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RESEARCH ARTICLE

Identification of phenol- and *p*-cresol-producing intestinal bacteria by using media supplemented with tyrosine and its metabolites

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One sentence summary: We newly identified phenol- and *p*-cresol-producing bacteria by culture-based screening, and elucidated phylogenetic distribution of phenol- and *p*-cresol-producers.

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ABSTRACT

To identify intestinal bacteria that produce phenols (phenol and *p*-cresol), we screened 153 strains within 152 species in 44 genera by culture-based assay using broth media supplemented with 200 μM each of tyrosine and its predicted microbial metabolic intermediates (4-hydroxyphenylpyruvate, DL-4-hydroxyphenyllactate, 3-(*p*-hydroxyphenyl)propionate, 4-hydroxyphenylacetate and 4-hydroxybenzoate). Phenol-producing activity was found in 36 strains and *p*-cresol-producing activity in 55 strains. Fourteen strains had both types of activity. Phylogenetic analysis based on the 16S rRNA gene sequences of strains that produced 100 μM or more of phenols revealed that 16 phenol producers belonged to the *Coriobacteriaceae*, *Enterobacteriaceae*, *Fusobacteriaceae* and *Clostridium* clusters I and XIVa; four *p*-cresol-producing bacteria belonged to the *Coriobacteriaceae* and *Clostridium* clusters XI and XIVa; and one strain producing both belonged to the *Coriobacteriaceae*. A genomic search for protein homologs of enzymes involved in the metabolism of tyrosine to phenols in 10 phenol producers and four *p*-cresol producers, the draft genomes of which were available in public databases, predicted that phenol producers harbored tyrosine phenol-lyase or hydroxyarylic acid decarboxylase, or both, and *p*-cresol producers harbored *p*-hydroxyphenylacetate decarboxylase or tyrosine lyase, or both. These results provide important information about the bacterial strains that contribute to production of phenols in the intestine.

Keywords: phenol; *p*-cresol; intestinal bacteria; tyrosine; metabolite; phylogenetic analysis

INTRODUCTION

The more than 100 trillion bacteria in the human intestinal tract form a complicated ecosystem (Bäckhed *et al.* 2005). These bacteria produce many metabolites that can either harm or benefit host health (Nicholson *et al.* 2012). Short-chain fatty acids, which are produced mainly through the fermentation of carbohydrates, not only are used as energy sources for the host's colonocytes but also have anti-inflammatory effects (Verbeke *et al.* 2015). Polyamines in the intestinal lumen enhance longevity and delay senescence (Kibe *et al.* 2014). Equol produced by intestinal microbiota reduces the risk of prostate cancer (Sugiyama *et al.*

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2013). In contrast to these beneficial metabolites, intestinal secondary bile acid concentrations are closely related to the incidence of colorectal cancer (Ajouz, Mukherji and Shamseddine 2014), and indole, which is a uremic toxin, promotes the progression of chronic kidney disease (Evenepoel *et al.* 2009; Ito and Yoshida 2014). Because of the increasing importance of metabolites to host health, many metabolomic analyses have been performed to identify novel factors. For example, it has been found that trimethylamine is a risk factor for cardiovascular disease (Wang *et al.* 2011). As shown in these studies, we are aware of the role of metabolites in host health, but few studies have attempted to identify the bacteria involved in producing each type of metabolite in the colon. Obtaining information about the bacteria producing these metabolites would provide new clues to our understanding of disease from the perspectives of morbidity risk evaluation and the establishment of prevention methods.

Phenols (phenol and *p*-cresol) are microbial metabolites produced from tyrosine (Windey, De Preter and Verbeke 2012). Phenol exhibits cytotoxicity and increases paracellular permeability *in vitro* (Verbeke *et al.* 2015); it acts as a promoter of skin cancer in an animal model (Boutwell and Bosch 1959). *p*-cresol exhibits cytotoxicity and genotoxicity and reduces endothelial barrier function *in vitro* (Andriamihaja *et al.* 2015; Verbeke *et al.* 2015). *p*-cresyl sulfate, a sulfate-conjugate of *p*-cresol, suppresses Th1-type cellular immune responses in mice (Shiba *et al.* 2014); an increase in its levels is associated with chronic kidney disease-associated events such as cardiovascular disease (Meyer and Hostetter 2012; Ito and Yoshida 2014). Furthermore, phenol and *p*-cresol suppress the differentiation of keratinocytes in humans and cause dermal disorders in mice (Iizuka *et al.* 2009a,b). Although studies focusing on the relationship between phenols and various diseases have been accumulating, to our knowledge there has been no comprehensive study to identify the bacteria contributing to phenol- and *p-cresol*-production, with the exception of reports focused only on the genus *Clostridium* or on limited species (Bone, Tamm and Hill 1976; Elsden, Hilton and Waller 1976; Smith and Macfarlane 1996).

Here, we screened bacteria producing phenol or *p*-cresol, or both, using 153 strains within 152 species in 44 genera—mainly of intestinal bacteria—to determine which strains had the ability to produce phenol or *p*-cresol or both. Strains that screened positive were analyzed to determine the relationship between the ability to produce phenols and phylogenetic classification. They were then genetically analyzed to predict their metabolic pathways from tyrosine to phenols.

MATERIALS AND METHODS

Chemicals

DL-4-hydroxyphenyllactic acid, 4-hydroxyphenylpyruvic acid, 4-hydroxyphenylacetic acid and 4-hydroxybenzoic acid were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Tyrosine and 3-(*p*-hydroxyphenyl)propionate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Substrate solution was prepared by dissolving these compounds together in 18 mM NaOH solution (final 2 mM each) and filtered for sterilization through a 0.20 μm cellulose acetate filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan).

Bacterial strains and culture conditions

The 153 bacterial strains and culture conditions used for screening are listed in Table 1. The 153 strains represented 152 species found in the human gut habitat and their phylogenetic relatives; they accounted for about 70% of the common species detected in human feces (Qin *et al.* 2010). Two types of media (rich medium and poor medium) were used for culture. Rich medium was used for its growth efficiency: modified Gifu anaerobic medium broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1% glucose; MRS broth (Nissui Pharmaceutical Co., Ltd.); Trypticase soy broth (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA); or peptone–yeast extract (PY) broth supplemented with 1% glucose was used. The PY broth (1 L) contained 5.0 g peptone, 5.0 g trypticase peptone, 10.0 g yeast extract, 0.5 g L-cysteine HCl • H_2O, 4.0 g Na_2CO_3, 7 mL 0.07% hemin solution, 1.0 mL 0.1% resazurin solution, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.4 g $NaHCO_3$, 0.08 g NaCl, 8 mg $CaCl_2$, 19 mg $MgSO_4 \bullet 7H_2O$ and 1 mg vitamin K_1 (pH 6.9). Basal Medium (Bone, Tamm and Hill 1976), which contains Tripticase Peptone (Becton, Dickinson and Company) instead of casein hydrolysate, was used as poor medium. As glucose supplementation can have critical effects on the production of phenols (Smith and Macfarlane 1996), basal medium that did not contain glucose as a carbon source was selected. The substrate solution described above was added to rich medium or poor medium to prepare test medium (final 200 μM each). Bacterial strains were pre-cultured in 4 mL of rich medium, and aliquots (40 μL) were inoculated into 4 mL of test media and incubated statically at 37°C for 6 days. An anaerobic chamber (N_2:CO_2:H_2 = 88:5:7) was used for culture, except in the case of three strains: *Cl. perfringens* YIT 6050^T and *Cl. difficile* YIT 10084^T were cultured under O_2 free N_2 gas, and *Staphylococcus epidermidis* YIT 6049^T was cultured aerobically.

