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Establishment of enterotype-specific antibodies for various diagnostic systems

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This study demonstrates that monoclonal antibodies can be developed to targeting specific gut bacteria prevalent in the Japanese population and the potential for creating a novel diagnostic system using these antibodies. In this study, we established specific antibodies against representative bacteria from the genera *Bacteroides*, *Faecalibacterium*, and *Prevotella* and showed that they could be detected using ELISA, flow cytometry, and western blot analysis. Furthermore, a technique to quantify target bacteria was developed by combining these antibodies in a sandwich ELISA, enabling the quantification of bacteria in human fecal samples. This technology serves as a foundational method for rapidly and easily measuring gut bacteria and is expected to evolve into a powerful tool for analyzing the impact of gut bacteria on health, as well as for personalized health management based on individual gut environments.

Keywords Monoclonal antibody, Commensal bacteria, Diagnostics

There are approximately 1000 species of bacteria in the gut, forming the gut microbiota. Traditional culture-based methods allowed for a partial understanding of the effects of gut bacteria on health, but the species that could be analyzed were limited. However, with the advent of next-generation sequencing (NGS) technologies, many bacteria that could not be identified through culture methods have been discovered, and numerous studies have reported associations between the composition of gut bacteria and specific health conditions[1]. For example, in our research on the Japanese population, we identified the potential of *Blautia wexlerae* to contribute to improvements in obesity and diabetes, and have elucidated parts of the underlying mechanisms[2].

As the relationship between diseases and the gut microbiota gains greater attention, it has been found that even healthy individuals exhibit variations in the composition of their gut microbiota. In this context, a method to classify the gut bacteria of healthy individuals into different types has been proposed, one of which is the concept known as "enterotypes," based on dominant bacterial groups[3,4]. The initially proposed enterotypes are classified into three categories, each considered to be related to long-term lifestyle habits, particularly diet. These three categories are the "*Prevotella* type," which is common among individuals who consume diets rich in grains and vegetables containing carbohydrates and dietary fiber; the "*Bacteroides* type," prevalent among those who consume more animal proteins and fats; and a third type in which other bacterial groups are dominant[3].

Against this backdrop, interest in the gut microbiota has increased not only in academic research but also socially, leading to the provision of services that analyze gut bacteria. Many of these services are based on genetic information obtained through NGS[1]. However, these analyses present challenges in terms of time

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and cost, making it difficult to conduct them routinely and continually[5]. Therefore, there is a growing need for the development of simpler, faster, and more affordable methods. Other methods for measuring gut bacteria include quantitative polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH)[6], and mass spectrometry[7], but in this study we focused on detection methods using antibodies. Antibodies have a proven track record not only in laboratory methods such as ELISA, flow cytometry, and western blot analysis, but also in simple measurement kits, such as latex agglutination tests and immunochromatography, making them a potential tool for widespread use[8].

When producing antibodies against bacteria or cells, it is common to identify molecules that are specific to the target and to then create antibodies against those molecules. However, at present, not all gut bacteria have had their unique molecules fully identified[9]. Therefore, in this study we used a method to establish antibodies that specifically react with gut bacteria by immunizing with whole bacteria, without limiting reactions to a specific target molecule. Previously, we focused on mast cells as immune cells involved in allergies and established antibodies by immunizing with whole mast cells. Unexpectedly, we found that mast cells expressed the extracellular ATP receptor P2X7, activation of which contributes to the development of inflammatory bowel disease[10]. Further analysis using these antibodies revealed that, in the skin, an environment is formed in which P2X7 expression in mast cells is suppressed, contributing to the maintenance of skin homeostasis[11]. More recently, we applied this technique to bacteria, establishing antibodies against *Campylobacter*, and identified the menaquinol cytochrome *c* reductase complex QcrC, which plays a key role in energy metabolism, as a labeled molecule. Treatment with these antibodies exhibited bacteriostatic effects through inhibition of energy metabolism, and we further reported that a vaccine targeting QcrC could induce protective immunity against *Campylobacter*[12].

In the present study, as the first step towards establishing antibodies specific to gut bacteria using our previously described method, we targeted *Bacteroides*, *Faecalibacterium*, and *Prevotella* species, which are prevalent in the gut of the Japanese population and are known to be among the determinants of enterotypes[3,13]. We established monoclonal antibodies (mAbs) against these bacteria and report examples of their use in ELISA, western blotting, and flow cytometry.

Results

Establishment of mAbs specific to enterotypes of the human gut microbiome

Our aim in this study was to establish antibodies targeting the three major bacterial genera that determine the enterotype of the human gut microbiome. According to our data from a study on healthy Japanese individuals, the specific average prevalence of each of the bacterial genera was 29% for *Bacteroides*, 8.2% for *Faecalibacterium*, and 5.4% for *Prevotella*, with significant variability among individuals (Supplementary Fig. 1). This variability suggests that the evaluation of detection systems using antibodies would be useful.

We investigated the dominant species within these genera and selected representative bacteria[14], specifically *Phocaeicola vulgatus* (known as *Bacteroides vulgatus* until 2020)[15], *Faecalibacterium duncaniae* (known as *Faecalibacterium prausnitzii* until 2022)[16], and *Segatella copri* (renamed from *Prevotella copri* in 2023)[17]. Reference strains of each species were obtained, and BALB/c mice were immunized with these strains. After confirming antibody production against the target bacteria, the spleen and inguinal lymph nodes were harvested from the mice, and B cells were fused with the mouse myeloma cell line P3U1 to create hybridomas. Of the antibodies produced by each hybridoma, we excluded those that cross-reacted with non-target bacteria, as determined by performing simultaneous ELISAs using the target bacteria and major gut bacteria (non-target controls) as antigens. In addition to the three aforementioned bacterial species, we used two species of bifidobacteria common in the Japanese population (*Bifidobacterium pseudocatenulatum* and *B. longum*), *Blautia wexlerae*, and *Akkermansia muciniphila* (which is gaining attention for its potential in weight control in Western countries) as negative controls. To ensure accurate assessment, the bacterial cells used as antigens were serially diluted. We successfully established two mAbs against *P. vulgatus* (PV-L2B7-117K1 and PV-S10F7-14K1), three mAbs against *F. duncaniae* (FD-S2D3-18K1, FD-L4F6-18K2, and FD-L5B6-33K2), and one mAb against *S. copri* (SC-L10B5-35K1) (Fig. 1A). All antibodies were specifically reactive to target bacteria without non-specific binding to the other bacteria, and all were of the IgG class, with subclasses including IgG2a, IgG2b, and IgG3, depending on the clone (Fig. 1A).

