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. 2012).
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 (Windel, 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 (Andramihaja 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 (izuka 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, Tam 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-hydroxyphenylactic 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 \( \mu \)m 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 tryptase peptone, 10.0 g yeast extract, 0.5 g L-cysteine HCl \( \cdot \) H\( \text{2} \)O, 4.0 g Na\( \text{2} \)CO\( \text{3} \), 7 mL 0.07% hemin solution, 1.0 mL 0.1% resazurin solution, 0.04 g K\( \text{2} \)HPO\( \text{4} \), 0.04 g KH\( \text{2} \)PO\( \text{4} \), 0.4 g NaHCO\( \text{3} \), 0.08 g NaCl, 8 mg CaCl\( \text{2} \), 19 mg MgSO\( \text{4} \) \( \cdot \) 7H\( \text{2} \)O and 1 mg vitamin K\( \text{3} \); pH 6.9. Basal Medium (Bone, Tamm and Hill 1976), which contains Trypticase 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 \( \mu \)M each). Bacterial strains were pre-cultured in 4 \( \mu \)L of rich medium, and aliquots (40 \( \mu \)L) were inoculated into 4 \( \mu \)L of test media and incubated statically at 37 \( ^\circ \)C for 6 days. An anaerobic chamber (N\( \text{2} \):CO\( \text{2} \):H\( \text{2} \) \( \text{2} \)8:5:7) was used for culture, except in the case of three strains: Cl. perfringens YIT 6050\( \text{a} \) and Cl. difficile YIT 10084\( \text{a} \) were cultured under \( \text{O} \)\( \text{2} \) free \( \text{N} \)\( \text{2} \) gas, and Staphylococcus epidermidis YIT 6049\( \text{a} \) 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 \( \text{g} \) for 5 min at 4 \( ^\circ \)C, and the supernatant was filtered through a 0.20 \( \mu \)m cellulose acetate acetate filter. Filters were diluted if necessary, and 225 \( \mu \)L of filtrate was mixed with 0.3 g sodium chloride, 180 \( \mu \)L of 1 N hydrochloric acid, 45 \( \mu \)L of 200 \( \mu \)M 4-isopropylphenol as an internal control and 450 \( \mu \)L of ethyl acetate, then vigorously vortexed for 30 s. The mixture was centrifuged at 2,350 \( \text{g} \) for 5 min at room temperature. The ethyl acetate layer was filtered by using 0.45 \( \mu \)m PTFE filter fiers (Uston 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 \( ^\circ \)C; mobile phase: 0.1% phosphoric acid: acetonitrile (75:25) mixture; flow rate: 1 mL/min; sample injection volume: 6 \( \mu \)L.

**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.
| No. | Species                                      | Registration No.          | Medium for culture                  |
|-----|---------------------------------------------|---------------------------|-------------------------------------|
| 1   | Acidaminococcus fermentans                 | YIT 6071T = ATCC 25085T   | modified GAM + 1% Glucose broth     |
| 2   | Acinetobacter baumannii                    | YIT 12295T = JCM 6841T   | Trypticase Soy broth                |
| 3   | Akkermansia muciniphila                    | YIT 11774T = ATCC BAA-835T | modified GAM + 1% Glucose broth     |
| 4   | Anaerococcus hydrogenesii                 | YIT 12837T = JCM 7635T   | modified GAM + 1% Glucose broth     |
| 5   | Anaerococcus vaginalis                     | YIT 11698T = DSM 7457T   | modified GAM + 1% Glucose broth     |
| 6   | Anaerostipes caccae                       | YIT 10168T = DSM 14662T  | modified GAM + 1% Glucose broth     |
| 7   | Anaerostipes hadras                        | YIT 10092T = DSM 3319T   | modified GAM + 1% Glucose broth     |
