MICROBIOLOGY

Bioactive polyamine production by a novel hybrid system comprising multiple indigenous gut bacterial strategies

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Metabolites of the intestinal microbiota are thought to be generated through metabolic pathways spanning multiple taxa of intestinal bacteria. We have previously shown that the level of putrescine, a polyamine found abundantly in the human intestinal lumen, is increased in the colonic lumen following administration of arginine and the probiotic *Bifidobacterium* sp.; however, the underlying mechanism remained poorly understood. We report a novel pathway for putrescine production from arginine through agmatine involving the collaboration of two bacterial groups, and triggered by environmental acidification (drop in pH to below 6.5 from neutral). This pathway comprises the acid tolerance system of *Escherichia coli*, representing bacteria that have an arginine-dependent acid resistance system; the energy production system of *Enterococcus faecalis*, representing bacteria that have an agmatine deiminase system; and the acid production system of the acid-producing bacteria, represented by *Bifidobacterium* spp. This pathway is unique in that it represents a relationship between the independent survival strategies of multiple bacteria.

INTRODUCTION

Metabolites derived from gut microbes are associated with host health and disease (*1*), and probiotic bacteria are expected to optimize the metabolite composition of the intestinal microbiota by affecting their biochemical reactions. Polyamines (for example, putrescine and spermidine) are important metabolites of intestinal bacteria, and putrescine is present in the intestinal lumen in 0.5 to 1 mM concentrations in healthy humans (*2*, *3*). These organic cations are found in almost all organisms (*4*, *5*) and are required for cell growth and proliferation. Polyamines promote animal health through induction of autophagy (*6*), suppression of inflammation (*7*), improvement of cognitive function (*8*), and suppression of the progression of heart failure (*9*). High levels of polyamines are also observed in tumors (*10*), and the selective inhibition of ornithine decarboxylase, a key enzyme in polyamine synthesis, is effective against malignancies, including colon cancer (*10*, *11*). Hence, whether or not polyamines in the intestinal lumen are harmful or beneficial for health, strategies to regulate and optimize their concentrations in the intestinal tract are likely to have practical applications. Our group explored the relationship between polyamines and prevention of senescence with the ultimate aim to create novel foods and/or technologies that facilitate bacterial putrescine production to increase the polyamine levels in elderly individuals who have weakened polyamine biosynthesis systems. In contrast, if the mechanism of polyamine production in the intestine is elucidated, then there is a possibility that polyamine concentration can be decreased in patients with colon cancer, for whom decreased polyamine levels in the colon have been found to be beneficial. As food-derived polyamines are absorbed before they reach the lower intestine (*12*), most polyamines in this region of the gut are produced by intestinal microbiota (*13*); therefore, regulation of microbial polyamine metabolism and transport is required to maintain polyamine concentrations at 0.5 to 1 mM, the normal level in healthy individuals, in the intestinal lumen of individuals with low level of intestinal luminal polyamines, represented by the elderly (*2*). However, the mechanisms underlying the up-regulation of colonic luminal polyamines following administration of arginine and/or probiotics (*14*, *15*) are poorly understood at the genetic level, precluding the regulation of gene expression to optimize the polyamine concentrations in the intestinal lumen.

Putrescine is biosynthesized in bacterial cells from agmatine, which is produced by decarboxylation of arginine by the arginine decarboxylases SpeA and AdiA. Two metabolic pathways for the conversion of agmatine to putrescine have been reported to date: The first is the agmatine ureohydrolase (SpeB) pathway, where agmatine is directly converted to putrescine; the second is the *N*-carbamoylputrescine pathway, which involves *N*-carbamoylputrescine production from agmatine by agmatine iminohydrolase and its conversion to putrescine by *N*-carbamoylputrescine amidohydrolase. In silico genome analysis of the 56 most abundant bacterial species in the human microbiome (*16*) has predicted that many species are unable to produce putrescine from arginine using their own enzymes, owing to incomplete synthetic pathways (*17*, *18*), in turn suggesting the existence of a metabolic pathway spanning multiple bacterial species in the gut. However, to our knowledge, there currently is no genetic proof for such a hybrid metabolic pathway. Moreover, even if a microorganism has the synthetic pathway of a specific metabolite, it does not necessarily secrete this metabolite. To confirm the release of a target metabolite from specific bacteria, it is necessary to analyze gene expression related to both the synthetic pathway and the transporters of the target metabolite. However, it is very difficult to identify links between expression in synthetic pathways and that of specific metabolite transporters using metagenomics and transcriptomics alone. Here, by using traditional cultivation and

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molecular biological techniques, we present evidence of novel hybrid pathways for the synthesis and release of target metabolites, and demonstrate that this system consists of independent pathways involving various bacterial species, rather than of pathways for metabolite synthesis and release within a single bacterial species.

Probiotics are defined as live microbial food supplements that confer a health benefit to the host by improving intestinal balance (*19*), such as by influencing the composition of metabolites derived from the intestinal microbiota. *Bifidobacterium* spp. is frequently used in probiotic products (*20*). Supplementation of *Bifidobacterium animalis* subsp. *lactis* has been shown to increase the colonic polyamine concentration (*15*). However, *Bifidobacterium* spp. do not have homologs of enzymes involved in polyamine biosynthesis; therefore, the increased concentration of polyamine in the intestinal lumen likely results from the activation of polyamine biosynthesis by indigenous gut microbiota.

Here, we used traditional cultivation bacterial culture methods, molecular biological techniques, and literature searches for combinations of commensal bacterial metabolic pathways to identify a novel putrescine production pathway, from arginine through agmatine, shared between two bacterial species: *Enterococcus faecalis* and *Escherichia coli*. Activity of this pathway is stimulated by environmental acidification (drop in pH to below 6.5 from neutral) induced by acid-producing bacteria, represented by *Bifidobacterium* spp. Putrescine, which is biosynthesized by the two bacteria, is a byproduct of two independent survival strategies in the acidic environment caused by acid-producing bacteria, represented by *Bifidobacterium* spp., namely, the acid resistance of *E. coli* and the energy production of *En. faecalis*.

RESULTS

Induction of putrescine production by mixed microbial cultures

First, we examined the difference between the effects of single and mixed bacterial cultures on putrescine production. The putrescine concentration in the culture supernatant from a mixed microbial culture including 14 species from the four main phyla in the human intestine in medium containing arginine was 8.5 mM, which was more than 10 times higher than the average concentration (0.21 mM; range from 0 to 1.02 mM) for each species cultured individually (Fig. 1A). To determine which bacteria were responsible for the induced putrescine production, we analyzed the putrescine concentration in medium from cocultured pairs of bacterial species. The highest concentration of 8.0 mM was found in medium from mixed *E. coli* and *En. faecalis* culture (Fig. 1B), and this concentration was over eight times higher than those observed when either species was cultured separately (fig. S1A). The putrescine production in *E. coli* and *En. faecalis* mixed culture was highest when the pH of the medium was 5.5 (fig. S1B), suggesting that the induction of putrescine production might be attributed to a bacterial acid resistance system. *E. coli* has an acid resistance system involving arginine and agmatine, while *En. faecalis* has an agmatine utilization pathway that contributes indirectly to mitigating growth medium acidification as a consequence of adenosine 5′-triphosphate (ATP) production (see below) (*21*); notably, agmatine is an intermediate between arginine and putrescine in these pathways.

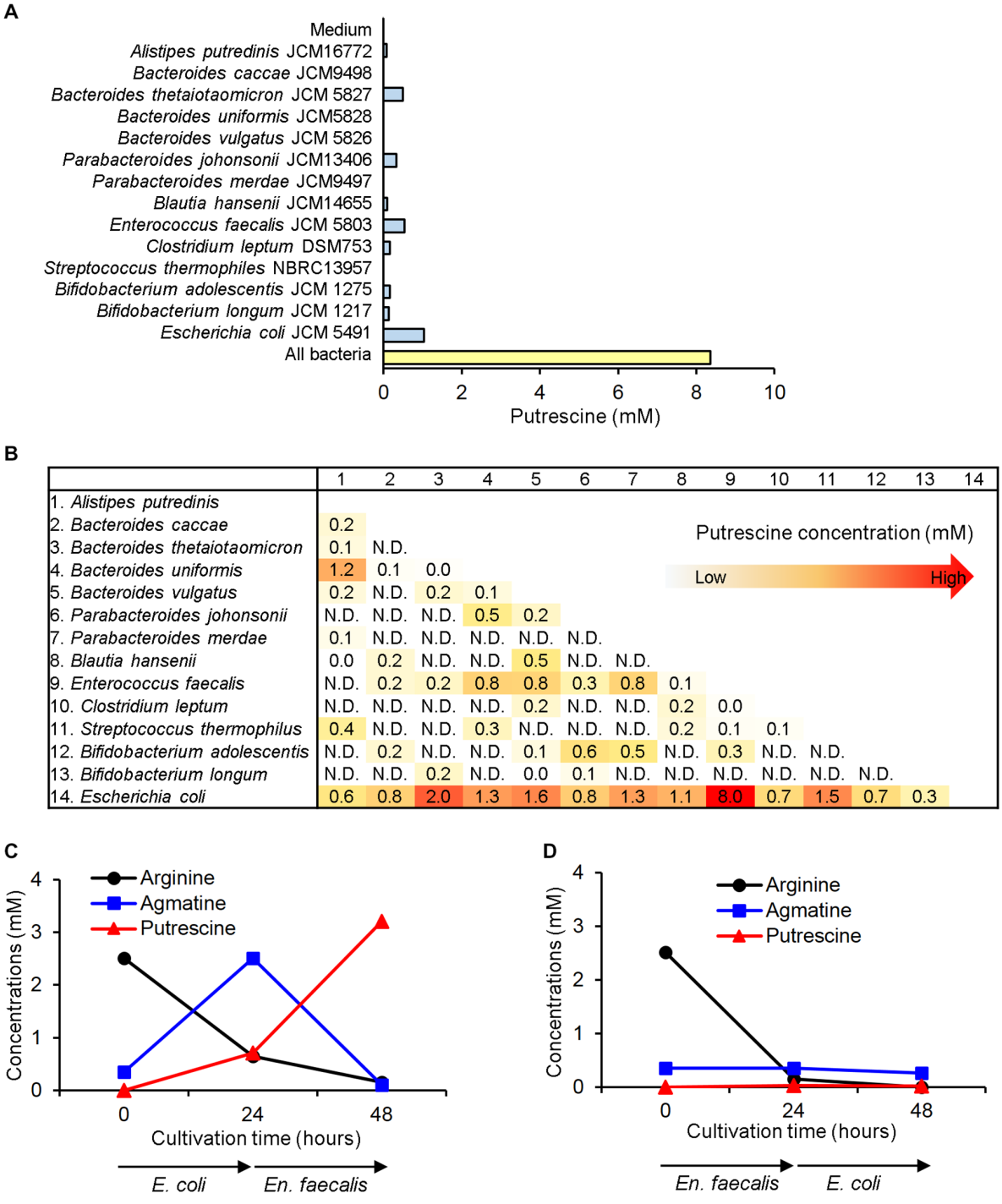
To assess the possibility that either *E. coli* or *En. faecalis* synthesizes putrescine from arginine via extracellular agmatine released by the other bacterium, we used the spent medium from culture of each bacterium to culture the other and quantified arginine, putrescine, and agmatine. During cultivation of *E. coli*, arginine decreased from 2.51 to 0.64 mM (26% of the initial concentration), agmatine increased from 0.35 to 2.51 mM, and putrescine increased from 0 to 0.71 mM (0 to 24 hours; Fig. 1C). When spent medium from *E. coli* cultivation was used to culture *En. faecalis*, agmatine decreased from 2.51 to 0.10 mM (3.9% of the initial concentration), while putrescine increased from 0.71 to 3.21 mM (24 to 48 hours; Fig. 1C). In contrast, when spent medium from *En. faecalis* culture was used to cultivate *E. coli*, neither agmatine nor putrescine production was observed, and arginine was almost completely absent (Fig. 1D). Together, these results suggest that induction of putrescine production from arginine by a mixed culture of *E. coli* and *En. faecalis* is due to the transmission of agmatine, a reactive intermediate between arginine and putrescine, from *E. coli* to *En. faecalis*.