Extraction and preparation of phenols from culture

Phenols were extracted by using a previously reported method, with partial modification (Niwa 1993). The bacterial culture was centrifuged at 20,400 *g* for 5 min at 4°C, and the supernatant was filtered through a 0.20 μm cellulose acetate filter. Filtrates were diluted if necessary, and 225 μL of filtrate was mixed with 0.3 g sodium chloride, 180 μL of 1 N hydrochloride, 45 μL of 200 μM 4-isopropylphenol as an internal control and 450 μL of ethyl acetate, then vigorously vortexed for 30 s. The mixture was centrifuged at 2,350 *g* for 5 min at room temperature. The ethyl acetate layer was filtered by using 0.45 μm PTFE filter vials (Thomson Instrument Company, Oceanside, California, USA), and the filtrate was subjected to HPLC analysis.

HPLC conditions

HPLC analysis was performed under the following conditions: pump: PU-2080 Plus (JASCO Corporation, Tokyo, Japan); column: L-column (Chemicals Evaluation and Research Institute, Tokyo, Japan); detector: FP-2025 Plus (excitation wavelength 260 nm and emission wavelength 305 nm); column temperature: 40°C; mobile phase: 0.1% phosphoric acid: acetonitrile (75:25) mixture; flow rate: 1 mL/min; sample injection volume: 6 μL.

Statistical analysis

Bacterial culture was performed three times independently. Bacterial strains were judged positive on screening if the concentrations of phenols in their cultures were significantly higher than those in uninoculated controls as background levels. Results were analyzed by using Student's *t*-test, and strains were considered positive if the *P*-value was less than 0.05.

Table 1. Bacterial strains used in this study, and culture conditions

No.	Species	Registration No.	Medium for culture
1	*Acidaminococcus fermentans*	YIT 6071^T = ATCC 25085^T	modified GAM + 1% Glucose broth
2	*Acinetobacter baumannii*	YIT 12295^T = JCM 6841^T	Trypticase Soy broth
3	*Akkermansia muciniphila*	YIT 11774^T = ATCC BAA-835^T	modified GAM + 1% Glucose broth
4	*Anaerococcus hydrogenalis*	YIT 12837^T = JCM 7635^T	modified GAM + 1% Glucose broth
5	*Anaerococcus vaginalis*	YIT 11698^T = DSM 7457^T	modified GAM + 1% Glucose broth
6	*Anaerostipes caccae*	YIT 10168^T = DSM 14662^T	modified GAM + 1% Glucose broth
7	*Anaerostipes hadrus*	YIT 10092^T = DSM 3319^T	modified GAM + 1% Glucose broth
8	*Bacteroides caccae*	YIT 10226^T = JCM 9498^T	modified GAM + 1% Glucose broth
9	*Bacteroides dorei*	YIT 12192	modified GAM + 1% Glucose broth
10	*Bacteroides eggerthii*	YIT 10227^T = DSM 20697^T	modified GAM + 1% Glucose broth
11	*Bacteroides fragilis*	YIT 6158^T = ATCC 25285^T	modified GAM + 1% Glucose broth
12	*Bacteroides ovatus*	YIT 6161^T = ATCC 8483^T	modified GAM + 1% Glucose broth
13	*Bacteroides plebeius*	YIT 12661	modified GAM + 1% Glucose broth
14	*Bacteroides stercoris*	ATCC 43183^T	modified GAM + 1% Glucose broth
15	*Bacteroides thethaiotaomicron*	YIT 6163^T = JCM 5827^T	modified GAM + 1% Glucose broth
16	*Bacteroides uniformis*	YIT 6164^T = JCM 5828^T	modified GAM + 1% Glucose broth
17	*Bacteroides vulgatus*	YIT 6159^T = ATCC 8482^T	modified GAM + 1% Glucose broth
18	*Bifidobacterium adolescentis*	YIT 4011^T = ATCC 15703^T	modified PYG broth
19	*Bifidobacterium animalis* subsp. *lactis*	YIT 4121^T = DSM 10140^T	modified PYG broth
20	*Bifidobacterium angulatum*	YIT 4012^T = ATCC 27535^T	modified PYG broth
21	*Bifidobacterium bifidum*	YIT 4039^T = DSM 20456^T	modified PYG broth
22	*Bifidobacterium breve*	YIT 4014^T = ATCC 15700^T	modified PYG broth
23	*Bifidobacterium catenulatum*	YIT 4016^T = ATCC 27539^T	modified PYG broth
24	*Bifidobacterium longum* subsp. *infantis*	YIT 4018^T = ATCC 15697^T	modified PYG broth
25	*Bifidobacterium longum* subsp. *longum*	YIT 4021^T = ATCC 15707^T	modified PYG broth
26	*Bifidobacterium pseudocatenulatum*	YIT 4072^T = JCM 1200^T	modified PYG broth
27	*Blautia coccoides*	YIT 6035^T = JCM 1395^T	modified GAM + 1% Glucose broth
28	*Blautia hansenii*	YIT 12129^T = DSM 20583^T	modified GAM + 1% Glucose broth
29	*Blautia hydrogenotrophica*	YIT 10080^T = DSM 10507^T	modified GAM + 1% Glucose broth
30	*Blautia producta*	YIT 6141^T = JCM 1471^T	modified GAM + 1% Glucose broth
31	*Blautia schinkii*	YIT 6177^T = DSM 10518^T	modified GAM + 1% Glucose broth
32	*Butyrivibrio crossotus*	YIT 10152^T = DSM 2876^T	modified GAM + 1% Glucose broth
33	*Citrobacter freundii*	YIT 6045^T = JCM 1657^T	Trypticase Soy broth
34	*Citrobacter koseri*	YIT 10117^T = JCM 1658^T	Trypticase Soy broth
35	*Clostridium aminophilum*	YIT 6167^T = DSM 10710^T	modified GAM + 1% Glucose broth
36	*Clostridium aminovalericum*	YIT 10174^T = JCM 11016^T	modified GAM + 1% Glucose broth
37	*Clostridium asparagiforme*	YIT 12840^T = DSM 15981^T	modified GAM + 1% Glucose broth
38	*Clostridium bifermentans*	YIT 6053^T = JCM 1386^T	modified GAM + 1% Glucose broth
39	*Clostridium butyricum*	YIT 10073^T = JCM 1391^T	modified GAM + 1% Glucose broth
40	*Clostridium celerecrescens*	YIT 6168^T = DSM 5628^T	modified GAM + 1% Glucose broth
41	*Clostridium clostridioforme*	YIT 6051^T = JCM 1291^T	modified GAM + 1% Glucose broth
42	*Clostridium cochlearium*	YIT 12837^T = JCM 1396^T	modified GAM + 1% Glucose broth
43	*Clostridium cocleatum*	YIT 6036^T = JCM 1397^T	modified GAM + 1% Glucose broth
44	*Clostridium difficile*	YIT 10084^T = JCM 1296^T	modified GAM + 1% Glucose broth
45	*Clostridium ghonii*	YIT 11479^T = JCM 1400^T	modified GAM + 1% Glucose broth
46	*Clostridium glycolicum*	YIT 6058^T = JCM 1401^T	modified GAM + 1% Glucose broth
47	*Clostridium hathewayi*	YIT 12259^T = DSM 13479^T	modified PYG broth
48	*Clostridium hylemonae*	YIT 12258^T = DSM 15053^T	modified PYG broth
49	*Clostridium indolis*	YIT 10077^T = JCM 1380^T	modified GAM +1% Glucose broth
50	*Clostridium innocuum*	YIT 10151^T = DSM 1286^T	modified GAM + 1% Glucose broth
51	*Clostridium leptum*	YIT 6169^T = DSM 753^T	modified GAM + 1% Glucose broth
52	*Clostridium limosum*	YIT 6061^T = JCM 1427^T	modified GAM + 1% Glucose broth
53	*Clostridium malenominatum*	YIT 12839^T = JCM 1405^T	modified GAM + 1% Glucose broth
54	*Clostridium nexile*	YIT 6170^T = ATCC 27757^T	modified GAM + 1% Glucose broth
55	*Clostridium orbiscindens*	YIT 10060^T = DSM 6740^T	modified GAM + 1% Glucose broth
56	*Clostridium oroticum*	YIT 6037^T = JCM 1429^T	modified GAM + 1% Glucose broth
57	*Clostridium paraputrificum*	YIT 10074^T = JCM 1293^T	modified GAM + 1% Glucose broth
58	*Clostridium perfringens*	YIT 6050^T = JCM 1290^T	modified GAM + 1% Glucose broth
59	*Clostridium ramosum*	YIT 10062^T = JCM 1298^T	modified GAM + 1% Glucose broth
60	*Clostridium saccharolyticum*	YIT 12747^T = DSM 2544^T	modified GAM + 1% Glucose broth
61	*Clostridium scindens*	YIT 6171^T = JCM 6567^T	modified GAM + 1% Glucose broth
62	*Clostridium sordellii*	YIT 6065^T = JCM 3814^T	modified GAM + 1% Glucose broth
63	*Clostridium sphenoides*	YIT 6059^T = JCM 1415^T	modified GAM + 1% Glucose broth