| 8   | Bacteroides caccae                        | YIT 10226T = JCM 9498T   | modified GAM + 1% Glucose broth     |
| 9   | Bacteroides dorei                          | YIT 12192                | modified GAM + 1% Glucose broth     |
| 10  | Bacteroides egerthii                       | YIT 11027T = DSM 20697T  | modified GAM + 1% Glucose broth     |
| 11  | Bacteroides fragilis                      | YIT 6158T = ATCC 25285T  | modified GAM + 1% Glucose broth     |
| 12  | Bacteroides ovatus                        | YIT 6161T = ATCC 8483T   | modified GAM + 1% Glucose broth     |
| 13  | Bacteroides plebeius                      | YIT 12661                | modified GAM + 1% Glucose broth     |
| 14  | Bacteroides stercoris                     | ATCC 43183T              | modified GAM + 1% Glucose broth     |
| 15  | Bacteroides thethaoaoticomicrobium        | YIT 6163T = JCM 5827T    | modified GAM + 1% Glucose broth     |
| 16  | Bacteroides uniformis                     | YIT 6164T = JCM 5828T    | modified GAM + 1% Glucose broth     |
| 17  | Bacteroides vulgatus                       | YIT 6159T = ATCC 8482T   | modified GAM + 1% Glucose broth     |
| 18  | Bifidobacterium adolescentis               | YIT 4011T = ATCC 15703T  | modified PYG broth                  |
| 19  | Bifidobacterium animalis                  | YIT 4121T = DSM 10140T   | modified PYG broth                  |
|     | subsp. lactis                              |                           |                                     |
| 20  | Bifidobacterium angulatum                 | YIT 4012T = ATCC 27535T  | modified PYG broth                  |
| 21  | Bifidobacterium bifidum                   | YIT 4039T = DSM 20456T   | modified PYG broth                  |
| 22  | Bifidobacterium breve                     | YIT 4014T = ATCC 15700T  | modified PYG broth                  |
| 23  | Bifidobacterium catenulatum               | YIT 4016T = ATCC 27539T  | modified PYG broth                  |
| 24  | Bifidobacterium longum subsp. infantis    | YIT 4018T = ATCC 15697T  | modified PYG broth                  |
|     | longum                                     |                           |                                     |
| 25  | Bifidobacterium longum subsp.             | YIT 4021T = ATCC 15707T  | modified PYG broth                  |
| 26  | Bifidobacterium pseudocatenulatum         |                           | modified PYG broth                  |
| 27  | Blautia cocoide                           | YIT 6035T = JCM 1395T    | modified GAM + 1% Glucose broth     |
| 28  | Blautia hansenii                          | YIT 12129T = DSM 20583T  | modified GAM + 1% Glucose broth     |
| 29  | Blautia hydrogenotrophica                 | YIT 10080T = DSM 10507T  | modified GAM + 1% Glucose broth     |
| 30  | Blautia producta                          | YIT 6141T = JCM 1471T    | modified GAM + 1% Glucose broth     |
| 31  | Blautia schinkii                          | YIT 6177T = DSM 10518T   | modified GAM + 1% Glucose broth     |
| 32  | Butyribrio crosbatus                      | YIT 10152T = DSM 2876T   | modified GAM + 1% Glucose broth     |
| 33  | Citrobacter freundii                      | YIT 6045T = JCM 1657T    | Trypticase Soy broth                |
| 34  | Citrobacter koseri                        | YIT 10117T = JCM 1658T   | Trypticase Soy broth                |
| 35  | Clostridium aminophilum                   | YIT 6167T = DSM 10710T   | modified GAM + 1% Glucose broth     |
| 36  | Clostridium aminovalericum                | YIT 10174T = JCM 11016T  | modified GAM + 1% Glucose broth     |
