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A report of 10 unrecorded bacterial species of Korea, belonging to the phylum *Firmicutes*

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To investigate the indigenous prokaryotic species diversity in Korea, various environmental samples from diverse ecosystems were examined taxonomically. The isolated bacterial strains were identified based on 16S rRNA gene sequences, and those exhibiting at least 98.7% sequence similarity with known bacterial species but never reported in Korea were selected as unrecorded species. As an outcome of this study, 10 unrecorded bacterial species belonging to the phylum *Firmicutes* were discovered from various sources such as soil, tidal flat, fresh water, sea water, kimchi and gut of *Fulvia mutica*. The unrecorded species were assigned to 7 different genera of 5 families, namely *Bacillus* and *Ornithinibacillus* of *Bacillaceae*, *Exiguobacterium* of *Exiguobacteriaceae*, *Brevibacillus* and *Paenibacillus* of *Paenibacillaceae*, *Staphylococcus* of *Staphylococcaceae*, and *Lactococcus* of *Streptococcaceae*. The selected isolates were subjected to further taxonomic characterization including the analysis of Gram reaction, cellular and colonial morphology, biochemical activities, and phylogenetic trees. The descriptive information on the 10 unrecorded species are provided.

Keywords: *Bacillales*, *Firmicutes*, *Lactobacillales*, unrecorded bacterial species

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Introduction

The phylum *Firmicutes* encompasses Gram-positive bacteria with low DNA G+C content (Gibbons and Murray, 1978). In contrast to the other Gram-positive bacterial phylum *Actinobacteria*, the G+C content of *Firmicutes* is generally less than 50 mol% (De Vos *et al*., 2009). Currently, the phylum contains 5 classes, namely *Bacilli*, *Clostridia*, *Erysipelotrichia*, *Negativicutes*, and *Thermolithobacteria*, encompassing 9 orders, 37 families, and >370 genera (Marchandin *et al*., 2010).

Firmicutes are diverse in terms of their morphology, physiology, and metabolic capabilities. Most of *Firmicutes* possess a rigid cell wall containing muramic acid in their cell wall but some contain teichoic acid (Rosenberg *et al*., 2014). Though *Firmicutes* are defined as Gram-positive low G+C group, the phylum includes a number of organisms whose cells stain Gram-negative like as class *Negativicutes* (Marchandin *et al*., 2010). The shape of cells in the phylum vary from long and slender, sometimes bent rods to short coryneform coccobacilli or spherical cells with or without flagella (De Vos *et al*., 2009). *Firmicutes* are aerobes, facultative or strict anaerobes. Most of *Firmicutes* are chemoorganotrophs, a few of them are anoxygenic photoheterotrophs, and grow well at neutral pH (De Vos *et al*., 2009).

The class *Bacilli* is the second largest class in the phylum *Firmicutes*, after the class *Clostridia*, and currently composed of 2 orders, *Bacillales* and *Lactobacillales*. The members of the order *Bacillales* form endospores and possess menaquinone 7 (MK-7) (De Vos *et al*., 2009). In contrast, the order *Lactobacillales* are

non-sporeforming and non-respiring, producing lactate with fermentative metabolism. Members of the order *Lactobacillales* can be easily found in milk products and the healthy microflora of human mucosal surfaces.

Members of the phylum *Firmicutes* has an important role in industrial and clinical aspect. The members of the genus *Clostridium* can cause food-poisoning, while the members of the order *Lactobacillales* are good sources of probiotics (Wood, 1998). Recently, the members of the phylum *Firmicutes* has been shown to be the largest portion of the gut flora and involved in fatty acid absorption and obesity (Backhed *et al*., 2004; Ley *et al*., 2006; Turnbaugh *et al*., 2008). The abundance of *Firmicutes* in the gut microflora can lead to an overall decrease in metabolic diversity and obesity (Ley *et al*., 2006; Le Chatelier *et al*., 2013).

In this study, bacteria belonging to Firmicutes were isolated from various sources such as soil, tidal flat, fresh water, sea water, kimchi and gut of *Fulvia mutica*. Through the phylogenetic analysis using 16S rRNA gene sequences, we recovered 10 species that could be recognized as unrecorded bacterial species.