Putrescine production by sequential reactions in *E. coli* and *En. faecalis*

E. coli has an acid resistance system composed of arginine decarboxylase (AdiA) and an arginine-agmatine antiporter (AdiC) (*22*); the equivalent function in *En. faecalis* is achieved by agmatine deiminase (AguA) and an agmatine-putrescine antiporter (AguD), although it was previously reported that acidic stress does not induce this system (*21*). We hypothesized that the two acid resistance systems of these individual bacteria could combine, resulting in induced putrescine production. In this hypothetical pathway (Fig. 2), arginine is taken up from the medium into *E. coli* cells by AdiC and converted to agmatine by AdiA/SpeA. The generated agmatine is then exported from the *E. coli* cells to the medium via AdiC. Next, *En. faecalis* takes up the agmatine from the medium using AguD. Agmatine is then converted to putrescine by the sequential actions of AguA and a putrescine carbamoyl transferase (AguB). The generated putrescine is exported from *En. faecalis* cells via AguD. Hence, the combination of these metabolic and transport pathways of the two bacteria could promote the production of putrescine.

To test this hypothesis, we deleted the *adiA* gene and complemented it in *E. coli*, which we then cocultured with wild-type *En. faecalis* (Fig. 3A). In LB medium containing 2 mM L-arginine, 1.5 g/liter D-glucose, and 0.5 g/liter L-cysteine-hydrochloride (LB-RGC medium), cocultures of wild-type *E. coli* (LKM10096) and wild-type *En. faecalis* (SK947) produced 1.82 mM putrescine in the supernatant. When *adiA*-deleted *E. coli* (SK914) was cocultured with wild-type *En. faecalis* (SK947), putrescine production was abolished. Coculture of *adiA*-complemented *E. coli* (SK912) and wild-type *En. faecalis* (SK947) completely restored putrescine production. Similarly, when *adiC*-deleted *E. coli* (LKM10097) was cocultured with wild-type *En. faecalis* (SK947), putrescine production was abolished, and in complementation experiments using *adiC*-complemented *E. coli* (LKM10100) and wild-type *En. faecalis* (SK947), putrescine production was completely restored (Fig. 3B).

Similarly, we investigated the role of the *aguD* gene in *En. faecalis* (Fig. 3C). When wild-type *E. coli* (LKM10096) was cocultured with *aguD*-deleted *En. faecalis* (SK948), the putrescine concentration decreased to 14% (0.26 mM) of the value when wild-type *E. coli* was cocultured with wild-type *En. faecalis* (SK947) (1.82 mM). In the complementation experiment using wild-type *E. coli* (LKM10096) and *aguD*-complemented *En. faecalis* (SK949), the putrescine concentration was restored to 0.68 mM (37% of the value from coculture



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *Alistipes putredinis*														
2. *Bacteroides caccae*	0.2													
3. *Bacteroides thetaiotaomicron*	0.1	N.D.												
4. *Bacteroides uniformis*	1.2	0.1	0.0											
5. *Bacteroides vulgatus*	0.2	N.D.	0.2	0.1										
6. *Parabacteroides johonsonii*	N.D.	N.D.	N.D.	0.5	0.2									
7. *Parabacteroides merdae*	0.1	N.D.	N.D.	N.D.	N.D.	N.D.								
8. *Blautia hansenii*	0.0	0.2	N.D.	N.D.	0.5	N.D.	N.D.							
9. *Enterococcus faecalis*	N.D.	0.2	0.2	0.8	0.8	0.3	0.8	0.1						
10. *Clostridium leptum*	N.D.	N.D.	N.D.	N.D.	0.2	N.D.	N.D.	0.2	0.0					
11. *Streptococcus thermophilus*	0.4	N.D.	N.D.	0.3	N.D.	N.D.	N.D.	0.2	0.1	0.1				
12. *Bifidobacterium adolescentis*	N.D.	0.2	N.D.	N.D.	0.1	0.6	0.5	N.D.	0.3	N.D.	N.D.			
13. *Bifidobacterium longum*	N.D.	N.D.	0.2	N.D.	0.0	0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		
14. *Escherichia coli*	0.6	0.8	2.0	1.3	1.6	0.8	1.3	1.1	8.0	0.7	1.5	0.7	0.3	

Fig. 1. Induction of putrescine production by mixed microbial cultures. (**A**) Extracellular putrescine concentrations in cocultures and individual cultures of 14 bacterial strains in GAM medium supplemented with 2 mM arginine. (**B**) Extracellular putrescine concentrations in cocultures of each pair of 14 bacterial strains (total 91 combinations). N.D. means not detected; that is, the levels fell below the minimum detectable amount. (**C**) Extracellular concentration of L-arginine, putrescine, and agmatine in the medium after culture of *E. coli* for 24 hours (0 to 24 hours), followed by culture of *En. faecalis* for another 24 hours (24 to 48 hours) in the same medium; *E. coli* was removed by filtration after 24 hours. (**D**) Extracellular concentrations of L-arginine, putrescine, and agmatine in the medium after culture of *En. faecalis* for 24 hours (0 to 24 hours), followed by culture of *E. coli* for another 24 hours (24 to 48 hours) in the same medium. *En. faecalis* was removed by filtration after 24 hours.

of wild-type *E. coli* and wild-type *En. faecalis*). These results demonstrated that a hybrid system composed of *E. coli* and *En. faecalis* enzymes and transporters transported and metabolized arginine to putrescine in the medium (Fig. 2).

The hybrid system for putrescine production by two bacteria in the mouse intestinal tract

To determine whether the putrescine production pathway (Fig. 2) is functional in the mouse intestine, we performed experiments using

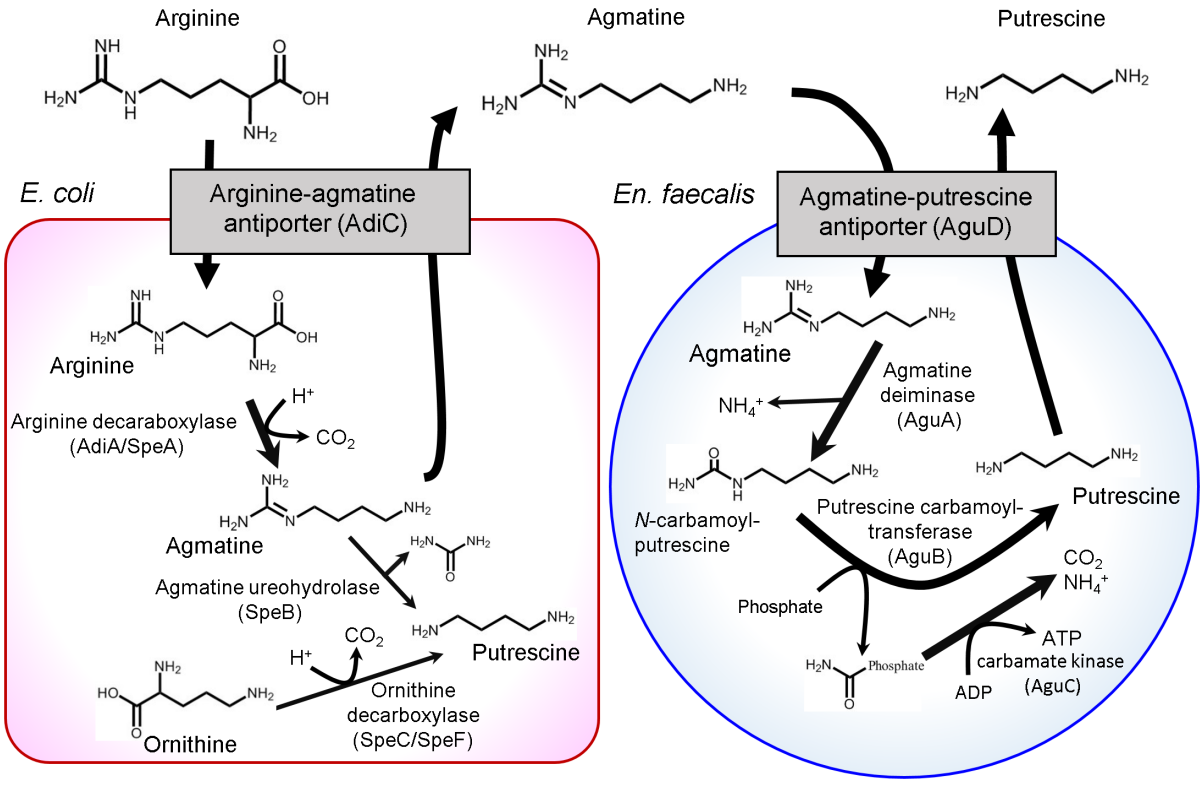


Fig. 2. Hypothetical putrescine production pathway consisting of the acid resistance system of *E. coli* and an ATP synthesis system of *En. faecalis*. A putrescine biosynthesis pathway consisting of sequential reactions accomplished by *E. coli* and *En. faecalis*. Details are shown in the text.