| 37  | Clostridium asparaginiforme               | YIT 12840T = DSM 19581T  | modified GAM + 1% Glucose broth     |
| 38  | Clostridium bifermentans                  | YIT 6053T = JCM 1386T    | modified GAM + 1% Glucose broth     |
| 39  | Clostridium butyricum                     | YIT 10079T = JCM 1391T   | modified GAM + 1% Glucose broth     |
| 40  | Clostridium celerstaris                    | YIT 6168T = DSM 5628T    | modified GAM + 1% Glucose broth     |
| 41  | Clostridium clodiumiforium                | YIT 6051T = JCM 1291T    | modified GAM + 1% Glucose broth     |
| 42  | Clostridium cohollearium                  | YIT 12837T = JCM 1396T   | modified GAM + 1% Glucose broth     |
| 43  | Clostridium coecalatum                    | YIT 6036T = JCM 1397T    | modified GAM + 1% Glucose broth     |
| 44  | Clostridium difficile                     | YIT 10084T = JCM 1296T   | modified GAM + 1% Glucose broth     |
| 45  | Clostridium ghonii                        | YIT 11479T = JCM 1400T   | modified GAM + 1% Glucose broth     |
| 46  | Clostridium glycolicum                    | YIT 6058T = JCM 1401T    | modified GAM + 1% Glucose broth     |
| 47  | Clostridium hathewayi                     | YIT 12259T = DSM 13479T  | modified PYG broth                  |
| 48  | Clostridium hylemonae                     | YIT 12258T = DSM 15053T  | modified PYG broth                  |
| 49  | Clostridium indolis                       | YIT 10077T = JCM 1380T   | modified GAM +1% Glucose broth      |
| 50  | Clostridium innocuum                      | YIT 10151T = DSM 1286T   | modified GAM + 1% Glucose broth     |
| 51  | Clostridium leptum                        | YIT 6169T = DSM 753T     | modified GAM + 1% Glucose broth     |
| 52  | Clostridium limosum                       | YIT 6061T = JCM 1427T    | modified GAM + 1% Glucose broth     |
| 53  | Clostridium malenominatum                 | YIT 12839T = JCM 1405T   | modified GAM + 1% Glucose broth     |
| 54  | Clostridium nectarile                     | YIT 6170T = ATCC 27757T  | modified GAM + 1% Glucose broth     |
| 55  | Clostridium orbiscindens                  | YIT 10060T = DSM 6740T   | modified GAM + 1% Glucose broth     |
| 56  | Clostridium oroticum                      | YIT 6037T = JCM 1429T    | modified GAM + 1% Glucose broth     |
| 57  | Clostridium paraputreficum                | YIT 10094T = JCM 1293T   | modified GAM + 1% Glucose broth     |
| 58  | Clostridium perfringens                   | YIT 6050T = JCM 1290T    | modified GAM + 1% Glucose broth     |
| 59  | Clostridium ramosum                       | YIT 11062T = JCM 1298T   | modified GAM + 1% Glucose broth     |
| 60  | Clostridium saccharolyticum               | YIT 12747T = DSM 2544T   | modified GAM + 1% Glucose broth     |
| 61  | Clostridium scindens                      | YIT 6171T = JCM 6567T    | modified GAM + 1% Glucose broth     |
| 62  | Clostridium sordellii                     | YIT 6065T = JCM 3814T    | modified GAM + 1% Glucose broth     |
| 63  | Clostridium sphenoides                    | YIT 6059T = JCM 1415T    | modified GAM + 1% Glucose broth     |
| No. | Species                                      | Registration No. | Medium for culture                |
|-----|---------------------------------------------|-----------------|-----------------------------------|
| 64  | Clostridium spiroforme                       | YIT 10342T = JCM 1432T | modified GAM + 1% Glucose broth   |
| 65  | Clostridium sporogenes                       | YIT 6605T = JCM 1416T | modified GAM + 1% Glucose broth   |