Materials and Methods

Bacterial strains were isolated from various environmental sources including soil, tidal flat, fresh water, sea water, kimchi and gut of *Fulvia mutica*. Each environmental sample was processed separately. R2A, marine agar 2216 (MA), or MRS agar were used as culture media and incubated at 25-30°C for 2-5 days. The designation of strains, source of isolation, culture media, and incubation conditions are summarized in Table 1. All strains were purified as single colonies and stored as 10-20% glycerol suspension at −80°C and as lyophilized ampoules.

Bacterial DNA extraction, PCR amplification, and gene sequencing were performed using standard procedures. Primers 27F and 1492R were used for PCR and sequencing of 16S rRNA gene. The 16S rRNA gene sequences were compared with other bacterial species with validly published names using the EzTaxon-e server (Kim *et al*., 2012). The cutoff value of 98.7% sequence similarity was employed for identification. Strains exhibiting 98.7% or higher sequence similarity with known bacterial species but never reported in Korea were selected as unrecorded species. For phylogenetic analyses, sequence alignments between the 16S rRNA gene sequences of the isolates and those of the reference type strains were carried out using EzEditor (Jeon *et al*., 2014). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983) and the phylogenetic trees were constructed by using the neigh-

Table 1. Summary of strains isolated belonging to the *Firmicutes* and their taxonomic affiliations.

Order	Family	Genus	Strain ID	NIBR ID	Most closely related species	Similarity (%)	Isolation source	Medium	Incubation conditions
Bacillales	*Bacillaceae*	*Bacillus*	MBM12	NIBRBA0000114787	*Bacillus oceanisediminis*	99.6	Fresh water	MA	25°C, 2d
		Ornithinibacillus	Eg81205	NIBRBA0000114941	*Ornithinibacillus contaminans*	95.3	Gut of *Fulvia mutica*	R2A	25°C, 3d
	Exiguobacteriaceae	*Exiguobacterium*	HMF2471	NIBRBA0000114995	*Exiguobacterium sibiricum*	99.7	Fresh water	MA	25°C, 3d
	Paenibacillaceae	*Brevibacillus*	NMD 3Y-3-3	NIBRBA0000114872	*Brevibacillus reuszeri*	99.8	Soil	R2A	30°C, 2d
		Paenibacillus	CH2-1	NIBRBA0000114887	*Paenibacillus lautus*	99.1	Tidal flat	R2A	30°C, 2d
			CF4	NIBRBA0000114895	*Paenibacillus oceanisediminis*	99.9	Tidal flat	R2A	30°C, 2d
			EgN2201	NIBRBA0000114940	*Paenibacillus pabuli*	98.6	Gut of *Fulvia mutica*	R2A	25°C, 3d
			CF7	NIBRBA0000114896	*Paenibacillus xylanilyticus*	99.8	Tidal flat	R2A	30°C, 2d
	Staphylococcaceae	*Staphylococcus*	MB2M14	NIBRBA0000114762	*Staphylococcus epidermidis*	99.9	Sea water	R2A	25°C, 2d
Lactobacillaes	*Streptococcaceae*	*Lactococcus*	Kef2	NIBRBA0000114817	*Lactococcus lactis* subsp. *cremoris*	99.9	Kimchi	MRS	25°C, 2d

bor-joining (Saitou and Nei, 1987) and maximum-likelihood algorithms (Felsenstein, 1993) implemented in MEGA 6.0 (Tamura *et al.,* 2013). The robustness of the inferred trees was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1,000 re-samplings.

Colonial morphology was observed on agar plates after the cells were cultivated to their stationary phase. Cellular morphology and cell size were examined by either transmission electron microscopy or scanning electron microscopy. Gram staining was performed using a Gram-staining kit or the standard procedures. Biochemical characteristics were tested by using API 20NE galleries (bioMérieux) according to the manufacturer's instructions.