In addition, when examining the reactivity of each clone, some clones showed weak reactivity (e.g., FD-L5B6-33K2 and SC-L10B5-35K1) (Fig. 1A). Because we suspected antigens were present not only on the bacterial surface but also inside the bacteria, an ELISA was performed using lysed bacterial samples. The reactivity of FD-L5B6-33K2 and SC-L10B5-35K1 to target bacteria was higher after lysis than in non-lysed conditions, (Fig. 1B), whereas the reactivity of the other clones was not changed (Supplementary Fig. 2). These results suggest that many of the antibodies we established recognize antigens expressed either on the bacterial surface or internally.

Flow cytometry detection of human gut microbiome enterotype

To enhance the applicability of these antibodies, we investigated their use in flow cytometry as one of the measurement methods. To maintain antigenicity, dead bacteria were fixed with Farmer's solution, a mixture of ethanol and acetic acid in a ratio of 7:3[18], and mAbs were reacted with the fixed bacteria. Binding was then confirmed by flow cytometry. Both mAbs targeting *P. vulgatus* reacted with the target bacteria, as did all three antibodies targeting *F. duncaniae* (Fig. 2A, B). The mAb targeting *S. copri* (SC-L10B5-35K1) weakly reacted with the target bacteria (Fig. 2C). During this screening process, we identified an antibody (SC-S10C3-49K1) that exhibited weaker reactivity than the SC-L10B5-35K1 antibody in direct ELISA against lysed bacteria (Fig. 1B and 3A), but reacted at the same level as SC-L10B5-35K1 in fluorescence-activated cell sorting (Fig. 3B). None of the antibodies reacted with non-target bacteria (Fig. 2 and supplementary Fig. 3), demonstrating the high specificity of these antibodies.

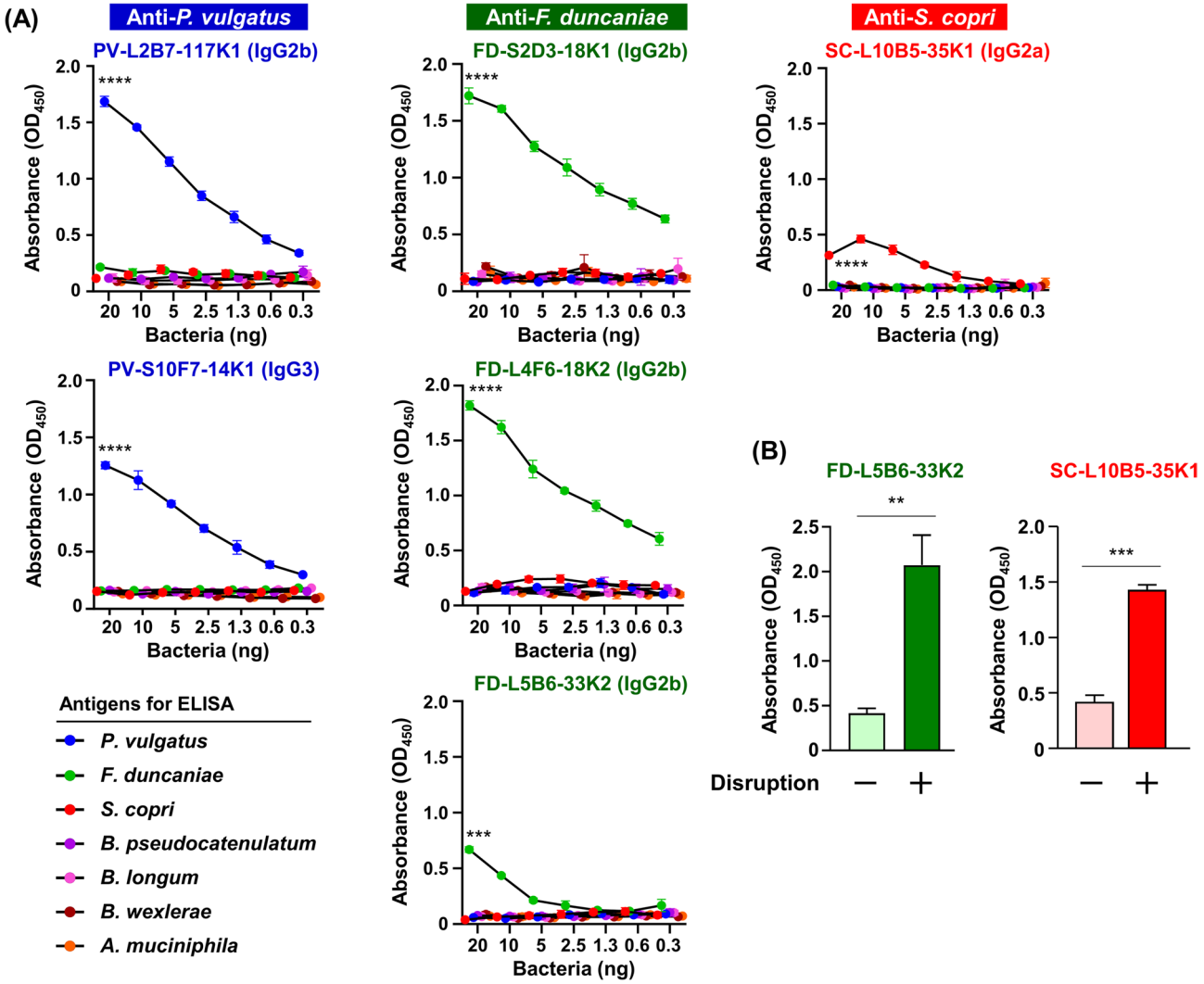

Fig. 1. Reactivity and specificity of monoclonal antibodies (mAbs) established in this study based on ELISA. (**A**) ELISA of mAbs that were specifically reactive to heat-killed representative bacteria of human gut microbiome enterotypes *Phocaeicola vulgatus* JCM5826, *Faecalibacterium duncaniae* JCM31915, and *Segatella copri* JCM13464, but not to other gut bacteria, including *Bifidobacterium pseudocatenulatum* JCM1200, *Bifidobacterium longum* subsp. *longum* JCM1217, *Blautia wexlerae* JCM31267, and *Akkermansia muciniphila* JCM33894. Representative data for selected clones PV-L2B7-117K1, PV-S10F7-14K1, FD-S2D3-18K1, FD-L4F6-18K2, FD-L5B6-33K2, and SC-L10B5-35K1 are shown. (**B**) Comparison of the reactivity of established mAbs that were specifically reactive to representative bacteria of human gut microbiome enterotypes *Faecalibacterium duncaniae* JCM31915 and *Segatella copri* JCM13464, as detected by ELISA, to bacterial cells with (+) or without (–) bead disruption. Representative data for selected clones FD-L5B6-33K2 and SC-L10B5-35K1 are shown. Data are the mean ± SD of two independent experiments. Statistical significance was determined using one-way ANOVA (**A**) and the Mann–Whitney U test (**B**). $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. OD_{450}, optical density at 450 nm.