| 66  | Clostridium symbiosum                        | YIT 11480T = JCM 1297T | modified GAM + 1% Glucose broth   |
| 67  | Clostridium tetanomorphum                   | YIT 12841T = DSM 4474T | modified GAM + 1% Glucose broth   |
| 68  | Clostridium xylanovorans                    | YIT 12130T = DSM 12503T | modified PYG broth               |
| 69  | Collinsella aerofaciens                     | YIT 10235T = DSM 3979T | modified GAM + 1% Glucose broth   |
| 70  | Coprococcus eutactus                        | YIT 10160T = ATCC 27759T | modified GAM + 1% Glucose broth   |
| 71  | Cronobacter sakazakii                       | YIT 10246T = JCM 1233T | Trypticase Soy broth             |
| 72  | Dorea formicigeners                         | YIT 10093T = DSM 3992T | modified GAM + 1% Glucose broth   |
| 73  | Edwardsiella tarda                          | YIT 10118T = JCM 1656T | Trypticase Soy broth             |
| 74  | Eggerthella lenta                           | YIT 6077T = ATCC 2555T | modified GAM + 1% Glucose broth   |
| 75  | Enterobacter aerogenes                       | YIT 6041T = JCM 1232T | Trypticase Soy broth             |
| 76  | Enterobacter cloacae                        | YIT 10255T = JCM 8722T | MRS broth                        |
| 77  | Enterococcus avium                          | YIT 10236T = GIFU 9960T | MRS broth                        |
| 78  | Enterococcus durans                         | YIT 2031T = ATCC 19433T | MRS broth                        |
| 79  | Enterococcus faecalis                       | YIT 2032T = ATCC 19434T | MRS broth                        |
| 80  | Enterococcus gilus                          | YIT 11114T = DSM 15689T | MRS broth                        |
| 81  | Enterococcus hirae                          | YIT 20047T = ATCC 8043T | MRS broth                        |
| 82  | Enterococcus malodoratus                    | YIT 11175T = JCM 8730T | MRS broth                        |
| 83  | Enterococcus mundtii                        | YIT 11176T = JCM 8731T | MRS broth                        |
| 84  | Enterococcus pseudoavus                     | YIT 11177T = JCM 8732T | MRS broth                        |
| 85  | Enterococcus raffinosus                      | YIT 11178T = JCM 8733T | MRS broth                        |
| 86  | Escherichia coli                            | YIT 6042T = JCM 1649T | Trypticase Soy broth             |
| 87  | Eubacterium biforme                         | YIT 6076T = ATCC 27806T | modified GAM + 1% Glucose broth   |
| 88  | Eubacterium cellulolactobios                 | YIT 12261T = ATCC 45171T | modified GAM + 1% Glucose broth   |
| 89  | Eubacterium cylindrones                      | YIT 10236T = DSM 3991T | modified GAM + 1% Glucose broth   |
| 90  | Eubacterium delicatum                       | YIT 10078T = DSM 3376T | modified GAM + 1% Glucose broth   |
| 91  | Eubacterium eligens                         | YIT 10064T = DSM 3353T | modified GAM + 1% Glucose broth   |
| 92  | Eubacterium hallii                          | YIT 11175T = JCM 8730T | modified GAM + 1% Glucose broth   |
| 93  | Eubacterium rectale                         | YIT 6082T = ATCC 39656T | modified GAM + 1% Glucose broth   |
| 94  | Eubacterium siraeum                         | YIT 10049T = DSM 3996T | modified GAM + 1% Glucose broth   |
| 95  | Eubacterium uniforme                        | YIT 12318T = ATCC 35992T | modified GAM + 1% Glucose broth   |
| 96  | Eubacterium ventriosum                      | YIT 10066T = ATCC 27560T | modified GAM + 1% Glucose broth   |