Results and Discussion

During the course of systematic study of diverse ecosystem in Korea, a number of previously unreported bacterial species were isolated. Based on 16S rRNA gene sequence similarity, 10 strains were identified as members of the class *Bacilli* of the phylum *Firmicutes*. The taxonomic composition and identification results are summarized in Table 1. One of the strain belongs to the family *Streptococcaceae* of the order *Lactobacillales*. The other 9 strains were assigned to the family *Paenibacillaceae* (5 strains), *Bacillaceae* (2 strains), *Staphylococcaceae* (1 strain), and *Exiguobacteriaceae* (1 strain) of the order *Bacillales*. At generic level, the strains were found to belong to 7 separate genera: *Paenibacillus* (4 species), *Brevibacillus* (1 species), *Bacillus* (1 species), *Ornithinibacillus* (1 species), *Exiguobacterium* (1 species), *Staphylococcus* (1 species), and *Lactococcus* (1 species). The identification of the isolates based on sequence similarity were supported by the phylogenetic trees. The neighbor-joining and maximum likelihood trees were almost identical in their tree topologies showing the close relationship of the isolates and type strains of validly published species (Fig. 1). The 10 isolates were Gram-staining-negative or positive, rod- or coccoid-shaped bacteria (Fig. 2). The detailed morphological and physiological characteristics are given in the strain descriptions.

As an outcome of this study, the diversity of bacterial species whose presence in Korean ecosystems has not been previously reported was discovered. The 10 isolates were identified as unreported species belonging to the phylum *Firmicutes*, and their phenotypic characteristics were examined through polyphasic taxonomic study. Accordingly, the following 10 species are reported as unrecorded species in Korea.

Description of *Bacillus oceanisediminis* MBM12

Cells are Gram-staining-positive, flagellated, and rod-shaped. Colonies are circular with entire margin, raised, and cream colored after 2 days on MA at 25°C. Positive for oxidase, nitrate reduction, and gelatinase activity. Negative for indole production, glucose fermentation, arginine dihydrolase, urease, esculin, or β-galactosidase activities. Uses D-glucose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, adipic acid, malic acid, and trisodium citrate as carbon sources, but not L-arabinose, D-mannose, capric acid, or phenylacetic acid. Strain MBM12 (=NIBRBA0000114787) was isolated from a fresh water sample, Busan, Korea.

Description of *Brevibacillus reuszeri* NMD 3Y-3-3

Cells are Gram-staining-negative, flagellated, and rod-shaped. Colonies are punctiform, dry, flat, undulate and white colored after 2 days on R2A at 30°C. Positive for gelatin hydrolysis. Negative for oxidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, esculin hydrolysis, or β-galactosidase. Uses D-glucose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, malic acid, and phenylacetic acid as carbon sources, but not L-arabinose, D-mannose, capric acid, adipic acid, or trisodium citrate. Strain NMD 3Y-3-3 (=NIBRBA0000114872) was isolated from a soil sample, Anseong, Korea.

Description of *Exiguobacterium sibiricum* HMF2471

Cells are Gram-staining-positive, flagellated, and rod-shaped. Colonies are circular with entire margin, convex, and bright orange colored after 3 days on R2A at 25°C. Positive for oxidase, glucose fermentation, esculin hydrolysis, gelatin hydrolysis, and β-galactosidase. Negative for nitrate reduction, indole production, arginine dihydrolase, or urease. Does not use D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid as a carbon source. Strain HMF2471 (=NIBRBA0000114995) was isolated from a water sample, Yongin, Korea.

Description of *Lactococcus lactis* subsp. *cremoris* Kef2

Cells are Gram-staining-positive, non-flagellated, and cocci-shaped. Colonies are circular with entire margin, raised, and white colored after 2 days on MRS agar at 25°C. Positive for arginine dihydrolase, esculin hydrolysis, and β-galactosidase. Negative for oxidase, nitrate reduction, indole production, glucose fermentation, urease, or gelatin hydrolysis. Does not use D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid,