gnotobiotic mice colonized with *E. coli* and *En. faecalis*. As putrescine production was not completely abolished when wild-type *E. coli* and *aguD*-deleted *En. faecalis* were cocultured (Fig. 3C), putrescine-deficient *E. coli* (pBelobac11/Δ*speB*Δ*speC*Δ*speF*) (SK930), which produces agmatine but not putrescine, owing to the deletion of all putrescine biosynthetic genes, was used for this experiment. When putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (SK947) were cocultured in LB-RGC medium, the putrescine concentration in the culture supernatant reached 2.8 mM (Fig. 3D). In contrast, when putrescine-deficient *E. coli* (SK930) and *aguD*-deleted *En. faecalis* (SK948) were cocultured, putrescine production was completely abolished. Coculture of putrescine-deficient *E. coli* (SK930) and *aguD*-complemented *En. faecalis* (SK949) led to putrescine production at a level of 21% of that in coculture of putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (SK947) (Fig. 3D). Next, we administered each pair of bacterial strains to germ-free mice and quantified putrescine concentrations in the contents of their ceca and colons. In gnotobiotic mice colonized with putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (SK930), the cecal putrescine concentration was 100 μM (Fig. 3E). In contrast, that in mice colonized with putrescine-deficient *E. coli* (SK930) and *aguD*-deleted *En. faecalis* (SK948) dropped to 46 μM but was restored to 99 μM in mice colonized with putrescine-deficient *E. coli* (SK930) and *aguD*-complemented *En. faecalis* (SK949) (Fig. 3E). Colonic luminal concentrations of putrescine showed a similar trend, although the differences were not statistically significant ($P = 0.079$) (Fig. 3F). These results indicated that a system composed of enzymes and transporters of *E. coli* and *En. faecalis* was functional in the mouse intestine.

Physiological importance of the hybrid system involving two bacterial species for putrescine production by *E. coli* and *En. faecalis*

The increased production of putrescine observed in this study appeared to result from a combination of the acid resistance systems of *E. coli* and *En. faecalis*. It is known that *E. coli* has arginine-dependent "acid resistance systems" (AR3) that exchange intracellular agmatine for extracellular arginine (*23*). When wild-type *E. coli* (MG1655) was monocultured in arginine-containing medium at different pH values, the agmatine concentration decreased gradually with a pH increase from 5 to 9 (bacterial growth was inhibited at pH 4 and 9) (fig. S2A), demonstrating that the production of agmatine from arginine in *E. coli* depends on this system. However, wild-type *En. faecalis* (V583) monocultured in medium at different pH values (5 to 8) containing agmatine exhibited no significant difference in putrescine production (bacterial growth was inhibited at pH 4 and 9) (fig. S2B), demonstrating that environmental pH did not influence putrescine production from agmatine in *En. faecalis*. In contrast, putrescine production by wild-type *En. faecalis* (SK947) was inhibited in the presence of glucose (fig. S2C), indicating that this pathway is involved in energy production. These results are consistent with those of a previous report that showed that this pathway is activated to obtain energy but is not strongly correlated with protection of bacterial cells against acidic stress (*21*). The viable count of wild-type *En. faecalis* (SK948) monocultured in medium containing 5 mM agmatine at pH 6 was significantly higher than that of *aguD*-deleted *En. faecalis* monocultured in the same medium (fig. S2D). Furthermore, the viable count of *En. faecalis* in coculture of wild-type

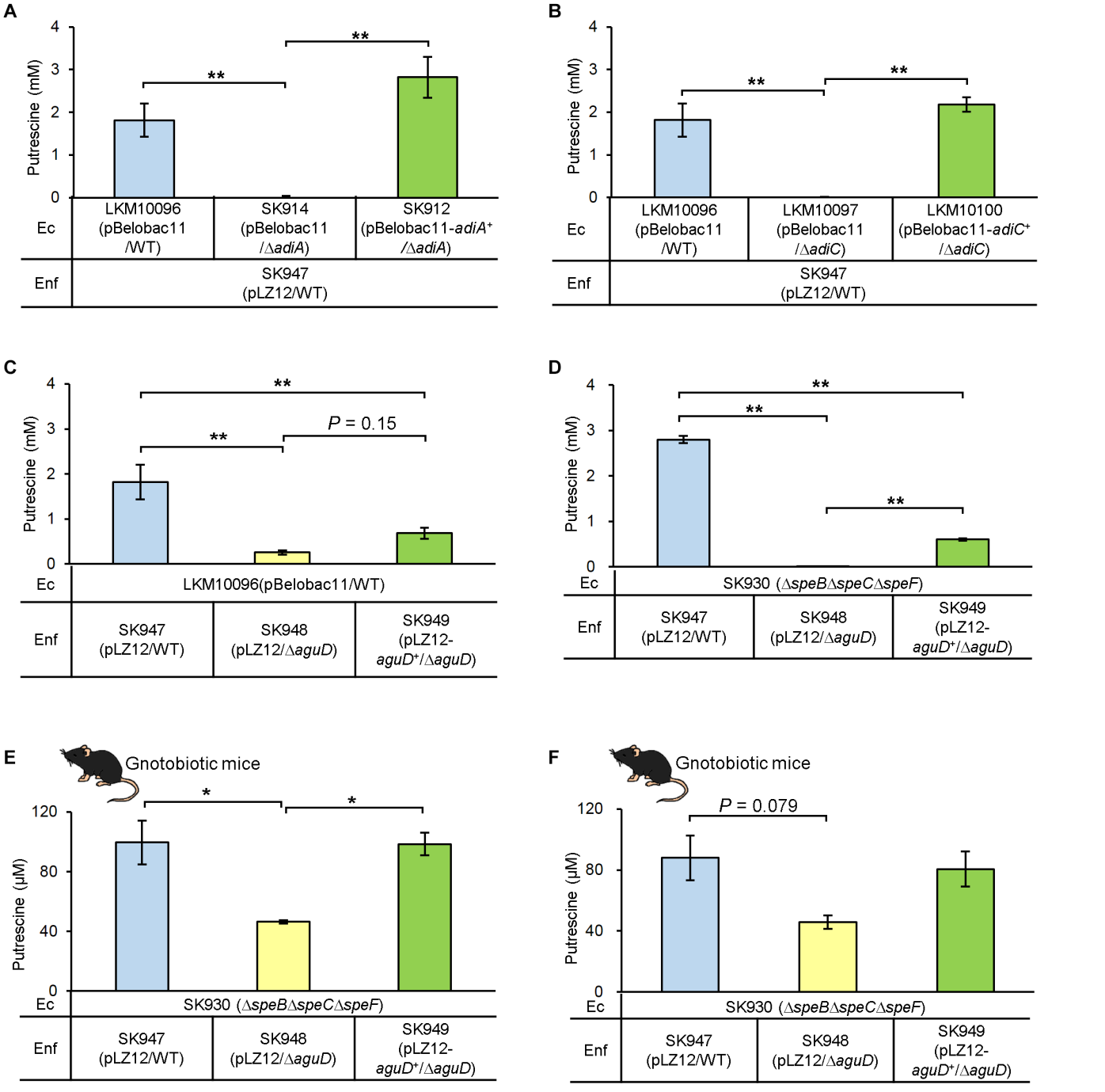


Fig. 3. Validation of the hypothesis of sequential reactions in *E. coli* and *En. faecalis* using bacteria whose genes encoding transporters and enzymes were deleted or complemented. (**A** and **B**) Extracellular putrescine concentration in cocultures of wild-type (WT) *En. faecalis* (Enf; SK947) and *E. coli* (Ec) including gene knockout mutants or complementary transformants of arginine decarboxylase (*adiA*) of *E. coli* (A) or arginine-agmatine antiporter (*adiC*) of *E. coli* (B). (**C**) Extracellular putrescine concentrations in cocultures of wild-type *E. coli* (LKM10096) and *En. faecalis* including the wild type, gene knockout mutants, or complementary transformants of agmatine-putrescine antiporter (*aguD*). (**D**) Extracellular putrescine concentration in cocultures of putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (SK947). Coculture of these bacteria was conducted under anaerobic conditions at 37°C for 24 hours in LB-RGC medium. (**E** and **F**) Putrescine concentrations in the content of cecal lumen (E) and colonic lumen (F) of gnotobiotic mice colonized with putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (SK947). Error bars represent SEs. **P* < 0.05 and ***P* < 0.01, one-way analysis of variance (ANOVA) with Tukey's test.

En. faecalis (SK947) with putrescine-deficient *E. coli* (SK930) was significantly higher than that in coculture of *aguD*-deleted *En. faecalis* (SK948) with putrescine-deficient *E. coli* (SK930) ($P < 0.05$). The viable count of *En. faecalis* was restored when *aguD*-complemented *En. faecalis* (SK949) was cocultured with putrescine-deficient *E. coli* (SK930) (fig. S2E). These results indicate that *En. faecalis* grows using agmatine produced by *E. coli* from arginine.