| 97  | Faecalibacterium prausnitzii                | YIT 10067T = ATCC 27768T | modified PYG broth               |
| 98  | Fusobacterium necrophorum                   | YIT 10362T = ATCC 25556T | modified GAM + 1% Glucose broth   |
| 99  | Fusobacterium necrophorum subsp. nucleatum  | YIT 10343T = JCM 3718T | modified GAM + 1% Glucose broth   |
| 100 | Fusobacterium necrophorum subsp. nucleatum  | YIT 6069T = JCM 8532T | modified GAM + 1% Glucose broth   |
| 101 | Fusobacterium russii                        | YIT 10363T = ATCC 25533T | modified GAM + 1% Glucose broth   |
| 102 | Fusobacterium varium                        | YIT 10363T = ATCC 25533T | modified GAM + 1% Glucose broth   |
| 103 | Hafnia alvei                                | YIT 10121T = JCM 1666T | Trypticase Soy broth             |
| 104 | Holdemania filiformis                       | YIT 1271T = JCM 1665T | modified GAM + 1% Glucose broth   |
| 105 | Klebsiella oxytoca                          | YIT 10122T = JCM 1665T | Trypticase Soy broth             |
| 106 | Klebsiella pneumoniae                       | YIT 6046T = JCM 1662T | Trypticase Soy broth             |
| 107 | Lactobacillus acidophilus                   | YIT 0700T = ATCC 4356T | MRS broth                        |
| 108 | Lactobacillus brevis                        | YIT 0705T = ATCC 14869T | MRS broth                        |
| 109 | Lactobacillus casei                         | YIT 0180T = ATCC 334T  | MRS broth                        |
| 110 | Lactobacillus fermentum                     | YIT 0081T = ATCC 14931T | MRS broth                        |
| 111 | Lactobacillus fructivorans                  | YIT 0235T = JCM 1117T  | MRS broth                        |
| 112 | Lactobacillus gasseri                       | YIT 0192T = DSM 20243T | MRS broth                        |
| 113 | Lactobacillus plantarum                    | YIT 0102T = ATCC 14917T | MRS broth                        |
| 114 | Lactobacillus reuteri                       | YIT 0197T = JCM 1112T  | MRS broth                        |
| 115 | Lactobacillus ruminis                       | YIT 0221T = JCM 1152T  | MRS broth                        |
| 116 | Lactobacillus sakei subsp. sakei            | YIT 0247T = JCM 1157T  | MRS broth                        |
| 117 | Lactobacillus sakei subsp. sakei            | YIT 014T = JCM 25556T | modified GAM + 1% Glucose broth   |
| 118 | Lactococcus garvieae                        | YIT 2071T = NCFB 2155T | MRS broth                        |
| 119 | Lactococcus lactis subs. lactic             | YIT 2008T = ATCC 19435T | MRS broth                        |
| 120 | Lactococcus plantarum                       | YIT 2061T = ATCC 43919T | MRS broth                        |
| 121 | Lactococcus raffinolactis                   | YIT 2061T = ATCC 43920T | MRS broth                        |
| 122 | Megasphaera elaidii                         | YIT 6083T = JCM 1772T | modified GAM + 1% Glucose broth   |
| 123 | Morganella morganii                         | YIT 10124T = JCM 1672T | Trypticase Soy broth             |
| 124 | Olsenella uli                               | YIT 12014T = JCM 1249T | modified GAM + 1% Glucose broth   |
| 125 | Parabacteroides distasonis                  | YIT 6162T = JCM 5825T | modified GAM + 1% Glucose broth   |
| 126 | Parabacteroides johnsonii                   | YIT 12680 | 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 expected to be obtained 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 (Page 1996). The 16S rRNA sequence of Desulfovibrio desulfuricans DSM 1296T was used as an outgroup.