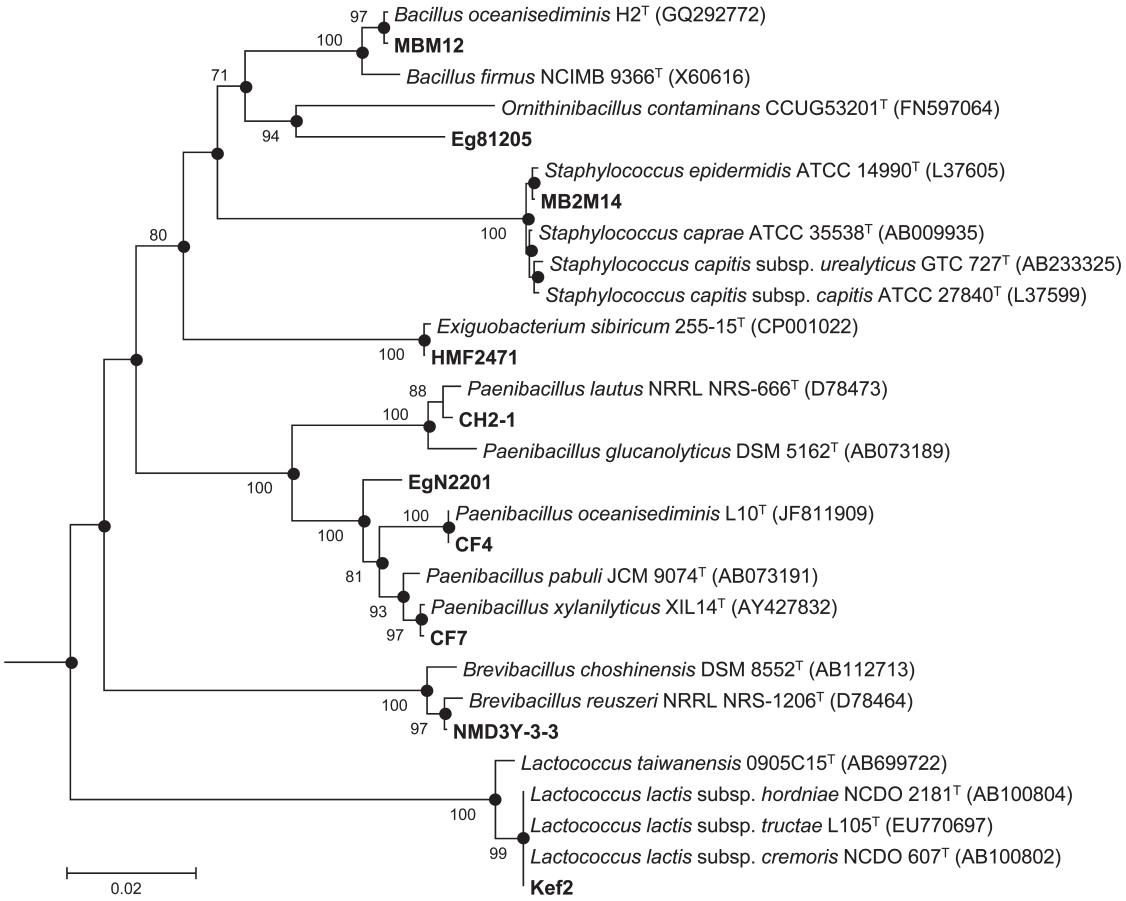


Fig. 1. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between the strains isolated in this study and their relatives of the class *Bacilli*. *Escherichia coli* ATCC 11775^T (X80725) was used as an outgroup. Filled circles indicate the nodes were also recovered in maximum-likelihood tree. Bootstrap values (>70%) are shown above nodes. Scale bar, 0.02 substitutions per nucleotide.