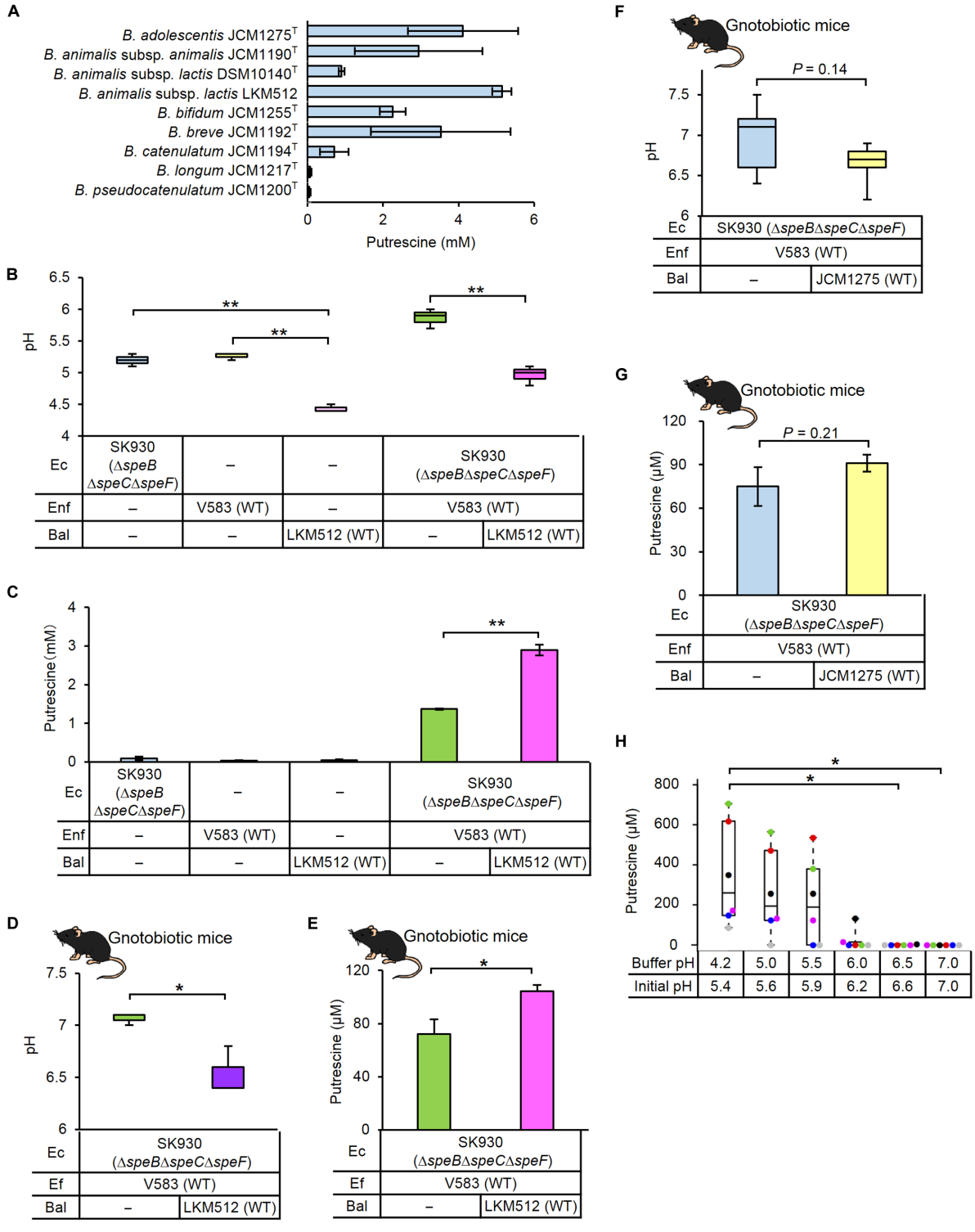


Fig. 4. Effects of acidification by bifidobacteria on putrescine production by *E. coli* and *En. faecalis*. (**A**) Extracellular putrescine concentration in cocultures of putrescine-deficient *E. coli* (SK930), wild-type *En. faecalis* (V583), and each *Bifidobacterium* sp. (**B** and **C**) Extracellular pH (B) and putrescine concentration (C) in cocultures of putrescine-deficient *E. coli* (SK930), wild-type *En. faecalis* (V583), and *B. animalis* subsp. *lactis* LKM512 (Bal). Coculture of these bacteria was conducted under anaerobic conditions at 37°C for 24 hours in LB medium containing 2 mM L-arginine, D-glucose (1.5 g/liter), galacto-oligosaccharide (5 g/liter), 2 mM $MgSO_4$, 60 mM NH_4Cl, and LB-RGC (0.5 g/liter) (pH 6.5). (**D** and **E**) Fecal pH (D) and putrescine concentration (E) in gnotobiotic mice colonized with putrescine-deficient *E. coli* (SK930), wild-type *En. faecalis* (V583), and *B. animalis* subsp. *lactis* LKM512. (**F** and **G**) Fecal pH (F) and putrescine (G) concentration in gnotobiotic mice inoculated with putrescine-deficient *E. coli* (SK930), wild-type *En. faecalis* (V583), and *B. adolescentis* JCM1275^T. (**H**) Extracellular putrescine concentration in human feces incubated at different pHs. Error bars represent SEs. *$P < 0.05$ and **$P < 0.01$, one-way ANOVA with Tukey's test, Student's *t* tests, and Steel-Dwass test.

Selection of *Bifidobacterium* strains that induce putrescine production through the hybrid system comprising *E. coli* and *En. faecalis*

We screened for *Bifidobacterium* spp. that strongly produced putrescine and added nine identified strains to the mixed cultures of the putrescine-deficient *E. coli* (SK930) strain and wild-type *En. faecalis* (SK947). We determined the concentrations of putrescine in the culture supernatants of these mixtures (Fig. 4A). Among cocultures tested, putrescine-deficient *E. coli* (SK930), wild-type *En. faecalis* (V583), and *B. animalis* subsp. *lactis* LKM512 produced the highest putrescine concentration. Moreover, putrescine concentrations in the presence of the two strains of *B. animalis* subsp. *lactis* (LKM512 and DSM10140^T) differed, indicating that induction of putrescine production by *Bifidobacterium* spp. is strain-dependent. *Bifidobacterium* spp. produce lactate and acetate (*24*), which decrease the pH in the intestinal lumen (*25*). Next, we investigated the mechanism by which *B. animalis* subsp. *lactis* LKM512 induced putrescine production in cocultures of *En. faecalis* and *E. coli*. The pH of culture supernatants from individually cultured putrescine-deficient *E. coli* (SK930), wild-type *En. faecalis* (V583), and *B. animalis* subsp. *lactis* LKM512 decreased from an initial value of 6.5 to approximately 5.2, 5.3, and 4.4, respectively, after 24 hours (Fig. 4B), and their putrescine concentrations were very low (Fig. 4C). However, the median pH of supernatants from cocultures of these three strains was 5.0, which was significantly lower than that of cocultures without LKM512 (pH 5.9) (Fig. 4B). Furthermore, the putrescine concentration in culture supernatants from cocultures of the three strains was more than twice that from cocultures without LKM512 ($P < 0.01$; Fig. 4C), suggesting that the acidic environment created by *B. animalis* subsp. *lactis* LKM512 probably induced putrescine production in cocultures of *E. coli* and *En. faecalis*. Nevertheless, it could be possible that factors other than environmental acidity are involved in this process because the extracellular putrescine concentration positively correlated with the number of both *E. coli* and *En. faecalis* in the coculture system (fig. S3A). Notably, in the culture with *B. animalis* subsp. *lactis* LKM512 or *B. adolescentis* JCM1275^T, which induced higher production of putrescine, large numbers of *E. coli* [$10^{8.5}$ colony-forming units (CFU)/ml or more] and *En. faecalis* ($10^{7.9}$ CFU/ml or more) were detected. However, the culture with *B. longum* JCM1217^T or *B. pseudocatenulatum* JCM1200^T, which induced only slight putrescine production, contained fewer than $^1/_{10}$ of the *E. coli* and *En. faecalis* (less than $10^{6.8}$ CFU/ml) than those with *B. animalis* subsp. *lactis* LKM512 or *B. adolescentis* JCM1275^T. All cultures reached pH 5.5 at which maximal putrescine production occurred within 12 hours (fig. S3B).

Induction of polyamine production in the feces of gnotobiotic mice by oral administration of *Bifidobacterium* spp.

To test the effect of the environment created by *Bifidobacterium* spp. on putrescine production in vivo, gnotobiotic mice were colonized with the putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (V583), with or without one of two *Bifidobacterium* spp. (*B. animalis* subsp. *lactis* LKM512 or *B. adolescentis* JCM1275^T). Compared with mice colonized with putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (V583) alone, the fecal pH (Fig. 4D) and putrescine concentrations (Fig. 4E) in mice additionally colonized with LKM512 were significantly lower ($P < 0.01$) and higher ($P < 0.05$), respectively. However, there was no significant difference in fecal pH (Fig. 4F) and putrescine concentration (Fig. 4G) between gnotobiotic mice colonized with and without *B. adolescentis* JCM1275^T. Although we confirmed the colonization of both bacteria, the number of *B. animalis* subsp. *lactis* LKM512 was higher than that of *B. adolescentis* JCM1275^T (fig. S4, A and B). These results suggested that the ability of *Bifidobacterium* spp. to increase putrescine depended on the ability of acidification of the intestinal luminal environment and that this ability differs among species.