Application to western blotting and identification of recognized molecules by proteome analysis

As a first step to identifying the molecules recognized by each mAb, we performed western blot analysis on the target bacteria. Of the antibodies reactive to *P. vulgatus*, PV-L2B7-117K1 showed two bands between 100 and 150 kDa (Fig. 4A). In contrast, PV-S10F7-14K1 recognized a molecule of approximately 15 kDa (Fig. 4A). Of the antibodies specific to *F. duncaniae*, FD-S2D3-18K1 and FD-L4F6-18K2 both recognized a molecule of approximately 18 kDa, suggesting that they recognize the same molecule. However, FD-L5B6-33K2 showed a band corresponding to a molecule of approximately 33 kDa (Fig. 4B). Of the antibodies specific to *S. copri*, SC-S10C3-49K1 recognized a molecule of approximately 49 kDa, whereas SC-L10B5-35K1 recognized a molecule of approximately 35 kDa (Fig. 4C).

Next, we immunoprecipitated the target bacterial suspensions, followed by proteome analysis of the samples[19] (Supplementary Table 1). As expected, there were several candidate proteins listed as molecules recognized by

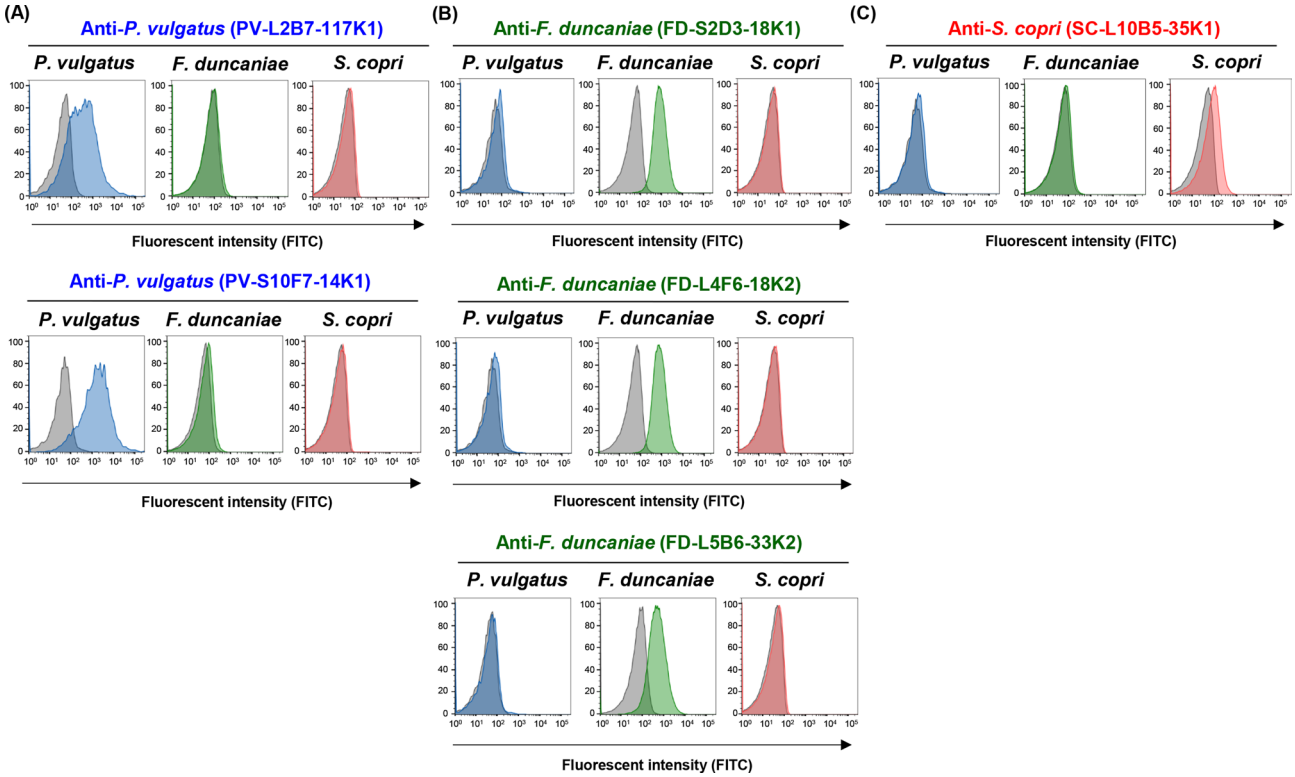

Fig. 2. Reactivity and specificity of monoclonal antibodies established in this study based on flow cytometry analysis. Reactivity of selected clones (PV-L2B7-117K1, PV-S10F7-14K1, FD-S2D3-18K1, FD-L4F6-18K2, FD-L5B6-33K2, and SC-L10B5-35K1) to representative bacteria of human gut microbiome enterotypes (**A**) *Phocaeicola vulgatus* JCM5826, (**B**) *Faecalibacterium duncaniae* JCM31915, and (**C**) *Segatella copri* JCM13464, as determined by flow cytometry. Representative histograms are shown for *P. vulgatus*, *F. duncaniae*, and *S. copri* strains treated with (blue, green or red) or without (gray) selected clones. Data are representative of two independent experiments. FITC, fluorescein isothiocyanate.