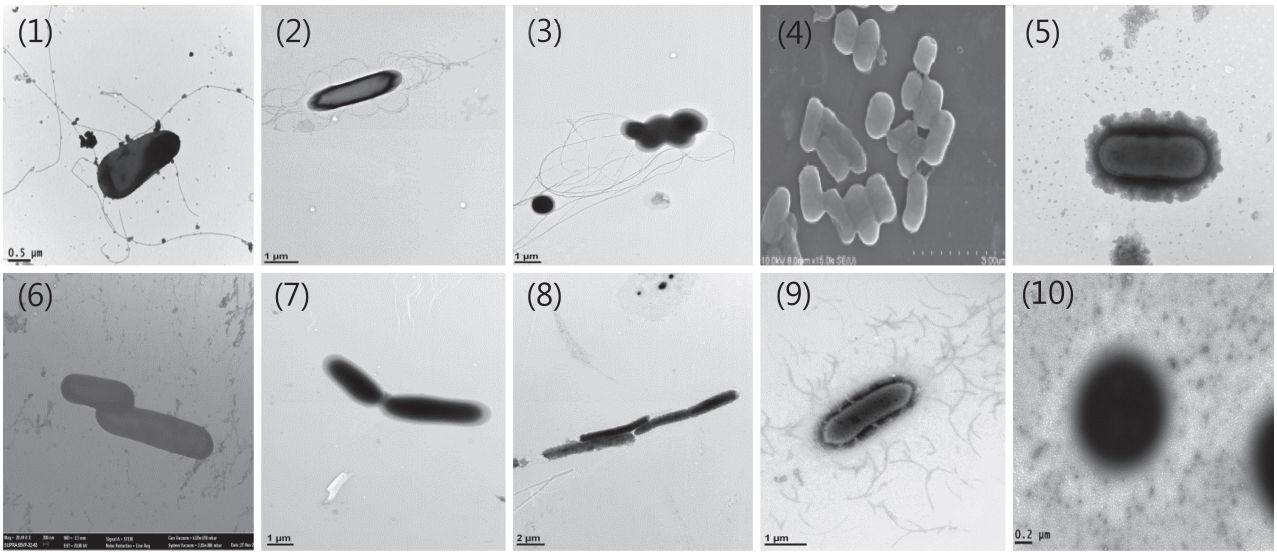


Fig. 2. Transmission electron micrographs or scanning electron micrographs of cells of the strains isolated in this study. Strains: 1, MBM12; 2, NMD 3Y-3-3; 3, HMF2471; 4, Kef2; 5, Eg81205; 6, EgN2201; 7, CF4; 8, CF7; 9, CH2-1; 10, MB2M14.

malic acid, trisodium citrate, or phenylacetic acid as a carbon source. Strain Kef2 (=NIBRBA0000114817) was isolated from a Kimchi, Daejeon, Korea.

Description of *Ornithinibacillus contaminans* Eg81205

Cells are Gram-staining-positive, non-flagellated, and cocci-shaped. Colonies are circular with entire margin, and cream colored after 3 days on MA supplemented with 10% NaCl at 25°C. Positive for nitrate reduction, esculin hydrolysis, and β-galactosidase. Negative for oxidase, indole production, glucose fermentation, arginine dihydrolase, urease, or gelatin hydrolysis. Does not use D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid as a carbon source. Strain Eg81205 (=NIBRBA0000114941) was isolated from gut of *Fulvia mutica*, Korea.

Description of *Paenibacillus pabuli* EgN2201

Cells are Gram-staining-positive, non-flagellated, and rod-shaped. Colonies are circular and beige colored after 3 days on NA at 25°C. Positive for nitrate reduction, glucose fermentation, esculin hydrolysis, and β-galactosidase. Negative for oxidase, indole production, arginine dihydrolase, urease, or gelatin hydrolysis. Uses D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose, and potassium gluconate as carbon sources, but not *N*-acetyl-glucosamine, capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid. Strain EgN2201 (=NIBRBA0000114940) was isolated from gut of *Fulvia mutica*, Korea.

Description of *Paenibacillus oceanisediminis* CF4

Cells are Gram-staining-negative, non-flagellated, and coccus-shaped. Colonies are punctiform with entire margin, raised, and white colored after 2 days on R2A at 30°C. Positive for esculin hydrolysis and β-galactosidase. Negative for oxidase, nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, or gelatin hydrolysis. Uses D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, and D-maltose as carbon sources, but not potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid. Strain CF4 (=NIBRBA 0000114895) was isolated from tidal flat, Ganghwa Island, Korea.

Description of *Paenibacillus xylanilyticus* CF7

Cells are Gram-staining-positive, non-flagellated, and rod-shaped. Colonies are punctiform, raised, undulate, and white colored after 2 days on R2A at 30°C. Positive for esculin hydrolysis and β-galactosidase. Negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, or gelatin hydrolysis. Uses D-glucose, L-arabinose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, and potassium gluconate as carbon sources, but not D-mannose, capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid. Strain CF7 (=NIBRBA0000114896) was isolated from tidal flat, Ganghwa Island, Korea.

Description of *Paenibacillus lautus* CH2-1

Cells are Gram-staining-negative, flagellated, and rod-shaped. Colonies are rhizoid, raised, undulate, and white colored after 2 days on R2A at 30°C. Positive for nitrate reduction, esculin hydrolysis, and β-galactosidase. Negative for oxidase, indole production, glucose fermentation, arginine dihydrolase, urease, or gelatin hydrolysis. Uses D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, and potassium gluconate as carbon sources, but not capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid. Strain CH2-1 (=NIBRBA0000114887) was isolated from tidal flat, Ganghwa Island, Korea.