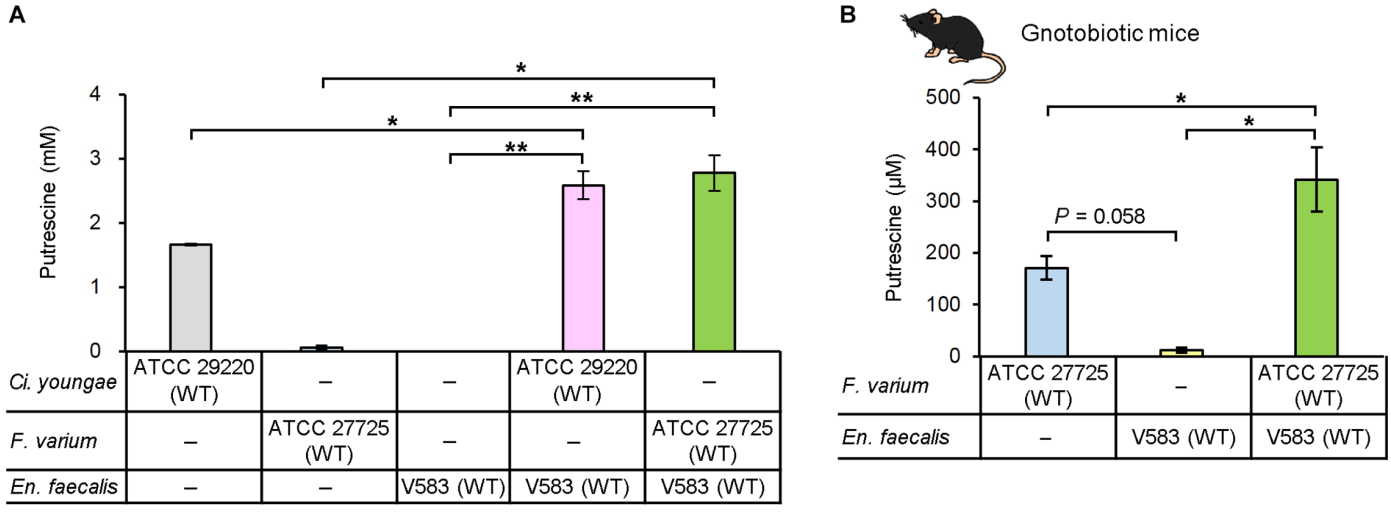
To investigate the potential effect of acidification of the intestinal luminal environment on putrescine production, we suspended human feces in buffers with different pH and incubated for 24 hours ($n = 6$). We increased the putrescine concentrations at pH 5.4 to 5.9 and observed the increase in all samples at pH 5.4, suggesting that acidification of the intestinal luminal environment might trigger putrescine production by intestinal bacteria (Fig. 4H). In an additional experiment ($n = 5$), wherein we suspended fecal samples in 0.1 N HCl to kill bacterial cells, putrescine concentration was slightly increased (fig. S5A), suggesting that the putrescine production in the fecal suspensions incubated at pH 5.4 to 5.9 (Fig. 4H) was not derived from the rupture of bacterial cells caused by the acidic conditions.

Putrescine production by collaboration of *En. faecalis* and other intestinal bacterial species

En. faecalis, which contributes to the second half of the putrescine production process identified in this study (Fig. 2), is a dominant intestinal bacterial species (*16*), and consequently, it is expected to play a significant role in metabolite flow in the intestinal lumen. However, *E. coli* is a relatively minor species in the intestine; therefore, to determine whether other intestinal bacteria can substitute for *E. coli* in the first half of putrescine production, we identified species that have AdiA and AdiC from a list of intestinal bacteria by in silico analyses (table S1). Two species identified by this screen, *Citrobacter youngae* ATCC 29220 and *Fusobacterium varium* ATCC 27725, were cocultured with wild-type *En. faecalis* (V583). When *Ci. youngae* ATCC 29220 and *En. faecalis* were cocultured, putrescine production tended to increase as compared to that in separate *Ci. youngae* culture (Fig. 5A). In addition, when *F. varium* ATCC 27725 and *En. faecalis* were cocultured, the putrescine concentration in the culture supernatant was more than 28 times higher than when *F. varium* was cultured alone. Furthermore, the concentration of putrescine in feces of gnotobiotic mice cocolonized with *F. varium* ATCC 27725 and wild-type *En. faecalis* (V583) was significantly higher ($P < 0.05$) than that of those monocolonized with wild-type *En. faecalis* (V583) or *F. varium* ATCC 27725 alone (Fig. 5B). These results indicated that several indigenous intestinal bacterial species that have AdiA and AdiC homologs contributed to the compound putrescine metabolic pathway. Although the agmatine production in *Ci. youngae* ATCC 29220 monoculture was highest when the pH of the medium was 5.0, that in *F. varium* ATCC 27725 monoculture was highest when the pH of the medium was 7.0 (fig. S6). This result showed that the AdiC/AdiA pathway of *Ci. youngae*, as well as that of *E. coli*, is activated by environmental acidification, whereas the pathway of *F. varium* is not.

Symbiont-symbiont co-occurrence networks and analysis of metatranscriptome of human feces

To analyze the coexistence of the three groups of bacteria identified in the current study as involved in putrescine production in the human intestinal lumen, we constructed a symbiont-symbiont co-occurrence network using previously reported human microbiome data from



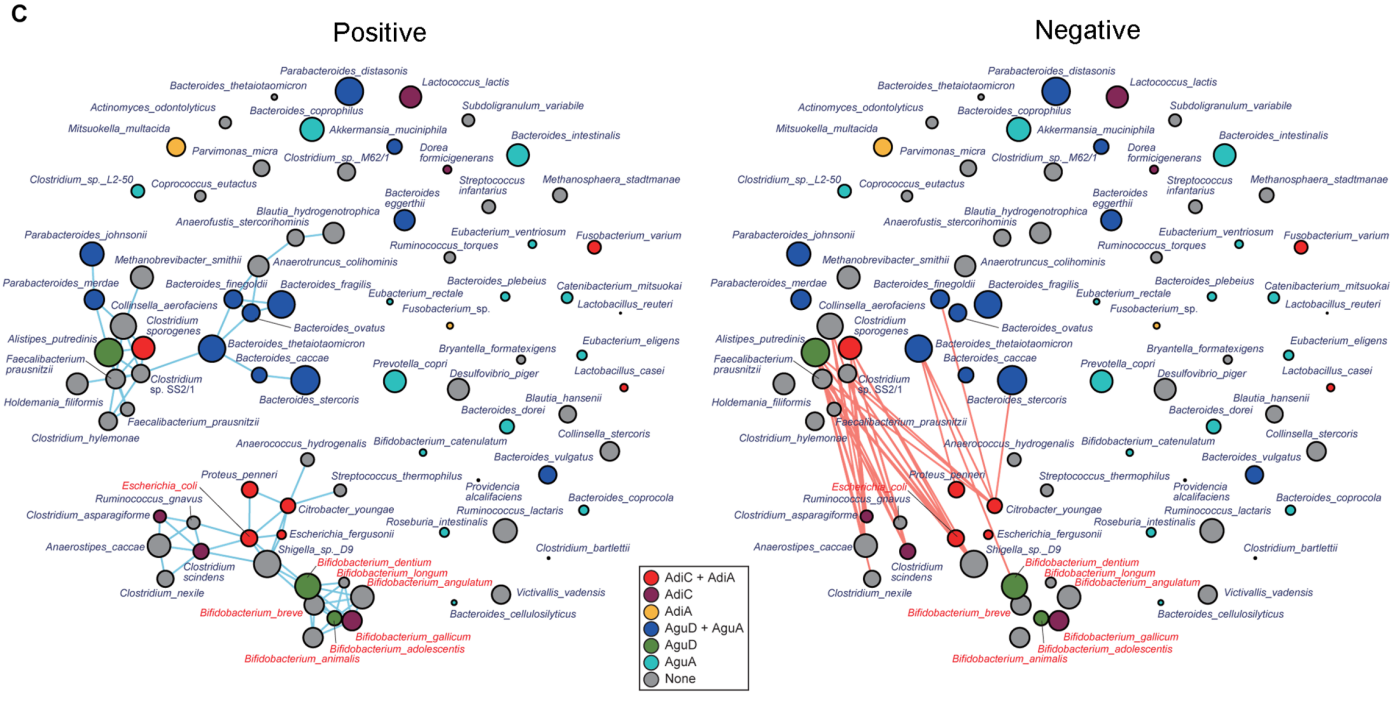


Fig. 5. General putrescine production pathway from arginine via agmatine in the human intestinal microbiome. (**A**) Extracellular putrescine concentration in monoculture of *Ci. youngae* ATCC 29220 or *F. varium* ATCC 27725 and in coculture of *Ci. youngae* ATCC 29220 or *F. varium* ATCC 27725 with wild-type *En. faecalis* (V583). (**B**) Fecal putrescine concentration in gnotobiotic mice inoculated with *F. varium* ATCC 27725 and wild-type *En. faecalis* (V583). Mono- and cocultures of these bacteria were conducted under anaerobic conditions at 37°C for 24 hours in LB-RGC medium. (**C**) Symbiont-symbiont co-occurrence networks of key genes in the putrescine production pathway using previously described human microbiome data from U.S. metropolitan areas (*26*). Blue and red lines between bacterial species indicate statistically significant aggregation and segregation, respectively. Names of *Bifidobacterium* spp. and *E. coli* are shown in red. The size of the circles represents the number of reads identified. Circle colors: red, bacterial species that have AdiC and AdiA; deep pink, bacterial species that have AdiC; gold, bacterial species that have AdiA; blue, bacterial species that have AguD and AguA; chartreuse, bacterial species that have AguD; cyan, bacterial species that have AguA; gray, other bacterial species. Error bars represent SE. **P* < 0.05 and ***P* < 0.01, one-way ANOVA with Tukey's test and Student's *t* tests.