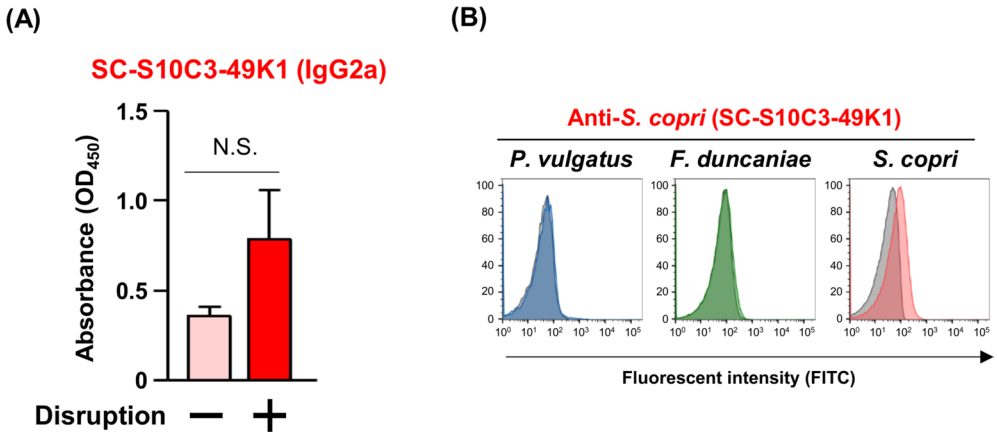

Fig. 3. Enhanced reactivity of SC-S10C3-49K1 monoclonal antibodies (mAbs) with access to cytoplasmic and cell-surface antigens. (**A**) Comparison of the reactivity of established mAbs that were specifically reactive to representative bacteria of human gut microbiome enterotypes *Segatella copri* JCM13464, as detected by ELISA, to bacterial cells with (+) or without (−) bead disruption. Representative data for selected clone SC-S10C3-49K1 are shown. Data are the mean ± SD of three independent experiments. Statistical significance was determined using the Mann–Whitney U test. OD_{450}, optical density at 450 nm. (**B**) Reactivity of selected clones to representative bacteria of human gut microbiome enterotypes *Phocaeicola vulgatus* JCM5826, *Faecalibacterium duncaniae* JCM31915, and *Segatella copri* JCM13464, as determined by flow cytometry. Representative histograms are shown for *P. vulgatus*, *F. duncaniae*, and *S. copri* strains treated with (blue, green or red) or without (gray) selected clones. Data are representative of two independent experiments. FITC, fluorescein isothiocyanate.

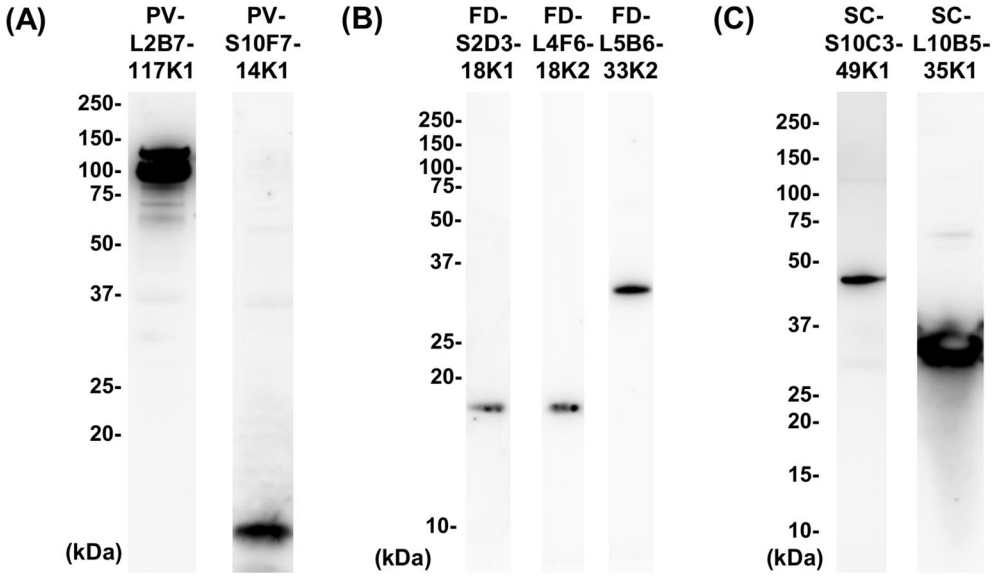

Fig. 4. Western blotting of target bacteria with the monoclonal antibodies (mAbs) established in this study to detect protein binding patterns. To identify whether distinct proteins were detected by the mAbs we isolated, we used them in western blotting against (**A**) *Phocaeicola vulgatus* JCM5826, (**B**) *Faecalibacterium duncaniae* JCM31915, and (**C**) *Segatella copri* JCM13464, as representatives of human gut microbiome enterotypes. Representative images are shown for the bands of lysates of *P. vulgatus*, *F. duncaniae,* or *S. copri* strains treated with selected clones. Data are representative of two independent experiments. The entire gel and blot are provided in the supplementary materials.

antibodies specific to *P. vulgatus* (Supplementary Table 1). Two bands were observed for the PV-L2B7-117K1 antibody in western blotting analysis; however, proteome analysis identified the DUF4988 domain-containing protein (UniProt: A6KZV4)[20] as a candidate from both bands (see Supplementary Table 1; data not shown). We subsequently constructed a plasmid expressing DUF4988 domain-containing protein and demonstrated that its protein expressed in *Escherichia coli* was recognized by PV-L2B7-117K1 (Fig. 5A). Using the same strategy, we found that PV-S10F7-14K1 recognizes TsaE (UniProt: A6L1K1; Fig. 5A).

Of the antibodies reactive to *F. duncaniae*, FD-S2D3-18K1 and FD-L4F6-18K2 were suggested to recognize the Tat pathway signal sequence domain protein (UniProt: C7H2H8), and this reactivity was confirmed in *E. coli* expressing the protein (Fig. 5B). In addition, FD-L5B6-33K2 was suggested to recognize 3-oxoacyl-[acyl-carrier-protein] synthase (FabH; UniProt: C7H9R4[21]; Supplementary Table 1 and Fig. 5B).

Of the antibodies reactive to *S. copri*, SC-S10C3-49K1 was suggested to recognize glutamate dehydrogenase (gdhA; UniProt: D1PC66[22]; Supplementary Table 1), and this reactivity was confirmed when the protein was expressed in *E. coli* (Fig. 5C). SC-L10B5-35K1 was shown to recognize the GGGtGRT protein (UniProt: A0AA90ZMQ0; Supplementary Table S1 and Fig. 5C).

Quantitative determination of bacteria in human stool samples using sandwich ELISA

We then used these antibodies in a sandwich ELISA to try to quantify the bacteria. As a counterpart to the PV-L2B7-117K1 antibody, we used PV-L1A6-117K2, which was identified during the screening process. Although the reactivity of PV-L1A6-117K2 in direct ELISA was not as high as that of PV-L2B7-117K1, it did recognize the same DUF4988 protein (Supplementary Fig. 4). Indeed, a dose-dependent sandwich ELISA was successfully established for the two antibodies against *P. vulgatus* (PV-L2B7-117K1 and PV-L1A6-117K2), with a detection limit of approximately 10^7 CFU (Fig. 6A, B). Similarly, for the two antibodies targeting the Tat pathway signal sequence domain protein of *F. duncaniae* (FD-S2D3-18K1 and FD-L4F6-18K2), we confirmed that a sandwich ELISA could be established with a detection limit of approximately 10^7 CFU.

