor kinase: a bacterial adrenergic receptor. *Proc Natl Acad Sci USA* 103: 10420–10425, 2006.
12. **Claustre J, Serusclat P, Peyrin L.** Glucuronide and sulfate catecholamine conjugates in rat and human plasma. *J Neural Transm* 56: 265–278, 1983.
13. **Clayburgh DR, Musch MW, Leitges M, Fu YX, Turner JR.** Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo. *J Clin Invest* 116: 2682–2694, 2006.

14. **Eisenhofer G, Kopin IJ, Goldstein DS.** Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacol Rev* 56: 331–349, 2004.
15. **Gaudin C, Ruget G, Selz F, Cuche JL.** Free and conjugated catecholamines in digestive tissues of rats. *Life Sci* 37: 1469–1474, 1985.
16. **Graef V, Furuya E, Nishikaze O.** Hydrolysis of steroid glucuronides with beta-glucuronidase preparations from bovine liver, Helix pomatia, and *E. coli*. *Clin Chem* 23: 532–535, 1977.
17. **Hazenberg MP, de Herder WW, Visser TJ.** Hydrolysis of iodothyronine conjugates by intestinal bacteria. *FEMS Microbiol Rev* 4: 9–16, 1988.
18. **Hernandez-Romero D, Sanchez-Amat A, Solano F.** A tyrosinase with an abnormally high tyrosine hydroxylase/dopa oxidase ratio. *FEBS J* 273: 257–270, 2006.
19. **Hughes DT, Sperandio V.** Inter-kingdom signalling: communication between bacteria and their hosts. *Nat Rev Microbiol* 6: 111–120, 2008.
20. **Ilett KF, Tee LB, Reeves PT, Minchin RF.** Metabolism of drugs and other xenobiotics in the gut lumen and wall. *Pharmacol Ther* 46: 67–93, 1990.
21. **Iwaeda T, Kuroki M, Ohta K, Ishimura S, Takahashi H, Watanabe H.** Development of fully automated catecholamine analyzer, HLC-8030. *J TOSOH Res* 32: 59–64, 1988.
22. **Kendall MM, Rasko DA, Sperandio V.** Global effects of the cell-to-cell signaling molecules autoinducer-2, autoinducer-3 an epinephrine in a luxS mutant of enterohemorrhagic *Escherichia coli*. *Infect Immun* 75: 4875–4884, 2007.
23. **Kim DH, Jin YH, Jung EA, Han MJ, Kobashi K.** Purification and characterization of beta-glucuronidase from *Escherichia coli* HGU-3, a human intestinal bacterium. *Biol Pharm Bull* 18: 1184–1188, 1995.
24. **Kravchenko VV, Kaufmann GF, Mathison JC, Scott DA, Katz AZ, Grauer DC, Lehmann M, Meijler MM, Janda KD, Ulevitch RJ.** Modulation of gene expression via disruption of NF-kappa B signaling by a bacterial small molecule. *Science* 321: 259–263, 2008.
25. **Lam R, App E, Nahirney D, Szkotak A, Vieira-Coelho M, King M, Duszyk M.** Regulation of Cl^- secretion by alpha-adrenergic receptors in mouse colonic epithelium. *J Physiol* 548: 475–484, 2003.
26. **Li Z, Schmauss C, Cuenca A, Ratcliffe E, Gershon M.** Physiological modulation of intestinal motility by enteric dopaminergic neurons and the D-2 receptor: analysis of dopamine receptor expression, location, development, and function in wild-type and knock-out mice. *J Neurosci* 26: 2798–2807, 2006.
27. **Lyte M.** Microbial endocrinology and infectious disease in the 21st century. *Trends Microbiol* 12: 14–20, 2004.
28. **Lyte M, Bailey MT.** Neuroendocrine-bacterial interactions in a neurotoxin-induced model of trauma. *J Surg Res* 70: 195–201, 1997.
29. **Lyte And Ernst S M.** Alpha-adrenergic and beta-adrenergic-receptor involvement in catecholamine-induced growth of gram-negative bacteria. *Biochem Biophys Res Commun* 190: 447–452, 1993.
30. **Lyte And Ernst S M.** Catecholamine induced growth of gram-negative bacteria. *Life Sci* 50: 203–212, 1992.
31. **Lyte M, Frank C, Green B.** Production of an autoinducer of growth by norepinephrine cultured *Escherichia coli* O157:H7. *FEMS Microbiol Lett* 139: 155–159, 1996.
32. **Matsumoto M, Kibe R, Ooga T, Aiba Y, Kurihara S, Sawaki E, Koga Y, Benno Y.** Impact of intestinal microbiota on intestinal luminal metabolome. *Sci Rep* 2: 233, 2012.