Table 1. Continued

No.	Species	Registration No.	Medium for culture
64	*Clostridium spiroforme*	YIT 10342^T = JCM 1432^T	modified GAM + 1% Glucose broth
65	*Clostridium sporogenes*	YIT 6060^T = JCM 1416^T	modified GAM + 1% Glucose broth
66	*Clostridium symbiosum*	YIT 11480^T = JCM 1297^T	modified GAM + 1% Glucose broth
67	*Clostridium tetanomorphum*	YIT 12841^T = DSM 4474^T	modified GAM + 1% Glucose broth
68	*Clostridium xylanovorans*	YIT 12130^T = DSM 12503^T	modified PYG broth
69	*Collinsella aerofaciens*	YIT 10235^T = DSM 3979^T	modified GAM + 1% Glucose broth
70	*Coprococcus eutactus*	YIT 10160^T = ATCC 27759^T	modified GAM + 1% Glucose broth
71	*Cronobacter sakazakii*	YIT 10246^T = JCM 1233^T	Trypticase Soy broth
72	*Dorea formicigenerans*	YIT 10093^T = DSM 3992^T	modified GAM + 1% Glucose broth
73	*Edwardsiella tarda*	YIT 10118^T = JCM 1656^T	Trypticase Soy broth
74	*Eggerthella lenta*	YIT 6077^T = ATCC 25559^T	modified GAM + 1% Glucose broth
75	*Enterobacter aerogenes*	YIT 6042^T = JCM 1235^T	Trypticase Soy broth
76	*Enterobacter cloacae*	YIT 6041^T = JCM 1232^T	Trypticase Soy broth
77	*Enterococcus avium*	YIT 10255^T = JCM 8722^T	MRS broth
78	*Enterococcus durans*	YIT 2036^T = GIFU 9960^T	MRS broth
79	*Enterococcus faecalis*	YIT 2031^T = ATCC 19433^T	MRS broth
80	*Enterococcus faecium*	YIT 2032^T = ATCC 19434^T	MRS broth
81	*Enterococcus gilvus*	YIT 11114^T = DSM 15689^T	MRS broth
82	*Enterococcus hirae*	YIT 2004^T = ATCC 8043^T	MRS broth
83	*Enterococcus malodoratus*	YIT 11175^T = JCM 8730^T	MRS broth
84	*Enterococcus mundtii*	YIT 11176^T = JCM 8731^T	MRS broth
85	*Enterococcus pseudoavium*	YIT 11177^T = JCM 8732^T	MRS broth
86	*Enterococcus raffinosus*	YIT 11178^T = JCM 8733^T	MRS broth
87	*Escherichia coli*	YIT 6044^T = JCM 1649^T	Trypticase Soy broth
88	*Eubacterium biforme*	YIT 6076^T = ATCC 27806^T	modified GAM + 1% Glucose broth
89	*Eubacterium cellulosolvens*	YIT 12261^T = ATCC 43171^T	modified GAM + 1% Glucose broth
90	*Eubacterium cylindroides*	YIT 10236^T = DSM 3983^T	modified GAM + 1% Glucose broth
91	*Eubacterium dolichum*	YIT 10081^T = DSM 3991^T	modified GAM + 1% Glucose broth
92	*Eubacterium eligens*	YIT 10078^T = DSM 3376^T	modified GAM + 1% Glucose broth
93	*Eubacterium hallii*	YIT 10064^T = DSM 3353^T	modified GAM + 1% Glucose broth
94	*Eubacterium rectale*	YIT 6082^T = ATCC 33656^T	modified GAM + 1% Glucose broth
95	*Eubacterium siraeum*	YIT 10049^T = DSM 3996^T	modified GAM + 1% Glucose broth
96	*Eubacterium uniforme*	YIT 12318^T = ATCC 35992^T	modified GAM + 1% Glucose broth
97	*Eubacterium ventriosum*	YIT 10066^T = ATCC 27560^T	modified GAM + 1% Glucose broth
98	*Faecalibacterium prausnitzii*	YIT 10067^T = ATCC 27768^T	modified PYG broth
99	*Fusobacterium necrogenes*	YIT 10362^T = ATCC 25556^T	modified GAM + 1% Glucose broth
100	*Fusobacterium necrophorum* subsp. *necrophorum*	YIT 10343^T = JCM 3718^T	modified GAM + 1% Glucose broth
101	*Fusobacterium nucleatum* subsp. *nucleatum*	YIT 6069^T = JCM 8532^T	modified GAM + 1% Glucose broth
102	*Fusobacterium russii*	YIT 10363^T = ATCC 25533^T	modified GAM + 1% Glucose broth
103	*Fusobacterium varium*	YIT 11855 = JCM 3722	modified GAM + 1% Glucose broth
104	*Hafnia alvei*	YIT 10121^T = JCM 1666^T	Trypticase Soy broth
105	*Holdemania filiformis*	YIT 12717	modified GAM + 1% Glucose broth
106	*Klebsiella oxytoca*	YIT 10122^T = JCM 1665^T	Trypticase Soy broth
107	*Klebsiella pneumoniae*	YIT 6046^T = JCM 1662^T	Trypticase Soy broth
108	*Lactobacillus acidophilus*	YIT 0070^T = ATCC 4356^T	MRS broth
109	*Lactobacillus brevis*	YIT 0076^T = ATCC 14869^T	MRS broth
110	*Lactobacillus casei*	YIT 0180^T = ATCC 334^T	MRS broth
111	*Lactobacillus fermentum*	YIT 0081^T = ATCC 14931^T	MRS broth
112	*Lactobacillus fructivorans*	YIT 0235^T = JCM 1117^T	MRS broth
113	*Lactobacillus gasseri*	YIT 0192^T = DSM 20243^T	MRS broth
114	*Lactobacillus plantarum*	YIT 0102^T = ATCC 14917^T	MRS broth
115	*Lactobacillus reuteri*	YIT 0197^T = JCM 1112^T	MRS broth
116	*Lactobacillus ruminis*	YIT 0221^T = JCM 1152^T	MRS broth
117	*Lactobacillus sakei* subsp. *sakei*	YIT 0247^T = JCM 1157^T	MRS broth
118	*Lactococcus garvieae*	YIT 2071^T = NCFB 2155^T	MRS broth
119	*Lactococcus lactis* subsp. *lactis*	YIT 2008^T = ATCC 19435^T	MRS broth
120	*Lactococcus plantarum*	YIT 2061^T = ATCC 43199^T	MRS broth
121	*Lactococcus raffinolactis*	YIT 2062^T = ATCC 43920^T	MRS broth
122	*Megasphaera elsdenii*	YIT 6063^T = JCM 1772^T	modified GAM + 1%Glucose broth
123	*Morganella morganii*	YIT 10124^T = JCM 1672^T	Trypticase Soy broth
124	*Olsenella uli*	YIT 12014^T = JCM 12494^T	modified GAM + 1%Glucose broth
125	*Parabacteroides distasonis*	YIT 6162^T = JCM 5825^T	modified GAM + 1%Glucose broth
126	*Parabacteroides johnsonii*	YIT 12680	modified GAM + 1%Glucose broth