For *S. copri*, it was not possible to obtain two antibodies that recognized the same molecule. Because epitope competition occurs when using the same clone for a sandwich ELISA, it was anticipated that it would not be suitable for this assay. However, for reasons that remain unclear, among the mAbs for *S. copri*, SC-S10C3-49K1 was able to detect and quantify *S. copri* with comparable sensitivity of the antibodies which bind to other bacteria (Fig. 6C). We then produced recombinant gdhA protein (an antigen recognized by the SC-S10C3-49K1 antibody), immunized mice, and collected serum to attempt a sandwich ELISA. Sera from two of three mice showed favorable results, but the reaction was weak in one mouse (Supplementary Fig. 5).

Next, we attempted to quantify the bacterial cells present in human fecal samples using a sandwich ELISA with these antibody pairs. Using lysed human fecal samples as antigens, we were able to successfully count bacteria with good accuracy (Fig. 7A). We found a high correlation between enterotype results obtained by the sandwich ELISA and the numbers calculated from a *16S* rRNA analysis (Fig. 7B).

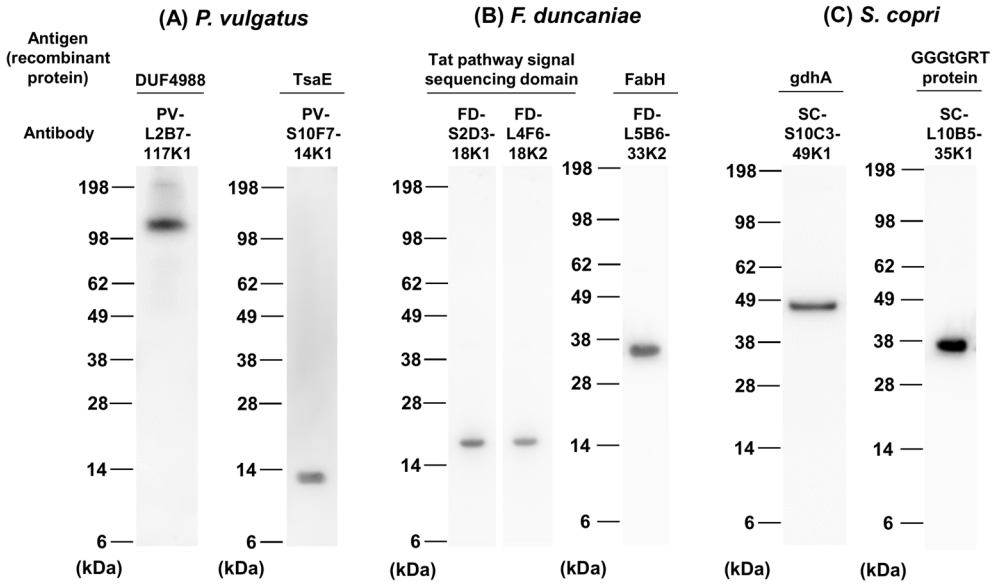

Fig. 5. Recognition of candidate proteins expressed from *Escherichia coli* by the monoclonal antibodies (mAbs) established in this study. Candidate target proteins were expressed in *E. coli* and subjected to western blotting with our established mAbs that were specifically reactive to (**A**) *Phocaeicola vulgatus* JCM5826, (**B**) *Faecalibacterium duncaniae* JCM31915, and (**C**) *Segatella copri* JCM13464. Representative images are shown. Data are representative of two independent experiments. The entire gel and blot are provided in the supplementary materials.

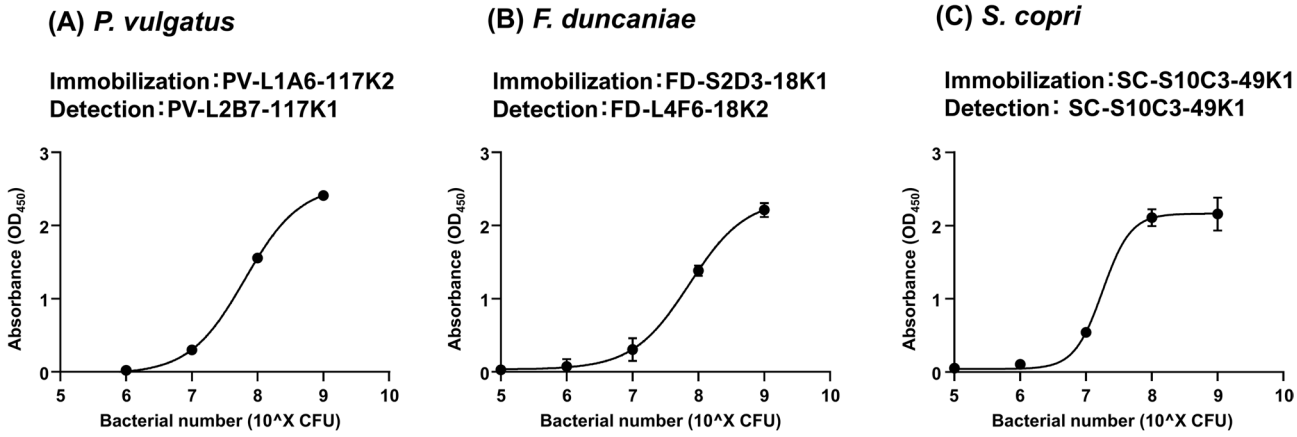

Fig. 6. Number–absorbance curves for the bacteria representative of human gut microbiome enterotypes. The monoclonal antibodies (mAbs) established in this study were used in sandwich ELISA with known numbers (CFU) of representative bacteria to create absorbance curves for (**A**) *Phocaeicola vulgatus* JCM5826, (**B**) *Faecalibacterium duncaniae* JCM31915, and (**C**) *Segatella copri* JCM13464. Representative data are shown for the sigmoid curve of absorbance and bacterial count of *P. vulgatus*, *F. duncaniae*, or *S. copri* strains. Data are presented as the mean ± SD and are representative of two independent experiments. OD_{450}, optical density at 450 nm.