33. **Nakano K, Yamaoka K, Hanami K, Saito K, Sasaguri Y, Yanagihara N, Tanaka S, Katsuki I, Matsushita S, Tanaka Y.** Dopamine induces IL-6-dependent IL-17 production via D1-like receptor on CD4 naive T cells and D1-like receptor antagonist SCH-23390 inhibits cartilage destruction in a human rheumatoid arthritis/SCID mouse chimera model. *J Immunol* 186: 3745–3752, 2011.
34. **Nohta H, Mitsui A, Ohkura Y.** Spectrofluorimetric determination of catecholamines with 1,2-diphenylethylenediamine. *Anal Chim Acta* 165: 171–176, 1984.
35. **Pacheco AR, Sperandio V.** Inter-kingdom signaling: chemical language between bacteria and host. *Curr Opin Microbiol* 12: 192–198, 2009.
36. **Reddy BS, Mangat S, Weisburger JH, Wynder EL.** Effect of high-risk diets for colon carcinogenesis on intestinal mucosal and bacterial beta-glucuronidase activity in F344 rats. *Cancer Res* 37: 3533–3536, 1977.
37. **Rhee SH, Pothoulakis C, Mayer EA.** Principles and clinical implications of the brain-gut-enteric microbiota axis. *Nat Rev Gastroenterol Hepatol* 6: 306–314, 2009.
38. **Rod And Midtvedt T T.** Origin of intestinal beta-glucuronidase in germfree, mono-contaminated and conventional rats. *Acta Pathol Microbiol Immunol Scand B* 85: 271–276, 1977.
39. **Roshchina VV.** Evolutionary considerations of neurotransmitters in microbial, plant, and animal cells. In: *Microbial Endocrinology*, edited by Lyte, Mark, Freestone PPE. New York: Springer, 2010, p. 17–52.
40. **Sarkar C, Basu B, Chakroborty D, Dasgupta PS, Basu S.** The immunoregulatory role of dopamine: an update. *Brain Behav Immun* 24: 525–528, 2010.
41. **Shibata O, Morooka H, Hara K, Nakamura H, Fukuzaki M, Gotoh Y, Ozaki M.** Importance of the liver and the digestive tract in the formation of catecholamine conjugates. *Masui* 37: 428–433, 1988.
42. **Shiga A, Sasaki T, Horii N.** Correlations among pH and Mg, Ca, P, Na, K, Cl^- and HCO_3^- contents of digesta in the gastro-intestinal tract of rats. *Nihon Juigaku Zasshi* 49: 973–979, 1987.
43. **Singh SK, Yamashita A, Gouaux E.** Antidepressant binding site in a bacterial homologue of neurotransmitter transporters. *Nature* 448: 952–956, 2007.
44. **Solano F, Garcia E, Perez D, Sanchez-Amat A.** Isolation and characterization of strain MMB-1 (CECT 4803), a novel melanogenic marine bacterium. *Appl Environ Microbiol* 63: 3499–3506, 1997.
45. **Strandberg K, Sedvall G, Midtvedt T, Gustafsson B.** Effect of some biologically active amines on the cecum wall of germfree rats. *Proc Soc Exp Biol Med* 121: 699–702, 1966.
46. **Sudo N, Aiba Y, Takaki A, Tanaka K, Yu XN, Oyama N, Koga Y, Kubo C.** Dietary nucleic acids promote a shift in Th1/Th2 balance toward Th1-dominant immunity. *Clin Exp Allergy* 30: 979–987, 2000.
47. **Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, Kubo C, Koga Y.** Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol* 558: 263–275, 2004.
48. **Sudo N, Sawamura SA, Tanaka K, Aiba Y, Kubo C, Koga Y.** The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 159: 1739–1745, 1997.
49. **Tsavkelova EA, Botvinko IV, Kudrin VS, Oleskin AV.** Detection of neurotransmitter amines in microorganisms with the use of high-performance liquid chromatography. *Dokl Biochem* 372: 115–117, 2000.
50. **Vaughan C, Aherne A, Lane E, Power O, Carey R, O'Connell D.** Identification and regional distribution of the dopamine D-1A receptor in the gastrointestinal tract. *Am J Physiol Regul Integr Comp Physiol* 279: R599–R609, 2000.
51. **Vlisidou I, Lyte M, van Diemen PM, Hawes P, Monaghan P, Wallis TS, Stevens MP.** The neuroendocrine stress hormone norepinephrine augments *Escherichia coli* O157:H7-induced enteritis and adherence in a bovine ligated ileal loop model of infection. *Infect Immun* 72: 5446–5451, 2004.
52. **Wang PC, Kuchel O, Buu NT, Genest J.** Catecholamine glucuronidation: an important metabolic pathway for dopamine in the rat. *J Neurochem* 40: 1435–1440, 1983.
53. **Ward FW, Coates ME.** Gastrointestinal pH measurement in rats: influence of the microbial flora, diet and fasting. *Lab Anim* 21: 216–222, 1987.
54. **Yoneda S, Alexander N, Vlachakis ND.** Enzymatic deconjugation of catecholamines in human and rat plasma and red blood cell lysate. *Life Sci* 33: 935–942, 1983.