Table 1. Continued

No.	Species	Registration No.	Medium for culture
127	*Parabacteroides merdae*	ATCC 43184^T	modified GAM + 1%Glucose broth
128	*Peptoniphilus asaccharolyticus*	YIT 10026^T = GIFU 7656^T	modified GAM + 1%Glucose broth
129	*Porphyromonas gingivalis*	YIT 12766^T = JCM 12257^T	modified GAM + 1%Glucose broth
130	*Prevotella denticola*	YIT 6131 = JCM 8528	modified GAM + 1%Glucose broth
131	*Prevotella intermedia*	YIT 12886^T = JCM 11150^T	modified GAM + 1%Glucose broth
132	*Prevotella melaninogenica*	YIT 6039^T = ATCC 25845^T	modified GAM + 1%Glucose broth
133	*Prevotella oris*	YIT 6134^T = JCM 8540^T	modified GAM + 1%Glucose broth
134	*Proteus mirabilis*	YIT 6047^T = JCM 1669^T	Trypticase Soy broth
135	*Proteus penneri*	YIT 10252^T = JCM 3948^T	Trypticase Soy broth
136	*Proteus vulgaris*	YIT 10335^T = DSM 13387^T	Trypticase Soy broth
137	*Providencia alcalifaciens*	YIT 10128^T = JCM 1673^T	Trypticase Soy broth
138	*Providencia rettgerii*	YIT 10108^T = DSM 4542^T	Trypticase Soy broth
139	*Pseudomonas aeruginosa*	YIT 6108^T = IFO 12689^T	Trypticase Soy broth
140	*Romboutsia lituseburensis*	YIT 10059^T = JCM 1404^T	modified GAM + 1% Glucose broth
141	*Roseburia faecis*	YIT 11921^T = DSM 16840^T	modified GAM + 1% Glucose broth
142	*Roseburia hominis*	YIT 11920^T = DSM 16839^T	modified GAM + 1% Glucose broth
143	*Roseburia intestinalis*	YIT 10172^T = DSM 14610^T	modified GAM + 1% Glucose broth
144	*Ruminococcus bromii*	YIT 6078^T = ATCC 27255^T	modified GAM + 1% Glucose broth
145	*Ruminococcus gnavus*	YIT 6176^T = ATCC 29149^T	modified GAM + 1% Glucose broth
146	*Ruminococcus lactaris*	YIT 10225^T = ATCC 29176^T	modified GAM + 1% Glucose broth
147	*Ruminococcus obeum*	YIT 6085^T = ATCC 29174^T	modified GAM + 1% Glucose broth
148	*Ruminococcus torques*	YIT 10159^T = ATCC 27756^T	modified GAM + 1% Glucose broth
149	*Staphylococcus epidermidis*	YIT 6049^T = ATCC 14990^T	Trypticase Soy broth
150	*Streptococcus mitis*	YIT 2069^T = GIFU 12458^T	MRS broth
151	*Streptococcus salivarius*	YIT 10260^T = JCM 5707^T	MRS broth
152	*Streptococcus thermophilus*	YIT 2037^T = ATCC 19258^T	MRS broth
153	*Veillonella parvula*	YIT 6072^T = GIFU 7884^T	modified GAM + 1% Glucose broth

Phylogenetic analysis

Sequences of the 16S rRNA genes of bacterial strains identical to, or the same species as, the strains used in this study were collected from the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) or GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Sequences were aligned by using Clustal X 2.1 (Larkin *et al.* 2007) and analyzed by using the Neighbor Joining method (Saitou and Nei 1987). The phylogenetic tree was visualized by using the TreeView 32 program (ver.1.6.6) (Page 1996). The 16S rRNA sequence of *Desulfovibrio desulfuricans* ATCC 29577^T was used as an outgroup.

Search for homologous protein

Files on the proteins that phenol- or *p*-cresol-producing bacteria were expected to have were obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/); the accession numbers of the derived genomes are listed in Tables 2 and 3. The amino acid sequences of tyrosine phenol-lyase (TPL) from *Citrobacter freundii* MT-10419 (Iwamori *et al.* 1991), TyrB (tyrosine aminotransferase) from *Escherichia coli* K-12 substr. MG1655 (Accession No. NP_418478), and ThiH (tyrosine lyase) from *E. coli* K-12 (Accession No. NP_418417) were used as queries. Homology searches between queries and obtained protein lists were performed by using GENETYX ver.11. Searches for proteins homologous to KpdB, KpdC and KpdD (*Klebsiella pneumoniae* decarboxylase) from *K. pneumoniae* NCTC 418 (Accession Nos. AAY57854, AAY57855 and AAY57856, respectively); HpdA, HpdB and HpdC (*p*-hydroxyphenylacetate decarboxylase) from *Cl. difficile* DSM 1296^T (Accession Nos. AJ543427, AJ543425 and AJ543426, respectively); FldH (phenyllactate dehydrogenase); FldBC (phenyllactate dehydratase); AcdA (acyl-CoA dehydrogenase) and PorA (pyruvate:ferredoxin oxidoreductase A) were performed by using MultiGeneBlast (Medema, Takano and Breitling 2013) with the default parameters. Amino acid sequences encoded by gene clusters consisting of *fldL*, *fldA*, *fldI*, *fldB*, *fldC*, *acdA*, *etfB*, *etfA*, *permease* and *fldH* from *Cl. sporogenes* ATCC 15579^T (Accession Nos. EDU39251 to 39261) were used as queries to identify homologs of FldH, FldBC and AcdA. Similarly, amino acid sequences encoded by *porA* from *Cl. sporogenes* ATCC 15579^T (Accession Nos. EDU39094 to 39096) were used to search for homologous proteins.