Discussion

In this study we established mAbs targeting gut bacteria commonly found in the Japanese population as a technical foundation for the rapid, affordable, and simple measurement of gut bacteria. These mAbs not only detected cultured bacteria in ELISA, flow cytometry, and western blot analyses, but also enabled the quantification of specific bacterial counts in human fecal samples via sandwich ELISA, in combination with additional antibodies. The proportions of the three bacteria identified with this method were comparable to those obtained using *16S* rRNA data, suggesting that this method could be useful in identifying enterotypes in the Japanese population. In addition, recent studies have shown that *P. vulgatus* modulates immune function[23] and that *F. duncaniae* could be a beneficial bacterium for human health[24]. Because the antibodies established in this study can be used

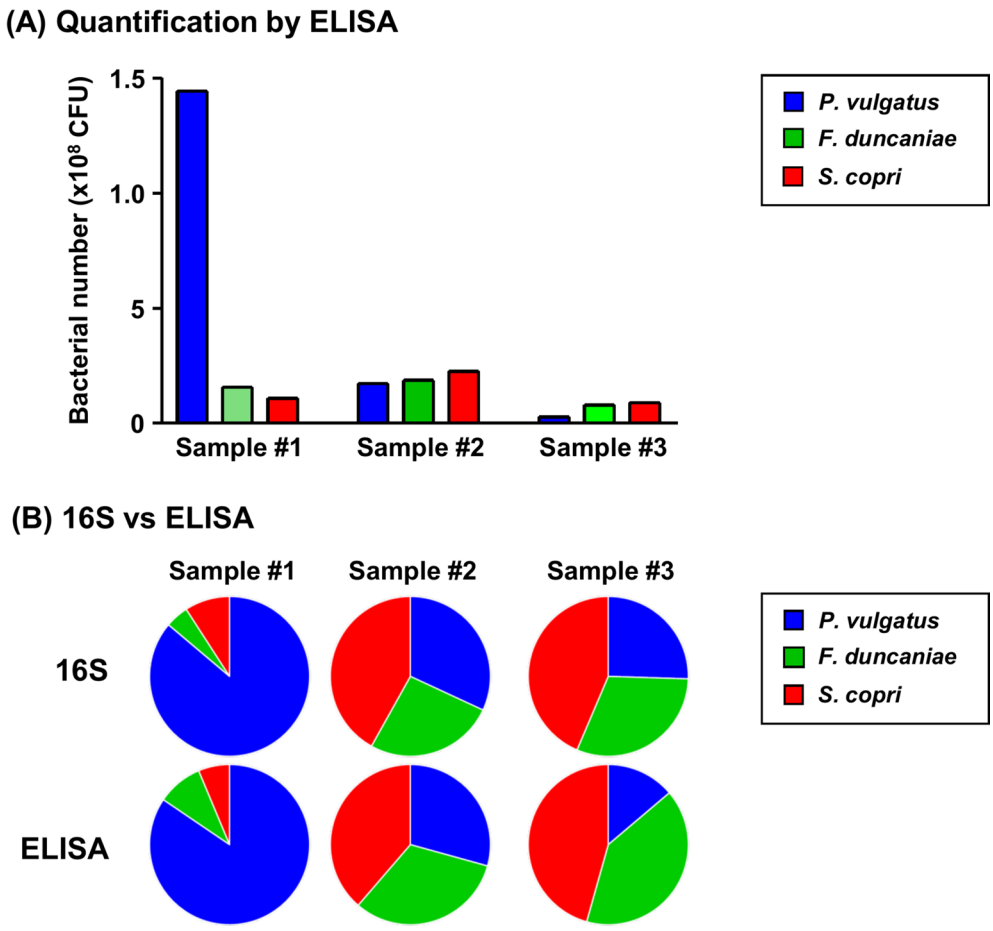

Fig. 7. Agreement between results of *16S* rRNA gene amplicon sequencing analysis and sandwich ELISA. (**A**) Bacterial numbers, as detected by sandwich ELISA, in human fecal samples using established monoclonal antibodies (mAbs) that were specifically reactive to representative bacteria of human gut microbiome enterotypes *Phocaeicola vulgatus* JCM5826, *Faecalibacterium duncaniae* JCM31915, and *Segatella copri* JCM13464. (**B**) Agreement between the results of *16S* rRNA gene amplicon sequencing analysis and sandwich ELISA of human fecal samples. The analysis was conducted using fecal samples from three different individuals. Data are representative of two independent experiments, confirming that our antibodies can be used to quantify these species of bacteria in human samples.

to develop immunochromatography and latex agglutination assays, they may be useful tools for the rapid and simple detection of beneficial bacteria.

It has been well established in previous studies[25,26], including our own one[12], that immunization with whole bacterial cells commonly yields antibodies against molecules expressed on the bacterial surface membrane. In the present study, we were able to generate several monoclonal antibodies reactive to surface antigens. Also, some antibodies are not applicable to flow cytometry, but showed increased ELISA reactivity after bacterial lysis, suggesting that they recognized molecules located inside the bacteria. They functioned effectively in western blot and ELISA, demonstrating their potential utility in diagnostic applications. Indeed, in the bacterial lysis condition, sandwich ELISA can be used for the enumeration of target bacteria as the detection limit of is approximately 10^7 CFU. It is known that bacterial loads in human feces typically range from 10^{10} to 10^{12} CFU per gram[27,28] and therefore, our assay sensitivity is within the practical range for quantifying target bacteria in human fecal samples. In these cases, ELISA does not specifically detect live bacteria because the bacterial cells are lysed during sample preparation, which is one of the limitations of this system. In this issue, while not all antibodies developed in this study are currently suitable for flow cytometry, we have confirmed that combining antibodies with commercially available Live/Dead bacterial staining kits allows us to distinguish viable from non-viable cells. We are currently working to expand the panel of antibodies applicable to flow cytometry and to optimize their use in combination with the Live/Dead bacterial staining system.

Although it is preferable that the antibody recognizes molecules expressed on the cell surface for flow cytometry, and dedicated equipment is required, it is expected that flow cytometry will be a useful technique because it can quickly measure the proportions and numbers of bacteria. Because cultured bacteria could be detected by flow cytometry using the antibodies we identified, it is anticipated that these antibodies could also be used to measure bacteria in fecal samples. In preliminary investigations, bacteria could be measured in some

human fecal samples, but not in others. One possibility is that the freezing of fecal samples led to the formation of ice crystals, which either destroyed the bacteria or disrupted the structure of the recognition molecules, making detection difficult[29]. Therefore, it is necessary to examine pretreatment conditions, such as the use of preservation solutions, for flow cytometry.

We also identified the molecules recognized by each mAb. Furthermore, after producing the identified molecules as recombinant proteins and immunizing mice, we examined the specificity of the resulting sera against bacteria. We found that some antibodies also reacted with other bacteria[30]. We also generated mAbs from these mice, but unfortunately these antibodies did not show specificity towards the target bacteria (data not shown). This is not surprising because the antigen molecules are expressed in other bacteria, resulting in cross-reactivity. It is likely that the initially established highly specific antibodies recognize unique regions of the bacteria, including the three-dimensional structure. In fact, by using whole bacterial immunization and unbiased screening, we were able to establish antibodies that recognize specific target bacteria. Recently, we used a similar strategy targeting *Campylobacter*, whereby we used whole bacterial immunization to establish unbiased antibodies. We successfully obtained an antibody that reacted with *Campylobacter jejuni* but not *Campylobacter coli*, and confirmed that the recognized molecule was QcrC, with the antibody discriminating based on a minor difference in the amino acid sequence of QcrC[12]. Interestingly, even among clones established from different mice, some recognized the same molecule; this has also been observed in the case of antibodies against other bacteria we are working on. These findings suggest that bacterial species–specific molecules are rare, and that most antibodies recognize highly immunogenic and specific sequences within shared molecules.