Description of *Staphylococcus epidermidis* MB2M14

Cells are Gram-staining-positive, non-flagellated, and coccus-shaped. Colonies are circular with entire margin, raised, and yellow colored after 2 days on R2A at 25°C. Positive for oxidase, nitrate reduction, urease, esculin hydrolysis, and β-galactosidase. Negative for indole production, glucose fermentation, arginine dihydrolase, or gelatin hydrolysis. Uses D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, malic acid, trisodium citrate, and phenylacetic acid as carbon sources, but not capric acid or adipic acid. Strain MB2M14 (=NIBRBA0000114762) was isolated from a sea water, Busan, Korea.

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References

Backhed, F., H. Ding, T. Wang, L.V. Hooper, G.Y. Koh, A. Nagy, C.F. Semenkovich and J.I. Gordon. 2004. The gut

microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 101(44):15718-15723.

De Vos, P., G.M. Garrity, D. Jones, N.R. Krieg, W. Ludwig, F.A. Rainey, K.H. Schleifer and W.B. Whitman. 2009. Bergey's manual of systematic bacteriology, New York: Springer.

Felsenstein, J. 1985. Confidence-limits on phylogenies - an approach using the bootstrap. Evolution 39(4):783-791.

Felsenstein, J. 1993. PHYLIP(phylogenetic inference package) version 3.5.1. Department of Genetics, University of Washington, Seattle, WA, USA.

Gibbons, N.E. and R.G.E. Murray. 1978. Proposals concerning higher taxa of bacteria. Int J Syst Bacteriol 28(1):1-6.

Jeon, Y.S., K. Lee, S.C. Park, B.S. Kim, Y.J. Cho, S.M. Ha and J. Chun. 2014. EzEditor: a versatile sequence alignment editor for both rRNA- and protein-coding genes. Int J Syst Evol Microbiol 64(2):689-691.

Kim, O.S., Y.J. Cho, K. Lee, S.H. Yoon, M. Kim, H. Na, S.C. Park, Y.S. Jeon, J.H. Lee, H. Yi, S. Won and J. Chun. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62(3):716-721.

Kimura, M. 1983. Rare variant alleles in the light of the neutral theory. Mol Biol Evol 1(1):84-93.

Le Chatelier, E., T. Nielsen, J. Qin, E. Prifti, F. Hildebrand, G. Falony, M. Almeida, M. Arumugam, J.M. Batto, S. Kennedy, P. Leonard, J. Li, K. Burgdorf, N. Grarup, T. Jorgensen, I. Brandslund, H.B. Nielsen, A.S. Juncker, M. Bertalan, F. Levenez, N. Pons, S. Rasmussen, S. Sunagawa, J. Tap, S. Tims, E.G. Zoetendal, S. Brunak, K. Clement, J. Dore, M. Kleerebezem, K. Kristiansen, P. Renault, T. Sicheritz-Ponten, W.M. de Vos, J.D. Zucker, J. Raes, T. Hansen, H.I.T.c. Meta, P. Bork, J. Wang, S.D. Ehrlich and O. Pedersen. 2013. Richness of human gut microbiome correlates with metabolic markers. Nature 500(7464):541-546.

Ley, R.E., D.A. Peterson and J.I. Gordon. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124(4):837-848.

Ley, R.E., P.J. Turnbaugh, S. Klein and J.I. Gordon. 2006. Microbial ecology - Human gut microbes associated with obesity. Nature 444(7122):1022-1023.

Marchandin, H., C. Teyssier, J. Campos, H. Jean-Pierre, F. Roger, B. Gay, J.P. Carlier and E. Jumas-Bilak. 2010. *Negativicoccus succinicivorans* gen. nov., sp. nov., isolated from human clinical samples, emended description of the family *Veillonellaceae* and description of *Negativicutes* classis nov., *Selenomonadales* ord. nov. and *Acidaminococcaceae* fam. nov. in the bacterial phylum *Firmicutes*. Int J Syst Evol Microbiol 60(6):1271-1279.

Rosenberg, E., E.F. DeLong, S. Lory, E. Stackebrandt and F. Thompson. 2014. The Prokaryotes, Springer.

Saitou, N. and M. Nei. 1987. The neighbor-joining method - a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406-425.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30(12):2725-2729.

Turnbaugh, P.J., F. Backhed, L. Fulton and J.I. Gordon. 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe 3(4):213-223.

Wood, B. 1998. Microbiology of Fermented Foods, Blackie, London.

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