RESULTS

Evaluation of phenol-producing ability

We determined the phenol concentrations in cultures of the 153 strains. The cultures of 36 strains had higher phenol concentrations than the background level (Fig. 1A). Of these 36 strains, 16 (*Cl. malenominatum* YIT 12839^T, *Cl. tetanomorphum* YIT 12841^T, *Fusobacterium varium* YIT 11855, *Morganella morganii* YIT 10124^T, *Cl. cochlearium* YIT 12837^T, *Cl. saccharolyticum* YIT 12747^T, *Citrobacter koseri* YIT 10117^T, *K. pneumoniae* YIT 6046^T, *Olsenella uli* YIT 12014^T, *Enterobacter aerogenes* YIT 6042^T, *Citrobacter freundii* YIT 6045^T, *Cronobacter sakazakii* YIT 10246^T, *K. oxytoca* YIT 10122^T, *En. cloacae* YIT 6041^T, *F. necrophorum* subsp. *necrophorum* YIT 10343^T and *F. russii* YIT 10363^T) exhibited phenol production at 100 μM or more in their cultures (Fig. 1C). They were calculated to convert at least half of the supplemented substrates, even if only one of the substrates were metabolized. The remaining 20 strains produced less than 100 μM of phenol in their cultures (Fig. 2A, blue).

We then determined the *p*-cresol concentrations in the cultures of the 153 strains. The *p*-cresol concentrations in the

Table 2. Predicted proteins homologous to enzymes involved in metabolic pathways from tyrosine to phenol

			% of identity / E-value				
Species	Strains	Genome (Accession No.)	TPL[a]	TyrB[b]	HadB[c]	HadC[d]	HadD[e]
Citrobacter freundii	YIT 6045^T	NZ_JMTA00000000	99/0.0	90/0.0	83/6e^{-98}	97/0.0	87/3e^{-37}
Clostridium saccharolyticum	YIT 12747^T	NC_014376	70/0.0	–	–	–	–
Cronobacter sakazakii	YIT 10246^T	NZ_CP011047	–	85/0.0	82/3e^{-91}	93/0.0	88/7e^{-38}
Enterobacter aerogenes	YIT 6042^T	NC_015663	–	88/0.0	92/2e^{-106}	98/0.0	92/7e^{-40}
Enterobacter cloacae	YIT 6041^T	NC_014121	–	87/0.0	90/4e^{-103}	96/0.0	94/6e^{-38}
Fusobacterium necrophorum	YIT 10343^T	NZ_FMXX00000000	76/0.0	–	–	–	–
Fusobacterium russii	YIT 10363^T	NZ_ARMK00000000	82/0.0	–	–	–	–
Klebsiella pneumoniae	YIT 6046^T	NZ_AJJI00000000	–	84/0.0	100/2e^{-114}	100/0.0	100/5e^{-42}
Morganella morganii	YIT 10124^T	NZ_BCZU00000000	90/0.0	66/0.0	–	–	–
Olsenella uli	YIT 12014^T	NC_014363	–	–	48/6e^{-127}	40/9e^{-12}	48/1e^{-48}

[a] Iwamori *et al.* 1991
[b] Accession No. NP_418478
[c] Accession No. AAY57854
[d] Accession No. AAY57855
[e] Accession No. AAY57856

Table 3. Predicted proteins homologous to enzymes involved in metabolic pathways from tyrosine to *p*-cresol

			% of identity / E-value				
Species	Strains	Genome (Accession No.)	ThiH[a]	TyrB[b]	HpdA[c]	HpdB[d]	HpdC[e]
Blautia hydrogenotrophica	YIT 10080^T	NZ_ACBZ00000000	–	–	57/2e^{-108}	55/0.0	42/5e^{-17}
Clostridium difficile	YIT 10084^T	NZ_AUOX00000000	36/8e^{-85}	–	99/0.0	100/0.0	100/9e^{-47}
Olsenella uli	YIT 12014^T	NC_014363	–	–	56/6e^{-109}	55/0.0	34/4e^{-8}
Romboutsia lituseburensis	YIT 10059^T	NZ_FNGW00000000	35/2e^{-82}	–	68/9e^{-133}	76/0.0	59/2e^{-28}

[a] Accession No. NP 418417
[b] Accession No. NP_418478
[c] Accession No. AJ543427
[d] Accession No. AJ543425
[e] Accession No. AJ543426

cultures of 55 strains were higher than the background level (Fig. 1B). *Blautia hydrogenotrophica* YIT 10080^T, *Cl. difficile* YIT 10084^T, *O. uli* YIT 12014^T and *Romboutsia lituseburensis* YIT 10059^T produced at least 100 μM of *p*-cresol (Fig. 1D). These four strains had markedly higher *p*-cresol production than the other 51, which produced less than 10 μM (Fig. 2B, blue).

Fourteen strains produced both phenol and *p*-cresol (*Anaerostipes hadrus* YIT 10092^T, *Bacteroides caccae* YIT 10226^T, *B. ovatus* YIT 6161^T, *B. vulgatus* YIT 6159^T, *Cl. celerecrescens* YIT 6168^T, *Cl. clostridioforme* YIT 6051^T, *Cl. cochlearium* YIT 12837^T, *Cl. indolis* YIT 10077^T, *Cl. innocuum* YIT 10151^T, *Cl. saccharolyticum* YIT 12747^T, *Cl. sphenoides* YIT 6059^T, *F. varium* YIT 11855, *O. uli* YIT 12014^T and *Veillonella parvula* YIT 6072^T). Of these strains, only *O. uli* YIT 12014^T produced both products at more than 100 μM; the others produced phenol or *p*-cresol, or both, at less than 10 μM.

Phylogenetic analysis of phenol-producing strains

All strains used in the screening were phylogenetically analyzed on the basis of the DNA sequences of the 16S rRNA gene. Phylogenetic tree analysis indicated that the phenol-producing strains were widely distributed in the *Enterobacteriaceae*, *Coriobacteriaceae*, *Bacteroidaceae*, *Prevotellaceae*, *Porphyromonadaceae*, *Fusobacteriaceae*, *Enterococcaceae* and *Lactobacillaceae*, as well as *Clostridium* clusters XVIII, XVI, IX, I and XIVa (Fig. 2A). The 16 strains that produced high levels of phenol (100 μM or more) belonged to specific families, namely the *Coriobacteriaceae*, *Enterobacteriaceae* and *Fusobacteriaceae*, along with *Clostridium* clusters I and XIVa. *p*-cresol-producing strains were dispersed across the *Bifidobacteriaceae*, *Coriobacteriaceae*, *Bacteroidaceae*, *Fusobacteriaceae* and *Lactobacillaceae*, along with *Clostridium* clusters XVI, IX, IV, I, XI, XIII and XIVa (Fig. 2B). Among them, four high *p*-cresol producers (100 μM or more) belonged to the specific family *Coriobacteriaceae*, or to *Clostridium* clusters XI and XIVa. The 14 strains that produced both phenol and *p*-cresol fell into the *Fusobacteriaceae*, *Coriobacteriaceae* or *Bacteroidaceae*, or *Clostridium* clusters XVI, IX, I and XIVa (Fig. 2). *O. uli* YIT 12014^T, which had strong ability to produce phenol and *p*-cresol, belonged to the *Coriobacteriaceae*.