When examining the recognized molecules, we found that the PV-L2B7-117K1 and PV-L1A6-117K2 antibodies, which are specific to *P. vulgatus*, recognize a DUF4988 domain-containing protein, a protein domain found in many bacteria[31]. "DUF" refers to "domain of unknown function," indicating that these proteins belong to a family whose functions have not yet been fully elucidated. With regard to the two protein bands between 75 and 150 kDa recognized by PV-L2B7-117K1 and PV-L1A6-117K2, we found that both bands likely correspond to DUF4988 domain-containing protein. Given that the theoretical molecular weight of DUF4988 domain-containing protein is 117 kDa, the presence of a lower molecular weight band implies that the antibody may recognize a truncated form or a degradation product of DUF4988. Furthermore, these two bands were observed in *Bacteroides* but not in recombinant *E. coli* expressing DUF4988 domain-containing protein. This suggests the possibility of a *Bacteroides*-specific mechanism affecting DUF4988 domain-containing protein, such as post-translational modification or processing, making it an intriguing subject for future research in addition to elucidating the biological function of DUF4988 domain-containing protein. The GGGtGRT protein, recognized by SC-L10B5-35K1 (UniProt: A0AA90ZMQ0), is a bacterial protein named after part of its amino acid sequence. Like DUF proteins, its precise function and biological role remain unclear. Future studies using PV-L2B7-117K1, PV-L1A6-117K2, and SC-L10B5-35K1 antibodies as tools may reveal the functions of these target proteins and their specific roles in different bacteria.

The PV-S10F7-14K1 antibody recognizes TsaE. In bacteria like *Bacteroides*, TsaE is part of a conserved protein complex including TsaB and TsaD that is involved in the threonylcarbamoyladenosine (t6A) modification at position 37 of tRNAs. This modification stabilizes the anticodon loop, ensuring accurate protein translation. TsaE interacts with TsaB and TsaD to regulate t6A synthesis, likely by controlling tRNA binding during the reaction sequence[32]. The FD-L5B6-33K2 antibody, which is specific to *F. duncaniae* among the species we examined, recognizes FabH. FabH, known as β-ketoacyl-ACP synthase III, catalyzes the reaction between acetyl-CoA and malonyl-ACP to produce a C4 acyl group, which is then used in the elongation of fatty acids. FabH has been reported to be present in many bacteria[21], and is being studied as a target for antibiotics[33]. Because other bacteria also possess these molecules, how each antibody exhibits specificity towards the target bacteria remains to be determined in future investigations.

The FD-S2D3-18K1 and FD-L4F6-18K2 antibodies specific to *F. duncaniae* were suggested to recognize the Tat pathway signal sequence domain protein (UniProt: C7H2H8). This protein is involved in the twin-arginine translocation (Tat) system, which transports fully folded proteins across the cell membrane. These proteins have a specific signal peptide with a twin-arginine (RR) motif that guides them through the Tat system. This pathway is critical for exporting proteins that require folding or cofactor addition in the cytoplasm before they can function outside the cell.

The SC-S10C3-49K1 antibody, which targets *S. copri*, recognizes *gdhA*, a gene encoding the enzyme glutamate dehydrogenase (GDH). This enzyme plays a crucial role in glutamate metabolism by catalyzing the reaction between ammonia and 2-oxoglutarate to produce glutamate[34,35]. GDH is widely expressed in various microorganisms and is used in industrial bioprocesses for its role in nitrogen metabolism[35]. The SC-S10C3-49K1 antibody could potentially serve as a tool to analyze the specific characteristics of GDH in different bacterial species given its diverse applications in microbial systems. Furthermore, the SC-S10C3-49K1 antibody could be used in a sandwich ELISA for the enumeration of bacteria. In a typical sandwich ELISA, the capture and detection antibodies need to recognize different epitopes like antibody pairs used in this study for *P. vulgatus* and *F. duncaniae*, they likely bind to different, non-overlapping epitopes. Therefore, using the same antibody may lead to epitope competition. However, there may be instances when a sandwich ELISA can function without competition, even when using identical antibodies, such as when (1) the antigen has multiple epitopes; (2) the antigen forms dimers, multimers, or cluster on membranes; and (3) the antigen is immobilized at a high density on the solid phase, allowing identical antibodies to bind to adjacent antigen molecules. Because we have established such antibodies by immunizing with whole bacteria, it is possible that antigens with these characteristics were preferentially selected as highly immunogenic molecules and became the targets of antibody production.

In this study we established mAbs using representative bacterial species from the *Bacteroides*, *Faecalibacterium*, and *Prevotella* genera, which are predominant in the gut microbiota of the Japanese population. These antibodies

did not show cross-reactivity with representative bacteria from other genera, but it remains unclear to what extent they may cross-react with other species within the same genus. To address this, we are currently developing a protocol using flow cytometry to sort bacteria bound to each antibody in human fecal samples for identification. We anticipate that this approach will clarify the extent of cross-reactivity.

At this stage, we have already demonstrated that the sandwich ELISA using these mAbs can quantify the target bacteria in human fecal samples. We are also in the process of developing antibodies to detect beneficial bacteria, such as *Bifidobacterium* and *Blautia*, abundant in the Japanese population, as well as *Akkermansia*, which is relatively rare in the Japanese population, although more abundant in non-Japanese individuals, and exhibits beneficial functions. Conversely, we are also working on antibodies to detect bacteria associated with inflammatory bowel disease and cancer, such as *Fusobacterium*. Although the aim of the present study was to demonstrate the feasibility of this strategy using a relatively small number of samples from Japanese individuals, we are also preparing to expand our antibody lineup and conduct validation studies in non-Japanese populations. To facilitate this, we are preparing the necessary ethics approvals and related procedures.