U.S. metropolitan areas (*26*). We identified *Bifidobacterium* spp. coexistent with bacterial species that have AdiC and/or AdiA (including *E. coli*) (Fig. 5C, left, and fig. S7, top). In addition, *Clostridium sporogenes*, which has both AdiC and AdiA, was found to coexist with several species of the genera *Bacteroides* and *Parabacteroides*, which have both AguA and AguD. However, these two coexistent clusters occurred separately. In addition, there was segregation between several bacterial species that have both AdiC and AdiA and those that have AguA and/or AguD (Fig. 5C, right, and fig. S7, bottom). The results of analyses using data from Venezuela and

Malawi (figs. S8 and S9) (*26*) were similar to those generated from U.S. fecal microbiomes. To confirm expression of the *adiA*, *adiC*, *aguA*, and *aguD* genes in colonic lumen of human, we analyzed the number of RNA sequences of these genes using previously reported metatranscriptome data obtained eight human feces (*27*). We observed expression of the *adiA*, *adiC*, and *aguA* genes derived from various bacterial species in all fecal samples (table S2), demonstrating that three of the four key genes of this metabolic system were activated in the human intestine. However, we did not find the expression of the *aguD* gene in this analysis. We also did not detect this gene (DNA sequence) in the metagenome data (table S2).

DISCUSSION

Metabolites of intestinal bacteria are considered to be synthesized by reactions within single bacterial cells or by sequential reactions in different bacterial species in the intestinal tract. Studies to experimentally identify enzymes involved in the synthesis of metabolites by intestinal bacteria are in progress and focus on pathways within individual bacterial cells (*28*–*30*). Only a few studies have explored the metabolic mechanisms involving the collaboration of more than two intestinal bacteria using genetically engineered bacterial strains. For example, in an excellent study describing the cross-feeding system of polysaccharides by intestinal microbes (*31*), gene deletions of one of two bacterial species involved in the cross-feeding demonstrated a symbiotic association between bacteria. Here, we have identified a novel hybrid system for putrescine production by two bacteria using deletion and complementation strains of both bacterial species involved.

This study has focused on the metabolism of the polyamine, putrescine, in the ecosystem of the intestinal lumen and has demonstrated genetically that independent metabolic pathways and transport systems of two intestinal bacterial species (*En. faecalis* and *E. coli*) can combine to induce the production of putrescine both in vitro and in vivo. In the in vivo experiment, the putrescine level in the colonic lumen was found to be insufficient, suggesting that the produced putrescine may be absorbed by the host. Further study is required to clarify this possibility. Putrescine production via this pathway does not depend on the specific combination of *En. faecalis* and *E. coli*, since pairings of *En. faecalis* and *Ci. youngae* or *F. varium*, which have AdiA and AdiC homologs, also produced putrescine. As there are many types of bacteria with homologs of the enzymes AdiA, AdiC, AguA, and/or AguD in the human colon, there are likely countless combinations that can produce putrescine via this pathway. The optimal pH to produce agmatine was different for *Ci. youngae* and *F. varium* in monoculture, indicating that the AdiC/AdiA system of some bacteria in the human gut is activated while that of others is not activated by environmental acidification (drop in pH to below 6.5 from neutral).

The induction of putrescine production observed in the present study was triggered by a resistance system of *E. coli*, which is induced to ensure survival in acidic environments, and the putrescine production depended on acidic pH. *E. coli* has two arginine decarboxylases, SpeA (*32*) and AdiA (*33*), and is able to biosynthesize agmatine. An operon consisting of *adiA* and *adiC* encodes an arginine-agmatine antiporter that exchanges agmatine inside the cell for arginine outside (*33*). In sequential reactions in *E. coli*, arginine imported from outside the cell by AdiC is decarboxylated by AdiA to generate agmatine with the consumption of protons, increasing the environmental pH. This alkalization system is induced in response to low pH and has been extensively studied as AR3 (*22*).

In *En. faecalis*, putrescine is synthesized from agmatine by sequential reactions catalyzed by AguA and AguB (*34*, *35*). As an agmatine biosynthetic pathway has not previously been reported in *En. faecalis*, and because homologs of agmatine biosynthetic enzymes are not present in *En. faecalis*, it is thought that this bacterium imports agmatine from outside the cell and converts it to putrescine. The uptake of agmatine is catalyzed by the agmatine-putrescine antiporter AguD, which exchanges putrescine inside the cell for agmatine outside (*36*). The pathway of agmatine catabolism to yield putrescine via agmatine deiminase consists of AguA, AguB, and AguD and can enhance the growth of *En. faecalis* by neutralizing the external medium (*21*). However, this pathway is not influenced by environmental pH but is induced by exogenous agmatine and completely suppressed by environmental glucose (fig. S1), corroborating the report of Suárez *et al.* (*21*) that suggested that the agmatine deiminase pathway of *En. faecalis* has a role not only in acid resistance but also in energy production.

In in silico analysis using human fecal microbiome data, among the three types of bacteria involved in putrescine production in the intestinal lumen, acid-producing *Bifidobacterium* spp. were coexistent with *E. coli* as representative bacteria that have systems that release agmatine, suggesting that agmatine production is most likely facilitated by AdiC/AdiA in response to acid stress caused by *Bifidobacterium* spp. *Cl. sporogenes*, which has AdiC and AdiA homologs, might play an important role in this putrescine biosynthetic pathway because only *Cl. sporogenes* coexists with several *Bacteroides* species, which have AguD and AguA homologs. Both coexistence and segregation were observed among bacterial species that have AdiC/AdiA and those that harbor AguD/AguA, which import agmatine and release putrescine, suggesting that the relationship between these two types of bacteria is not specific and they do not depend on each other. In other words, this relationship is not a specific cosurvival strategy. In this analysis, no existing cluster of the three types of bacteria–those with AdiC/AdiA, those with AguD/AguA, and *Bifidobacterium* spp.–was found. This may be because only sequenced bacterial genomes were included. In the future, if the number of bacteria with sequenced genomes derived from microbiomes of other ethnic groups increases, then clusters harboring these three bacterial types might be found. Furthermore, the possible involvement of other acid-producing bacteria in environmental acidification should also be considered. Analysis of metatranscriptomic data of human feces obtained from published articles revealed that three of the four key genes of this metabolic system (AdiA, AdiC, and AguA) are activated in the human intestine. AguD gene expression was not found in these data. Because this gene was also not detected in the metagenome data, the absence of AguD expression might be caused by the absence of AguD-harboring bacteria from the fecal samples used in this study, rather than by down-regulation of gene expression. The AguD gene can be detected in DNA sequences of some human feces samples in the database of the National Institutes of Health Human Metagenome Project (www.hmpdacc.org/hmp/), by searching the Joint Genome Institute Integrated Microbial Genomes and Mircobiomes system (JGI-IMG/M: https://img.jgi.doe.gov/). Therefore, there is a possibility that AguD gene expression may be detected in feces with high putrescine concentrations. Further studies involving fecal metatranscriptome analysis after putrescine concentration determination are needed to confirm this notion.

The well-known benefits of *Bifidobacterium* spp. depend on acidification of the luminal environment by acetate and lactate

production by these bacteria. For example, constipation can be alleviated by the activation of peristaltic movement in the digestive tract stimulated by these short-chain fatty acids (*37*). In addition, beneficial effects occur via the improvement of gut microbiota and the beneficial metabolites produced by *Bifidobacterium* spp. For example, *Bifidobacterium* spp. were shown to protect their hosts from enteropathogenic infections through acetate production in the intestinal tract (*38*). However, there are no previous reports showing that short-chain fatty acids derived from *Bifidobacterium* spp. induce the production of other metabolites by indigenous bacteria. Here, we describe a novel mechanism whereby acidification of the intestinal environment by *Bifidobacterium* spp. facilitates the synthesis and export of a polyamine by coculture of *E. coli* and *En. faecalis* in vitro and in vivo. The findings of the present study indicate the need for a major paradigm shift away from the concept that *Bifidobacterium* spp. only generate direct health benefits toward the idea that they facilitate indirect benefits through modification of metabolite generation by the gut microbiota in the large intestine. Putrescine production was inhibited by mixed cultures containing several *Bifidobacterium* spp. (*B. longum* and *B. pseudocatenulatum*) that suppressed growth of *E. coli* and *En. faecalis*. This finding suggests that only those *Bifidobacterium* spp. that do not suppress commensal bacteria, especially bacteria that have AdiC/AdiA or AguD/AguA, might be involved in inducing putrescine production via this hybrid system.

In conclusion, we identified a novel pathway for putrescine production from arginine through agmatine involving collaboration of two different bacterial species, triggered by environmental acidification (drop in pH to below 6.5 from neutral) in hosts harboring acid-producing bacteria in the gut. This pathway consists of independent strategies in the individual contributing bacteria: the acid tolerance system of *E. coli* (arginine-dependent acid resistance system) and the energy production system of *En. faecalis* (agmatine deiminase system), in the context of acid production by acid-producing bacteria, represented here by *Bifidobacterium* spp. (fig. S10).

We believe that similar interspecies connections are likely to exist in the production of other metabolites by intestinal microbiota. It will be important to consider this possibility in future analyses of metabolite production by gut microbial communities. It should be noted that novel pathways for the production of specific metabolites via microbial networks cannot be readily identified from metagenome data alone because, even if a microorganism has the synthetic pathway of a specific metabolite, it does not necessarily release this metabolite.

MATERIALS AND METHODS

Bacterial strains and plasmids

Most gut microbial species belong to four major phyla: Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes. We selected 14 intestinal bacterial species that are the predominant species in human microbiota (*16*) from these four phyla (table S3). The strains, plasmids, and primers used in this study are listed in table S4.