Prediction of metabolic pathways in phenol-producing strains

Three enzymes are involved in the initial or final steps of metabolic pathways from tyrosine to phenol: TPL, which metabolizes tyrosine to phenol in one step; TyrB, which metabolizes tyrosine to 4-hydroxyphenylpyruvate; and Had (hydroxyarylic acid decarboxylase), which metabolizes 4-hydroxybenzoate to phenol (Fig. 3A and B). Their activities were examined by using TPL from *C. freundii* MT-10419 (Iwamori *et al.* 1991), TyrB from

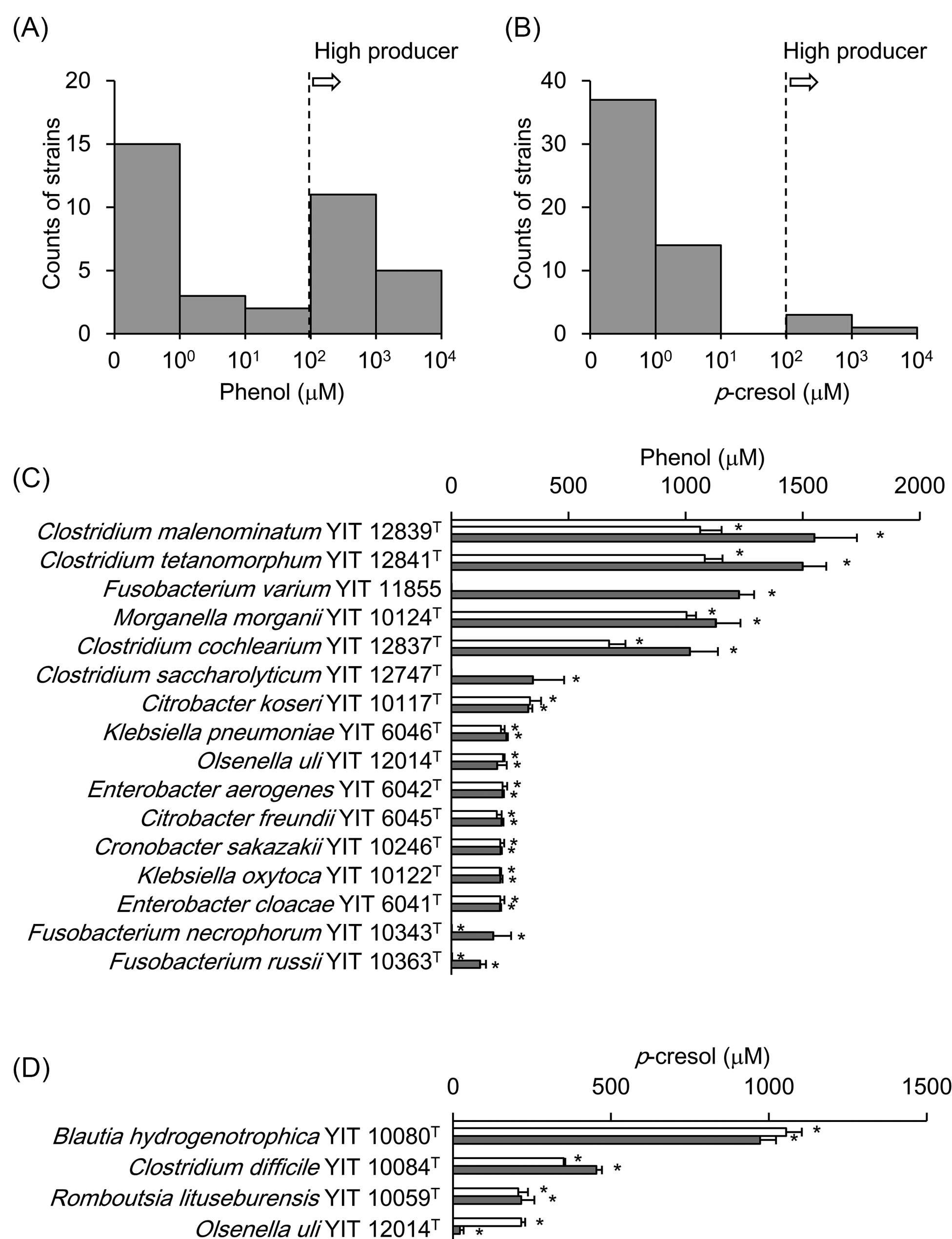

Figure 1. Evaluation of phenol and *p*-cresol production ability in 153 screened strains
One-hundred fifty three strains were cultured in rich or poor medium for 6 days. The counts of **(A)** phenol-positive strains and **(B)** *p*-cresol-positive strains are shown as histograms. The concentrations of **(C)** phenol and **(D)** *p*-cresol produced in culture by high producers are shown. White bars indicate results using rich-medium; gray bars indicate those using basal medium. Error bars indicate standard deviations. Asterisks represent $P < 0.05$ as analyzed by Student's *t*-test (increased compared with uncultured control medium).

E. coli strain K-12 (Kuramitsu *et al.* 1985), and Had from *K. pneumoniae* NCTC 418 (Lupa 2005), respectively. We then analyzed 10 strains with high phenol-producing ability, namely *C. freundii* YIT6045ᵀ, *Cl. saccharolyticum* YIT 12747ᵀ, *C. sakazakii* YIT 10246ᵀ, *En. aerogenes* YIT 6042ᵀ, *En. cloacae* YIT 6041ᵀ, *F. necrophorum* subsp. *necrophorum* YIT 10343ᵀ, *F. russii* YIT 10363ᵀ, *K. pneumoniae* YIT 6046ᵀ, *M. morganii* YIT 10124ᵀ and *O. uli* YIT 12014ᵀ, the draft genomes of which had already been sequenced, to determine whether homologous proteins of TPL, TyrB or Had were encoded in their genomes. A search for homologs of TPL derived from *C. freundii* MT-10419 revealed that homologs were encoded in the genomes of *C. freundii* YIT 6045ᵀ (99% identity of amino acid sequences), *Cl. saccharolyticum* YIT 12747ᵀ(70%), *F. necrophorum* subsp. *necrophorum* YIT 10343ᵀ(76%), *F. russii* YIT 10363ᵀ(82%) and *M. morganii* YIT 10124ᵀ (90%) (Table 2). Similarly, we found that homologs of TyrB from *E. coli* strain K-12 were encoded in the genomes of *C. freundii* YIT 6045ᵀ (90% identity of amino acid sequences), *C. sakazakii* YIT 10246ᵀ (85%), *En. aerogenes* YIT 6042ᵀ(88%), *En. cloacae* YIT 6041ᵀ (87%), *K. pneumoniae* YIT 6046ᵀ (84%) and *M. morganii* YIT 10124ᵀ (66%) (Table 2). Had activity depended on three clusters encoded in the *hadBCD* operon and a cell lysate of *E. coli* transformed with *kpdBCD*; the *hadBCD* operon derived from *K. pneumoniae* NCTC 418 can metabolize 4-hydroxybenzoate to phenol (Lupa 2005). Thus, homologs

Figure 2. Phylogenetic analysis of phenol or *p*-cresol producing bacteria
DNA sequences of 16S rRNA from 153 strains were subjected to phylogenetic analysis using Clustal X 2.1 and phylogenetic trees were constructed. **(A)** Phenol- or **(B)***p*-cresol-producing strains are colored red (strains that produced at least 100 μM product) or blue (strains that produced less than 100 μM product). Strains in black font are phenol non-producers. Cluster no. represents the *Clostridium* 16S rRNA phylogenic cluster number (Collins *et al.*1994). Accession numbers used for analysis are displayed according to the name of each species, respectively.