Search for homologous protein

Files on the proteins that phenol- or p-cresol-producing bacteria were expected to have been 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 1296T (Accession Nos. AJ543427, AJ543425 and AJ543426, respectively), FldH (phenyllactate dehydrogenase), FldBC (phenyllactate dehydratase), AcyA (acyl-CoA dehydrogenase) and PorA (pyruvate:ferredoxin oxidoreductase A) were performed by using MultiGeneBlast (Medema, Takano and Breiling 2013) with the default parameters. Amino acid sequences encoded by gene clusters consisting of fldA, fldD, fldB, fldF, fdiA, acyA, efb, etfA, permease and fldH from Cl. sporogenes ATCC 15579T (Accession Nos. EDU39251 to 39261) were used as queries to identify homologs of FldH, FldBC and AcyA. Similarly, amino acid sequences encoded by porA from Cl. sporogenes ATCC 15579T (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. malenomina grum YIT 10124T, Fusobacterium variium YIT 11855, Morganella morganii YIT 10124T, Cl. cohe/earium YIT 12837T, Cl. saccharolyticum YIT 12747T, Citrobacter koseri YIT 10117T, K. pneumoniae YIT 6046T, Olsenella uli YIT 12014T, Enterobacter aerogenes YIT 6042T, Citrobacter freundii YIT 6045T, Cronobacter sakazakii YIT 10246T, K. oxytoca YIT 10122T, En. cloacae YIT 6041T, F. necrophorum subsp. necrophorum YIT 10343T and F. rurii YIT 10363T) 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

| Species                     | Strains (Accession No.) | Genome (Accession No.) | % of identity / E-value |
|-----------------------------|-------------------------|------------------------|-------------------------|
| Citrobacter freundii       | YIT 6045<sup>T</sup>   | NZ_JMTA000000000       | 99/0.0 90/0.0 83/6<sup>-98</sup> 97/0.0 87/3<sup>-37</sup> |
| Clostridium saccharolyticum| YIT 12747<sup>T</sup>   | NC_014376              | 70/0.0 – – – –         |
| Cronobacter sakazakii      | YIT 10246<sup>T</sup>   | NZ_CP011047            | – 85/0.0 82/3<sup>-91</sup> 93/0.0 88/3<sup>-38</sup> |
| Enterobacter aerogenes      | YIT 6042<sup>T</sup>   | NC_015663              | – 88/0.0 92/2<sup>-106</sup> 98/0.0 92/7<sup>-40</sup> |
| Enterobacter cloacae       | YIT 6041<sup>T</sup>   | NC_014121              | – 87/0.0 90/4<sup>-103</sup> 96/0.0 94/6<sup>-38</sup> |
| Fusobacterium necrophorum  | YIT 10343<sup>T</sup>   | NZ_FJMX000000000       | 76/0.0 – – – –         |
| Fusobacterium russii       | YIT 10363<sup>T</sup>   | NZ_ARMK000000000       | 82/0.0 – – – –         |
| Klebsiella pneumoniae      | YIT 6046<sup>T</sup>   | –                      | – 84/0.0 100/2<sup>-114</sup> 100/0.0 100/5<sup>-42</sup> |
| Morganella morganii        | YIT 10124<sup>T</sup>   | NZ_BCZU000000000       | 90/0.0 66/0.0 – – – – |
| Olsenella uli              | YIT 12014<sup>T</sup>   | NC_014363              | – – 48/6<sup>-127</sup> 40/9<sup>-12</sup> 48/1<sup>-48</sup> |

*Accession No. AAY57856*

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

| Species                     | Strains (Accession No.) | Genome (Accession No.) | % of identity / E-value |
|-----------------------------|-------------------------|------------------------|-------------------------|
| Blautia hydrogenoferophica  | YIT 10080<sup>T</sup>   | NZ_ACBV000000000       | – – 57/2<sup>-108</sup> 55/0.0 42/5<sup>-37</sup> |
| Clostridium difficile       | YIT 10084<sup>T</sup>   | NZ_AOUX000000000       | 36/8<sup>-45</sup> – 99/0.0 100/0.0 100/5<sup>-47</sup> |
| Olsenella uli               | YIT 12014<sup>T</sup>   | NC_014363              | – 56/6<sup>-109</sup> 55/0.0 34/4<sup>-3</sup> |
| Romboutsia lituseburensis   | YIT 10059<sup>T</sup>   | NZ_FNGW000000000       | 35/2<sup>-42</sup> – 68/9<sup>-133</sup> 76/0.0 59/2<sup>-38</sup> |

*Accession No. AAY57856*

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, as well as with Clostridium clusters XVI, IV, XV, IX, I, 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<sup>T</sup>, 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

*Coriobacteriaceae* YIT 12014<sup>T</sup> and *Bacteroidaceae* YIT 10246<sup>T</sup> and *Romboutsia lituseburensis* YIT 10059<sup>T</sup>.