In *E. coli*, P1 transduction (*39*) was used to transfer chromosomes with gene deletions Δ*adiC* (JW4076) and Δ*adiA* (JW5731) from the Keio collection (*40*) into a wild-type MG1655 background to generate SK900 (Δ*adiA*:: FRT-*kan*$^+$-FRT) and SK902 (Δ*adiC*:: FRT-*kan*$^+$-FRT). The plasmid pCP20 (*41*) was then introduced to eliminate the kanamycin resistance cassettes, generating SK901 (Δ*adiA*:: FRT) and SK903 (Δ*adiC*:: FRT).

pBelobac11-*adiC*$^+$ was constructed as follows: A 1725–base pair (bp) DNA fragment including *adiC* and a 300-bp region upstream of this gene was amplified by polymerase chain reaction (PCR) using KOD-Plus (Toyobo Co. Ltd.) polymerase, "adiC_300bp-up_for" and "adiC_term_rev" as primers, and genomic DNA of wild-type *E. coli* (MG1655) as a template. The amplified fragment was cloned into pBelobac11 digested with Hpa I. The cloned region was sequenced, demonstrating that it contained no mutations.

pBelobac11-*adiA*$^+$ was constructed as follows: A 2598-bp DNA fragment including *adiA* and a 300-bp upstream region was amplified by PCR using KOD-Plus polymerase, "adiA_300bp-up_for" and "adiA_term_rev" as primers, and genomic DNA of wild-type *E. coli* (MG1655) as template. The amplified fragment was cloned into pBelobac11 digested with Hpa I. The cloned region was sequenced, and there were no mutations.

A derivative of the temperature-sensitive plasmid pLT06 (*42*), pLT06-Δ*aguD*, was used for allelic exchange of *aguD* in *En. faecalis*, following methods described previously (*43*, *44*). Briefly, a sequence extending from −1 to −1042, with reference to the *aguD* start codon, and a second fragment extending from +1 to +1096 relative to the *aguD* stop codon were amplified by PCR. The two amplicons were concatenated using PCR, and the resulting 2140-bp DNA fragment was cloned into the Eco RI site of pLT06 to generate pLT06-Δ*aguD*. pLT06-Δ*aguD* was introduced into V583, resulting in SK932. A strain with a single-crossover event in the region flanking *aguD* on the chromosome of SK932 was screened on THB plates (Becton, Dickinson and Company) supplemented with chloramphenicol (10 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 120 μg/ml) after incubation at 42°C to eliminate the temperature-sensitive plasmid, pLT06-Δ*aguD*, generating SK934. The single crossover was confirmed in a temperature- and chloramphenicol-resistant blue colony by PCR. Then, a strain with a double-crossover event was generated by two passages of culture in medium without chloramphenicol and screened on MM9YEG plates (*45*) containing X-gal (120 μg/ml) and *p*-chloro-phenylalanine (*p*-Cl-Phe; 10 μg/ml) to generate SK937. The double crossover generating Δ*aguD* was confirmed in a *p*-Cl-Phe–resistant white colony by PCR. The manipulated chromosomal region in SK937 was sequenced to confirm that no unintended mutations had been introduced.

pLZ12-*aguB*$^+$*D*$^+$ was constructed as follows: A 2985-bp DNA fragment, including the *aguBD* operon and a 500-bp region upstream of *aguB* on the chromosome of wild-type *En. faecalis* (V583), was amplified by PCR using PrimeSTAR polymerase (Takara Bio Inc.), the primers Clone_agcB+0. 5K_FWD and Clone_agcD+COMPL, and genomic DNA of wild-type *En. faecalis* (V583) as a template. The amplified fragment was cloned into pLZ12 (*46*) digested with Bam HI. The cloned region was sequenced, and no mutations were identified.

Screening of polyamine-producing bacteria

We searched for combinations of intestinal bacterial species that produced polyamines from L-arginine among 14 different intestinal bacteria; in total, 91 combinations were tested. Strains were each precultured in suitable medium at 37°C for 24 hours under anaerobic conditions. *Bacteroides vulgatus*, *Ba. thetaiotaomicron*, *Ba. caccae*, *Ba. uniformis*, *Parabacteroides merdae*, *Pa. johnsonii*, *En. faecalis*, and *E. coli* were precultured in GAM Broth (Nissui Pharmaceutical Co. Ltd.). *Alistipes putredinis* was precultured in Eggerth-Gagnon (EG) broth (JCM medium 14). *Blautia hansenii* was precultured in peptone

yeast glucose broth (JCM medium 676). *Cl. leptum* was precultured in PY+X broth (DSMZ medium 104c). *Streptococcus thermophiles* was precultured in M17 broth (Merck KGaA). *B. adolescentis* and *B. longum* were precultured in blood liver (BL) broth (Nissui Pharmaceutical Co. Ltd.). Each bacterial preculture was inoculated at a final optical density at 600 nm (OD_{600}) of 1.0×10^{-2} in gifu anaerobe medium (GAM) broth containing 2 mM L-arginine. Strains were grown under anaerobic conditions at 37°C for 24 hours. Culture supernatants were collected by centrifugation at 9100*g* for 10 min, and the putrescine concentrations were measured.

Mixed cultures of *E. coli* and *En. faecalis*

Strains were grown under anaerobic conditions at 37°C for 24 hours in LB medium (Becton, Dickinson and Company) containing 2 mM L-arginine, 1.5 g/liter D-glucose, and 0.5 g/liter L-cysteine-hydrochloride (LB-RGC medium) in screw-top test tubes with butyl rubber inner plugs (Sanshin Industrial Co. Ltd.). The gas-phase portions in screw-top test tubes were replaced with N_2/CO_2 (80:20, v/v) before autoclaving (115°C, 15 min). Chloramphenicol was added to the medium at a final concentration of 10 μg/ml, where required. *E. coli* precultures were harvested after 24 hours and inoculated at a final OD_{600} of 1.0×10^{-2}. *En. faecalis* was harvested after 24 hours and inoculated at a final OD_{600} of 1.0×10^{-5}. After 24 hours of mono- or coculture, culture supernatants were collected by centrifugation (9100*g*, 10 min), and the putrescine concentration was determined. To measure viable bacterial counts of *En. faecalis*, aliquots (10 μl) were removed and serially diluted, 20 μl of each dilution was plated on KF Streptococcus Agar (Oxoid), and the CFU were counted.

Incubation using culture supernatants from *E. coli* or *En. faecalis*

Strains were grown under anaerobic conditions at 37°C for 24 hours in LB-RGC medium in screw-top test tubes with butyl rubber inner plugs. The gas-phase portions in the screw-top test tubes were replaced with N_2/CO_2 (80:20, v/v) before autoclaving (115°C, 15 min). *E. coli* and *En. faecalis* precultures were harvested after 24 hours and inoculated at a final OD_{600} of 1.0×10^{-2}. *E. coli* or *En. faecalis* was cultured for 24 hours, and culture supernatants were collected by centrifugation (1500*g*, 10 min) and sterilized through commercial filter membrane units (Millex-GV, 0.22 μm; polyvinylidene difluoride, 33 mm; Merck Millipore). The other strain was then cultured for 24 hours in the sterilized culture supernatant, and L-arginine, agmatine, and putrescine concentrations were measured in the final culture supernatant.

Monoculture of *E. coli*, *En. faecalis*, *Ci. youngae*, and *F. varium*

Precultures were harvested and inoculated at a final OD_{600} of 1.0×10^{-2} in the media described below. Wild-type *E. coli* (MG1655) was cultured under anaerobic conditions at 37°C for 24 hours in LB-RGC medium (pH 4.0 to 9.0). Wild-type *En. faecalis* (V583) was cultured in LB medium containing L-cysteine-hydrochloride (0.5 g/liter) and 5 mM agmatine (pH 4.0 to 9.0) or in LB medium containing glucose (0 to 3 g/liter), L-cysteine-hydrochloride (0.5 g/liter), and 5 mM agmatine (pH 6.0). Culture supernatants were collected by centrifugation (9100*g*, 10 min), and the putrescine and agmatine concentrations were measured. *Ci. youngae* ATCC 29220 and *F. varium* ATCC 27725 were cultured under anaerobic conditions at 37°C for 24 hours in LB-RGC medium (pH 4.0 to 9.0). Culture supernatants were collected by centrifugation (9100*g*, 10 min), and the agmatine and putrescine concentrations and pH were measured. For viable bacterial counts of *Bifidobacterium* spp., aliquots (10 μl) were removed and serially diluted, 20 μl of each dilution was plated on TOS propionate agar (Yakult Pharmaceutical Ind. Co. Ltd.), and the CFU were counted.

Mixed cultures of *E. coli*, *En. faecalis*, and *Bifidobacterium* spp.

We prepared mixed cultures of putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (V583) and of putrescine-deficient *E. coli* (SK930), wild-type *En. faecalis* (V583), and *Bifidobacterium* spp. Strains were grown under anaerobic conditions at 37°C for 24 hours in LB medium containing 2 mM L-arginine, D-glucose (1.5 g/liter), galacto-oligosaccharide (5 g/liter), 2 mM $MgSO_4$, 60 mM NH_4Cl, and L-cysteine-hydrochloride (0.5 g/liter; pH 6.5) in screw-top test tubes with butyl rubber inner plugs. The gas-phase portions in the screw-top test tubes were replaced with N_2/CO_2 (80:20, v/v) before autoclaving (115°C, 15 min). *E. coli* precultures were harvested and inoculated at a final OD_{600} of 1.0×10^{-3} in the above-mentioned medium, *En. faecalis* precultures were inoculated at a final OD_{600} of 1.0×10^{-5}, and each *Bifidobacterium* sp. was inoculated at a final OD_{600} of 1.0×10^{-2}. Culture supernatants were collected by centrifugation (9100*g*, 10 min), and the putrescine concentration and pH were measured. For viable bacterial counts of *E. coli*, *En. faecalis*, and *Bifidobacterium* spp., aliquots (10 μl) were removed and serially diluted, 20 μl of each dilution was plated on deoxycholate agar (Merck KGaA), KF Streptococcus Agar (Oxoid), and TOS propionate agar (Yakult Pharmaceutical Ind. Co. Ltd.), respectively, and the CFU were counted.