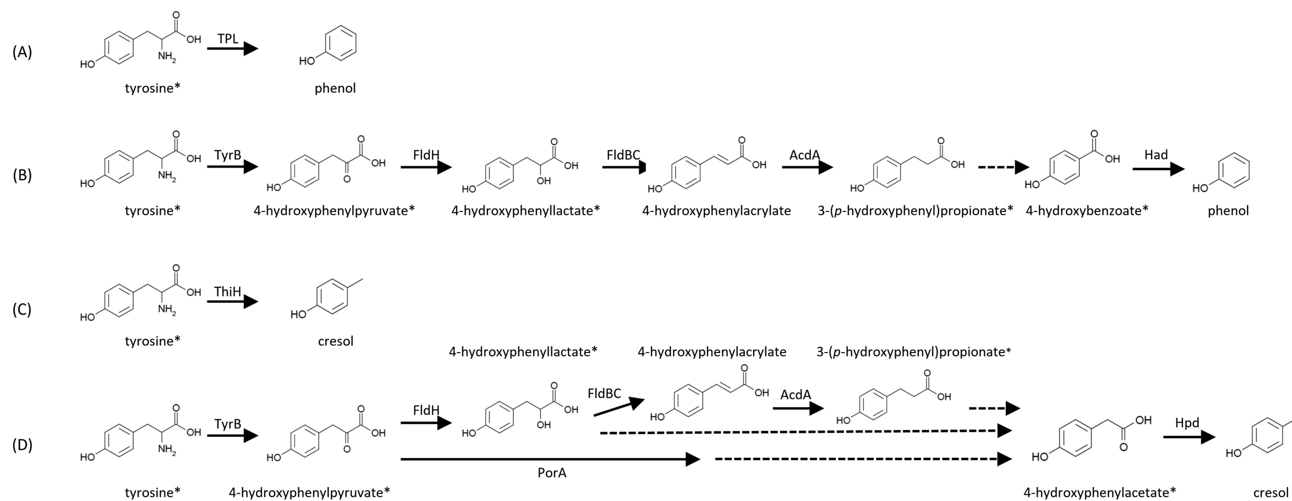

Figure 3. Metabolic pathways from tyrosine to phenol and *p*-cresol
Metabolic pathways from tyrosine to phenol **(A, B)** and *p*-cresol **(C, D)** are shown as indicated by previous reports (Enei *et al.*1973; Gelfand and Steinberg 1977; Kriek *et al.*2007; Windey, De Preter and Verbeke 2012; Dodd *et al.*2017). Known enzymes—tyrosine phenol-lyase (TPL), tyrosine aminotransferase B (TyrB), phenyllactate dehydrogenase (FldH), phenyllactate dehydratase (FldBC), acyl-CoA dehydrogenase (AcdA), hydroxyarylic acid decarboxylase (Had), tyrosine lyase (ThiH), pyruvate:ferredoxin oxidoreductase A (PorA) and hydroxyphenylacetate decarboxylase (Hpd)—are shown near the arrows for each step. Steps with unidentified enzymes are indicated by dotted lines. Compounds used in this study are marked with asterisks.

of KpdBCD were found to be encoded in the genome of *C. freundii* YIT 6045^T, *C. sakazakii* YIT 10246^T, *En. aerogenes* YIT 6042^T, *En. cloacae* YIT 6041^T and *K. pneumoniae* YIT 6046^T with more than 80% identity of amino acid sequences; in the case of *O. uli* YIT 12014^T there was 40% to 48% identity (Table 2). The three homologs were encoded on these genomes in the order of *HadB*, *HadC* and *HadD*, except in the case of *O. uli* YIT 12014^T, the three homologs of which were encoded on the genome in the order of *hadC*, *hadD* and *hadB*; the ORF encoding cation transporter was inserted between *hadD* and *hadB* (Fig. S1A, Supporting Information). FldBC homologs and AcdA homologs were not detected in the genomes of these six *hadBCD*-operon-positive strains (data not shown).

Prediction of metabolic pathways in *p*-cresol-producing bacteria

TyrB and Hpd, which metabolize 4-hydroxyphenylacetate to *p*-cresol, and ThiH, which metabolizes tyrosine to *p*-cresol in one step, are metabolic enzymes that act in metabolic pathways from tyrosine to *p*-cresol (Fig. 3C and D). We therefore examined whether TyrB, Hpd or ThiH homologous proteins were found in all four strains (*B. hydrogenotrophica* YIT 10080^T, *Cl. difficile* YIT 10084^T, *O. uli* YIT 12014^T and *R. lituseburensis* YIT 10059^T) with high *p*-cresol-producing ability. We used information already reported on their draft genome sequences. No proteins with more than 30% amino acid sequence identity to TyrB of *E. coli* strain K-12 were found. In *Cl. difficile* DSM 1296^T, three enzymes—HpdA, an activating enzyme; HpdC, a large subunit; and HpdC, a small subunit—are responsible for Hpd activity and are encoded in the *hpdBCA* operon (Andrei *et al.* 2004). Homologs of HpdBCA were identified in all four strains, with more than 30% identity of amino acid sequences (Table 3). In all four strains, the three homologs were encoded in a line in the order of *hpdB*, *hpdC* and *hpdA* (Fig. S1B, Supporting Information). ThiH from *E. coli* strain K-12 metabolizes tyrosine to dehydroglycine as the first step of the thiamine synthesis pathway, and *p*-cresol is formed as a by-product of this step (Kriek *et al.* 2007). We then found ThiH homologs encoded by the genome of *Cl. difficile* YIT 10084^T (36% amino acid sequence identity) and *R. lituseburensis* YIT 10059^T (35%) (Table 3). Analysis of homologs of other enzymes involved revealed that all four strains harbored FldH or PorA or both (data not shown). FldBC homologs were identified in *B. hydrogenotrophica* YIT 10080^T and *O. uli* YIT 12014^T. No AcdA homologs were identified in any strain (data not shown).

DISCUSSION

Screening conditions

To identify phenol- and *p*-cresol-producing bacteria, we used two major strategies. First, we supplemented the culture media with metabolic intermediates. Some of the supplemented intermediates—for example, 4-hydroxyphenyllactate and 4-hydroxyphenylacetate—are formed by intestinal bacteria *in vitro* (Smith and Macfarlane 1996; Beloborodova *et al.* 2012), suggesting that phenol- and *p*-cresol-producing bacteria further metabolize these intermediates in the intestinal environment. *Cl. difficile* YIT 10084^T and *O. uli* YIT 12014^T, which lacked a gene encoding TyrB in their genomes, might produce phenols from 4-hydroxyphenylpyruvate, 4-hydroxyphenyllactate, 3-(*p*-hydroxyphenyl)propionate, 4-hydroxybenzoate or 4-hydroxyphenylacetate as initial substrates in the intestine (Fig. 3B and D). Considering the complicated nature of the intestinal ecosystem, adding predicted metabolic intermediates to the culture media for screening was an effective strategy.