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, Clostridium tetanomorphum YIT 12841T, Fusobacterium varium YIT 11855, Morganella morganii YIT 10124T, Clostridium cocleareum YIT 12837T, Clostridium saccharolyticum YIT 12747T, Citrobacter koseri YIT 10117T, Klebsiella pneumoniae YIT 6046T, Olsenella uli YIT 12014T, Enterobacter aerogenes YIT 6042T, Citrobacter freundii YIT 6045T, Cronobacter sakazakii YIT 10246T, Klebsiella oxytoca YIT 10122T, Enterobacter cloacae YIT 6041T, Fusobacterium necrophorum YIT 10343T, Fusobacterium russii YIT 10363T, Blautia hydrogenotrophica YIT 10080T, Clostridium difficile YIT 10084T, Romboutsia lituseburensis YIT 10059T, Olsenella uli YIT 12014T.

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 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 (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 (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 (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 (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).
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 phylogenetic 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), phenyl-lactate dehydrogenase (FdhB), phenyllactate dehydratase (FdhBC), 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. freudii YIT 6045T, C. sakazakii YIT 10246T, En. aerogenes YIT 6042T, En. cloacae YIT 6041T and C. pneumoniae YIT 6046T with more than 80% identity of amino acid sequences; in the case of O. uli YIT 12014T there was 40% to 48% identity (Table 2). The three homologs were found on these genomes in the order of HpdB, HpdC and HpdD, except in the case of O. uli YIT 12014T, 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). FldBBC 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 10800T, Cl. difficile YIT 10084T, O. uli YIT 12014T and R. lituseburensis YIT 10059T) with high p-cresol-producing ability. We used information already reported on their draft genome sequences. No protein with more than 30% amino acid sequence identity to TyrB of E. coli strain K-12 were found. In Cl. difficile DSM 1296T, three enzymes—HpdA, an activating enzyme; HpdC, a large subunit; and HpdD, 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 hpdD (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 (Krieck et al. 2007). We then found ThiH homologs encoded by the genome of Cl. difficile YIT 10084T (36% amino acid sequence identity) and R. lituseburensis YIT 10059T (35%) (Table 3). Analysis of homologs of other enzymes involved revealed that all four strains harbored FldH or PorA or both (data not shown). FldBBC homologs were identified in B. hydrogenotrophica YIT 10800T and O. uli YIT 12014T. No AcdA homologs were identified in any strain (data not shown).

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 12839T, Cl. tetanomorphum YIT 12841T and Cl. cochlearium YIT 12837T—have already been reported to produce phenol (Elsden, Hilton and Waller 1976). Moreover, K. pneumoniae YIT 6046T, En. cloacae YIT 6041T and M. morganii YIT 10124T are known as phenol-producing bacteria at the species level (Patel and Grant 1969; Volkerova et al. 2001; Matsui et al. 2006; Ishizu et al. 2009b). The phenol-producing ability of C. freundii YIT 6045T 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 the first time as phenol producing. Among the 55 p-cresol-producing strains identified in this study, B. longum subsp. infantis YIT 4018T, Cl. difficile YIT 10084T, Cl. paraputrificum YIT 10074T and F. necrophorum YIT 10362T have already been examined for their ability to produce p-cresol (Bone, Tamm and Hill 1976; Elsdon, 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; Ishizu et al. 2009a; b, Windey, De Preter and Verbeke 2012; Ito and Yoshida 2014; Shiba et al. 2014; Andriamiarisoa 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 related 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 12747T, F. necrophorum subsp. necrophorum YIT 10343T, F. russii YIT 10363T, and M. organii YIT 10124T used TPL-dependent pathways; C. sakazakii YIT 10246T, E. aerogenes YIT 6042T, En. cloacae YIT 6041T, K. pneumoniae YIT 6046T and O. uli YIT 12014T used Had-dependent pathways, and C. freundii YIT 6045T 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. hydrogenotrophicica YIT 10080T and O. uli YIT 12014T could utilize Hpd-dependent pathways, whereas Cl. difficile YIT 10084T and R. lituseburensis YIT 10059T 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 12839T, Cl. tetanomorphum YIT 12841T, F. varium YIT 11855, Cl. cochlearium YIT 12837T, C. roseri YIT 10117T and K. oxytoca YIT 10122T) 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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