Colonization of gnotobiotic mice

Germ-free mice (C57BL/6) were purchased from Sankyo Labo Service Co. Inc. and were bred at Kyodo Milk Industry Co. Ltd. Mice were housed in flexible film plastic isolators with sterilized bedding and provided with sterilized water [containing chloramphenicol (10 μg/ml), where required] and sterilized commercial CMF pellets (Oriental Yeast Co. Ltd.) ad libitum. Six-week-old germ-free mice were used in all experiments and were divided into three groups ($n = 3$ per group): a control group colonized with putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (SK947), a knockout group colonized with putrescine-deficient *E. coli* (SK930) and *aguD*-deleted *En. faecalis* (SK948), and a complement group colonized with putrescine-deficient *E. coli* (SK930) and *aguD*-complemented *En. faecalis* (SK949). The experimental schedule is presented in fig. S11A. First, germ-free mouse feces were collected. Then, the three groups of mice were gavaged with phosphate-buffered saline (PBS) containing different *En. faecalis* strains (1.0×10^9 CFU). After 20 hours, mouse feces were collected, and all groups of mice were gavaged with PBS containing putrescine-deficient *E. coli* (SK930) (1.0×10^{10} CFU). After 20 hours, feces were collected. The mice were sacrificed, the colonic and cecal contents were obtained, and the putrescine concentration of these samples was measured.

In experiments to test the effects of three types of bacteria, 6-week-old germ-free C57BL/6 mice were divided into two groups ($n = 3$ per group): one colonized with two types of bacteria [putrescine-deficient *E. coli* (SK930) and wild-type *En. faecalis* (V583)] and one with three types of bacteria [putrescine-deficient *E. coli* (SK930), wild-type *En. faecalis* (V583), and *B. animalis* subsp. *lactis* (LKM512)]. The experimental schedule is presented in fig. S11B. First, germ-free mice feces were collected, and then, the first group of mice was gavaged with PBS containing putrescine-deficient *E. coli* (SK930) ($1.0 \times$

10^{10} CFU) and wild-type *En. faecalis* (V583) (3.0×10^9 CFU), while the latter group was gavaged with PBS containing putrescine-deficient *E. coli* (SK930) (1.0×10^{10} CFU), wild-type *En. faecalis* (V583) (3.0×10^9 CFU), and *B. animalis* subsp. *lactis* LKM512 (1.0×10^8 CFU). After 20 hours, feces were collected, and the polyamine concentrations and pH were measured.

To test the effects of colonization with *F. varium* and *En. faecalis*, experimental mice were divided into two groups ($n = 3$ per group). The experimental schedule is presented in fig. S11C. One group was gavaged with PBS containing *F. varium* ATCC 27725 (5.0×10^8 CFU) on the first day and wild-type *En. faecalis* (V583) (3.0×10^9 CFU) on the second day. A second group of mice was gavaged with PBS containing wild-type *En. faecalis* (V583) (3.0×10^9 CFU) on the first day and *F. varium* ATCC 27725 (5.0×10^8 CFU) on the second day. After the administration of bacteria (20 hours), mouse feces were collected, and the putrescine concentrations were measured. All animal experiments were approved by the Kyodo Milk Animal Use Committee (permit numbers 2015-01 and 2016-10) and were in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Academies Press.

Culture of human feces at various pH values

Feces were obtained from six volunteers and suspended in nine volumes of 100 mM MES buffer (Dojindo Laboratories) (pH 4.2, 5.0, 5.5, 6.0, 6.5, and 7.0). The suspensions were incubated for 24 hours under anaerobic conditions. Polyamine concentrations were measured in the culture supernatants.

In an additional experiment, feces were obtained from five volunteers and suspended in nine volumes of 100 mM HCl or 100 mM MES buffer (Dojindo Laboratories) (pH 4.2, 5.0, 6.0, and 7.0). The suspensions were incubated for 24 hours under anaerobic conditions. Polyamine concentrations and pH were measured in the culture supernatants. For viable bacterial counts, aliquots (10 μl) were removed and serially diluted; 20 μl of each dilution was plated on EG agar (JCM medium 14), BL agar (Nissui Pharmaceutical Co. Ltd.), and GAM agar (Nissui Pharmaceutical Co. Ltd.); and the CFUs were counted.

Determination of polyamine, L-arginine, and agmatine concentrations

The polyamine, L-arginine, and agmatine concentrations in culture supernatants were measured, as described by Reguera *et al.* (*47*), with some modifications, using an Acquity ultra-performance liquid chromatography system with an FLR Detector (Waters), as previously described (*14*).

In silico pathway analysis using BLAST

Protein BLAST (*48*) analysis was performed using protein sequence data from 126 bacterial strains present in the human gut (*26*). The accession IDs and sources of the protein sequences used for the BLAST search, as well as the identity scores, are listed in table S1.

Mixed cultures of *F. varium* and *Ci. youngae*

Similar to the mixed culture of *E. coli* and *En. faecalis*, *F. varium* and *Ci. youngae* were grown in LB-RGC medium under anaerobic conditions at 37°C for 24 hours. *F. varium* and *Ci. youngae* were harvested after 24 hours of preculture and inoculated at a final OD_{600} of 1.0×10^{-2}. After 24 hours of culture, culture supernatants were collected by centrifugation (9100*g*, 10 min), and the putrescine concentration was determined.

Symbiont-symbiont co-occurrence network analysis

Corresponding bacterial operational taxonomic units (OTUs) for each of the 126 bacterial species genome sequences were identified among the 16*S* ribosomal RNA data of Yatsunenko *et al.* (*26*) using the program Minimo (*49*). Corresponding 16*S* OTUs were found for 84 of the 126 bacteria. Patterns of co-occurrence among the 84 bacteria were examined using the sparse correlations for compositional data (SparCC) method (*50*). A positive correlation coefficient in the SparCC analysis represents a pair of bacteria potentially sharing environmental preferences and/or mutually interacting with each other, while a negative correlation coefficient represents diverged environmental preferences and/or competitive or antagonistic interactions. Only links with absolute correlation coefficients >0.3 were included in the networks.

Analysis of the expression of AdiA, AdiC, AguA, and AguD genes using metatranscriptomic data

The expression of AdiA, AdiC, AguA, and AguD genes in the metatranscriptome of human feces was analyzed using the metatranscriptomic data of Franzosa *et al.* (*27*).

Statistical analyses

Extracellular and fecal putrescine concentrations, pH, and viable bacterial counts were compared between the groups with the Student's *t* test, one-way ANOVA (followed by Tukey's test), Steel-Dwass test, or Wilcoxon signed-rank test. Comparisons at weeks 0 and 12 were tested using paired *t* test or Wilcoxon signed-rank test. Student's *t* test and one-way ANOVA (followed by Tukey's test) were performed using SPSS version 22 (IBM), and other analyses were performed using R statistical software version 3.4.2 or SPSS version 22.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/6/eaat0062/DC1

fig. S1. Induction of putrescine production by coculture of *E. coli* and *En. faecalis*.
fig. S2. Effects of extracellular pH and glucose concentration on bacterial metabolism in monocultures of *En. faecalis* and *E. coli*.
fig. S3. Viable bacterial counts and change of extracellular pH in cocultures of putrescine-deficient *E. coli* (SK930), wild-type *En. faecalis* (V583), and each *Bifidobacterium* sp.
fig. S4. Viable bacterial counts in feces of gnotobiotic mice.
fig. S5. Extracellular putrescine concentration and total viable bacterial counts in human feces incubated at different pH values ($n = 5$).
fig. S6. Effect of pH on extracellular agmatine concentration in monocultures of *E. coli*, *Ci. youngae*, and *F. varium*.
fig. S7. Symbiont-symbiont co-occurrence networks of key genes in the putrescine production pathway using previously described human microbiome data from U.S. metropolitan areas.
fig. S8. Symbiont-symbiont co-occurrence network patterns of key genes in the putrescine production pathway using previously described human microbiome data from Venezuela.
fig. S9. Symbiont-symbiont co-occurrence network patterns of key genes in the putrescine production pathway using previously described human microbiome data from Malawi.
fig. S10. Mechanistic model of a novel pathway for putrescine production from arginine through agmatine via the collaboration of three different bacterial species.
fig. S11. Outline of gnotobiotic mouse experiments.
table S1. List of species expressing homologs of the enzymes AdiA and AdiC, as determined by in silico analyses of 126 bacterial strains present in the human gut.
table S2. Detection of RNA sequences of AdiA, AdiC, AguA, and AguD by metatranscriptomic analysis of human feces.
table S3. List of bacteria used for screening of polyamine producing bacteria.
table S4. Strains, plasmids, and oligonucleotides used in this study.

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Acknowledgments: We are grateful to B. A. Duerkop, and L. Hooper from the University of Texas Southwestern Medical Center, Dallas for technical advice and for the gift of pLZ12. We acknowledge L. E. Hancock, University of Kansas, for providing pLT06. We are grateful to Y. Doi, Okayama University of Science, for technical advice regarding electroporation of *En. faecalis*. We thank A. Yamashita and A. Nakamura of Kyodo Milk Industry Co. Ltd. for supporting this study. **Funding:** This work was funded by Kyodo Milk Industry Co. Ltd. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. This study was partially supported by Grants-in-Aid from the Institute for Fermentation, Osaka (to S.K.). **Author contributions:** Y.K. performed the experiments in Figs.3 to 5 (A and B), wrote the Materials and Methods, and drew the figures. K.M. hypothesized and performed the experiments in Fig.1. H.T. performed the symbiont-symbiont co-occurrence network analysis and edited the manuscript. R.K. performed and analyzed the experiment in Fig.1 and table S2. Y.B. supervised this study. S.K. designed and prepared the genetically modified bacterial experiments, analyzed the data, and wrote the manuscript. M.M. conceived the study, analyzed the data, and wrote the manuscript. **Competing interests:** Kyodo Milk Industry Co. Ltd., which funded this research, has commercial products (yogurt and bacterial powder) containing *B. animalis* subsp. *lactis* LKM512 that is related to the content of this article to declare. The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 15 January 2018
Accepted 18 May 2018
Published 27 June 2018
10.1126/sciadv.aat0062

Citation: Y. Kitada, K. Muramatsu, H. Toju, R. Kibe, Y. Benno, S. Kurihara, M. Matsumoto, Bioactive polyamine production by a novel hybrid system comprising multiple indigenous gut bacterial strategies. *Sci. Adv.* **4**, eaat0062 (2018).

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Sci Adv **4** (6), eaat0062.
DOI: 10.1126/sciadv.aat0062