Second, we considered that other factors in the media might have affected phenol-production ability. Enei *et al.* (1973) reported that the presence of glucose in culture media suppressed TPL production in *Erwinia herbicola* ATCC 21434. Indeed, some TPL-positive strains, as represented by *Cl. saccharolyticum* YIT 12747^T and *F. russii* YIT 10363^T, produced much more phenol in glucose-limited media (poor media) than glucose-supplemented media (rich media) (Fig. 1C). On the other hand, glucose-limited media might be disadvantageous to growth. For example, *O. uli* YIT 12014^T produced less *p*-cresol in poor medium than in rich medium (Fig. 1D), possibility because of this growth limitation. Thus it is a reasonable strategy to use both rich and poor media supplemented with tyrosine metabolites.

Identification of strains producing phenols

This study newly found 29 strains with phenol-producing potential and 51 with *p*-cresol-producing potential. Of the 36 phenol-positive strains, three—*Cl. malenominatum* YIT 12839^T, *Cl. tetanomorphum* YIT 12841^T and *Cl. cochlearium* YIT 12837^T—have already been reported to produce phenol (Elsden, Hilton and Waller 1976). Moreover, *K. pneumoniae* YIT 6046^T, *En. cloacae* YIT 6041^T and *M. morganii* YIT 10124^T are known as phenol-producing bacteria at the species level (Patel and Grant 1969; Valkova *et al.* 2001; Matsui *et al.* 2006; Iizuka *et al.* 2009b). The phenol-producing ability of *C. freundii* YIT 6045^T had not been reported but had been surmised, because the phenol-forming activity of the purified TPL gene product from *C. freundii* species has been well characterized (Chandel and Azmi 2013). To our knowledge, the remaining 29 strains were identified here for first time as phenol producing. Among the 55 *p*-cresol-producing strains identified in this study, *B. longum* subsp. *infantis* YIT 4018^T, *Cl. difficile* YIT 10084^T, *Cl. paraputrificum* YIT 10074^T and *F. necrogenes* YIT 10362^T have already been examined for their ability to produce *p*-cresol (Bone, Tamm and Hill 1976; Elsden, Hilton and Waller 1976; Smith and Macfarlane 1996). Here, we identified, for the first time, the remaining 51 strains as *p*-cresol-producing bacteria.

An abundance of strong producers of phenols in the intestine could affect the host's health. The 16 phenol producers with high activity belonged to the *Fusobacteriaceae*, *Enterobacteriaceae* or *Coriobacteriaceae*, or to *Clostridium* clusters I and XIVa, and the four *p*-cresol producers with high activity belonged to the *Coriobacteriaceae* or to *Clostridium* cluster XI or XIVa. Kaur, Das and Mande (2017) have reported a relationship between the abundance of specific bacterial groups or specific putrefaction pathways in the intestine and the host's stage of colorectal cancer. The information from our study could be a new clue to understanding diseases associated with phenols (Boutwell and Bosch 1959; Iizuka *et al.* 2009a; b, Windey, De Preter and Verbeke 2012; Ito and Yoshida 2014; Shiba *et al.* 2014; Andriamihaja *et al.* 2015; Verbeke *et al.* 2015). For this purpose, we need to examine whether fecal concentrations of phenols are related to the intestinal counts of phenol- and *p*-cresol-producing clusters. Furthermore, clinical studies are needed to investigate whether the occurrence of diseases associated with phenols is relevant to the abundance of intestinal producers of phenols.

Metabolic pathways from tyrosine to phenols

The metabolic pathways by which bacteria produce phenols are linked to the possession of pathway-related metabolic enzymes. In the genomes of 10 of the strong phenol producers analyzed here (Table 2; genome information for the remaining six was not available in the public database), homologs of TPL or Had were encoded, suggesting that each strain used pathways relevant to the enzymes they possessed (Fig. 3A and B). *Cl. saccharolyticum* YIT 12747^T, *F. necrophorum* subsp. necrophorum YIT 10343^T, *F. russii* YIT 10363^T, and *M. morganii* YIT 10124^T used TPL-dependent pathways; *C. sakazakii* YIT 10246^T, *En. aerogenes* YIT 6042^T, *En. cloacae* YIT 6041^T, *K. pneumoniae* YIT 6046^T and *O. uli* YIT 12014^T used Had-dependent pathways, and *C. freundii* YIT 6045^T used both TPL- and Had-dependent pathways. None of the Had-positive strains harbored FldBC homologs, indicating that these strains could use 3-(*p*-hydroxyphenyl)propionate or 4-hydroxybenzoate as initial metabolic substrates. More detailed analysis is needed to clarify the enzymes involved in the unknown parts of the Had-dependent pathways (Fig. 3B).

All four strong *p*-cresol-producing bacteria are predicted to harbor homologs of ThiH or Hpd that are involved in the final steps of *p*-cresol production. (Fig. 3C and D). This result suggests that ThiH or Hpd, or both, are key enzymes in producing *p*-cresol in these strains. We can predict from the genomic analysis that *B. hydrogenotrophica* YIT 10080^T and *O. uli* YIT 12014^T could utilize Hpd-dependent pathways, whereas *Cl. difficile* YIT 10084^T and *R. lituseburensis* YIT 10059^T could use both Hpd- and ThiH-dependent pathways. The lack of TyrB homologs and the presence of Hpd homologs in the four abovementioned strains suggest that these strains utilize tyrosine metabolites such as 4-hydroxyphenylpyruvate, 4-hydroxyphenyllactate, 3-(*p*-hydroxyphenyl)propionate or 4-hydroxyphenylacetate as initial substrates (Fig. 3D). This information could be a clue to identifying the metabolic scheme of *p*-cresol formation.

Revealing overall metabolic pathways is important for understanding intestinal microbial ecology. Draft genome sequencing of six strains not analyzed in this study (*Cl. malenominatum* YIT 12839^T, *Cl. tetanomorphum* YIT 12841^T, *F. varium* YIT 11855, *Cl. cochlearium* YIT 12837^T, *C. koseri* YIT 10117^T and *K. oxytoca* YIT 10122^T) is needed. We also need to identify the currently unknown enzymes involved in the metabolism of phenols.

Limitations of this study

This screening took into account the intestinal environment, but there were three major limitations. First, the number of strains examined was limited from the perspective of the diversity of intestinal bacteria. Second, because the ability to produce phenols was evaluated in only one representative strain of each species, we did not consider variations in the ability to produce phenols among strains within a species. Third, the results of this *in vitro* screening might not always reflect the ability to produce phenols in the intestinal environment. Despite these limitations, this study was meaningful in that we were able to relate producers of phenols to clusters by phylogenetic analysis. This should give new insights into production of phenols in the intestine from the perspective of molecular genetics.

CONCLUSION AND FUTURE PERSPECTIVES

We identified 36 phenol-producing bacteria and 55 *p*-cresol-producing bacteria. Strong phenol producers belonged to the *Coriobacteriaceae*, *Enterobacteriaceae*, *Fusobacteriaceae* and *Clostridium* clusters I and XIVa, and strong *p*-cresol producers belonged to the *Coriobacteriaceae* and *Clostridium* clusters XI and XIVa. Such information on phenol- and *p*-cresol-producing bacteria should help identify the relationships between microbiota and host disease, as well as the underlying mechanisms.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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