The antibodies developed in this study focus on gut bacteria specific to the Japanese population; however, it is important to consider developing antibodies that target the gut microbiota of different populations because microbial composition can vary significantly across different regions and ethnic groups. We believe that by following similar protocols, it will be possible to detect and measure these bacteria in fecal samples. Compared with conventional analysis, such as *16S* rRNA metagenomic analysis, the assay we have described here does not require specialized equipment, such as next-generation sequencers. Instead, it uses ELISA, a widespread detection technique, which significantly reduces costs and labor while enabling rapid and straightforward testing. Even with the current sensitivity, measurement is feasible on a scale used in health checkups, and thus the development of diagnostic kits is also underway. By expanding these technologies, we expect to develop a system that allows anyone to easily measure bacteria of interest, contributing to the maintenance and promotion of personal health.

Methods

Study approval

Animal experiments were approved by the Animal Care and Use Committees of the National Institutes of Biomedical Innovation, Health, and Nutrition (Approval no. DSR04-37R7 and DSR04-38R7) and were conducted in accordance with their guidelines. The collection of human fecal samples was approved by the Ethics Committee of the National Institutes of Biomedical Innovation, Health, and Nutrition and was conducted in accordance with their guidelines (Approval no. 154 and 177). Informed consent was obtained from all participants. This study was conducted and reported in accordance with the ARRIVE guidelines.

Bacterial culture

P. vulgatus (JCM5826), *F. duncaniae* (JCM31915), *S. copri* (JCM13464), *B. pseudocatenulatum* (JCM1200), *B. longum* subsp. longum (JCM1217), *B. wexlerae* (JCM31267), and *A. muciniphila* (JCM33894) were provided by the RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan. All bacterial strains were cultured aerobically at 37 °C for 48 h in an anaerobic chamber (Bactron 300; Toei Kaisha, Tokyo, Japan). Precultured bacterial cells were transferred to fresh liquid medium to reach an optical density at 600 nm (OD_{600}) of 0.1, and then cultured overnight at 37 °C. For all experiments, *P. vulgatus*, *F. duncaniae*, *S. copri*, *B. pseudocatenulatum*, *B. longum* subsp. *longum*, and *B. wexlerae* were cultured anaerobically in YCFA medium (composition available at https://www.jcm.riken.jp/cgi-bin/jcm/jcm_grmd?GRMD=1130). *A. muciniphila* was cultured anaerobically in Bacto Brain Heart Infusion medium (Becton Dickinson).

Lysis and bead disruption of bacteria

To prepare lysed and bead-disrupted bacteria, the bacteria were suspended in 1 mL B-PER complete bacterial protein extraction reagents (Thermo Scientific) and 0.5 g of 0.1-mm glass beads (BioSpec Products). The mixture was mechanically disrupted three times by bead beating using a Cell Destroyer PS1000 (Bio Medical Science, Tokyo, Japan) at 4260 rpm for 50 s at room temperature. After centrifugation at 13,040 × *g* for 5 min at 4 °C to remove bubbles, the beads, pellet, and supernatant were mixed and filtered through a 100-µm cell strainer (FALCON). The supernatant was collected and used for experiments (e.g., direct or sandwich ELISA).

Development of hybridomas and screening for specific clones

Female BALB/c mice (age 8–10 weeks) were purchased from CLEA Japan (Tokyo, Japan). *P. vulgatus*, *F. duncaniae*, and *S. copri* were heat killed by incubation at 60–70 °C for 30 min. These heat-killed bacteria were suspended in phosphate-buffered saline (PBS) at a concentration of 2×10^{10} CFU/mL, and the cell suspensions were mixed 1:1 with Sigma Adjuvant System (Sigma). Mice were immunized once or several times by footpad injection with 100 µL of the mixture (10^9 CFU/mouse). Then, 7–14 days after injection, mice were killed by cervical dislocation under 2% isoflurane (FUJIFILM) anesthesia. Popliteal lymph nodes, inguinal lymph nodes, and the spleen were collected, and cell fusion using P3U1 myeloma cells was performed as described previously[10]. Culture supernatants were tested for specific antibodies 14 days after fusion by ELISA, as described previously[12]. Positive clones were separated by limiting dilution, and mAbs were purified using Protein G Sepharose 4 Fast Flow (Cytiva). Reactivity and specificity were confirmed by ELISA.

ELISA

For ELISA, 96-well immunoplates (Thermo Fisher Scientific) were coated with 100 µL lyophilized heat-killed bacteria (0.2 mg/mL) diluted with PBS or bacterial cells suspended in B-PER solution and disrupted using glass beads at 4 °C overnight. The plates were blocked with 1% (w/v) bovine serum albumin (Nacalai Tesque)

in PBS for 2 h at room temperature to prevent non-specific binding. After the plates had been washed with PBS containing 0.05% Tween 20 (PBS-T; Nacalai Tesque), culture supernatants were added to the wells and the plates were incubated for 2 h at room temperature. The plates were then washed with PBS-T and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotech) for 1 h at room temperature. After the incubation, the plates were again washed with PBS-T, after which tetramethylbenzidine peroxidase substrate was added to the wells and the plates were incubated for 2 min. Absorbance was then measured at 450 nm using an iMark microplate reader (Bio-Rad).

Sandwich ELISA

For the sandwich ELISA, 96-well immunoplates were coated with 100 µL capture antibody (10 µg/mL) diluted with PBS and incubated overnight at 4 °C. The plates were then blocked with 1% (w/v) bovine serum albumin in PBS for 2 h at room temperature. The plates were then washed with PBS-T. B-PER-treated bacteria or human feces mechanically disrupted by bead beating were suspended in 1% BSA-PBS-T and added to the wells (100 µL/well), followed by incubation at 37 °C for 1 h. The plates were then washed in PBS-T, biotin-labeled antibody (0.5 µg/mL) diluted in 1% BSA-PBS-T was added to the wells (100 µL/well), and the plates were incubated for 1 h at 37 °C. Biotin labeling of the antibody was performed using a Biotin Labeling Kit-NH_2 (DOJINDO) in accordance with the manufacturer's instructions. The plates were then washed with PBS-T, HRP-conjugated streptavidin diluted in 1% BSA-PBS-T (1:10,000 dilution) was added to the wells (100 µL/well), and the plates were incubated for 1 h at room temperature. After the plates had been washed with PBS-T, tetramethylbenzidine peroxidase substrate was added to the wells and the plates were incubated for 2 min. Absorbance was then measured at 450 nm using an iMark microplate reader (Bio-Rad).

Sandwich ELISA using human fecal samples

Human fecal samples were defrosted and then weighed to 0.2 g. To isolate bacteria from human fecal samples, weighed feces were placed on a 100-µm cell strainer and 3 mL PBS was added. The weighed feces were then gently rubbed using the rubber stopper of a syringe. The sample solution that passed through the 100-µm filter was transferred to a 15-mL tube and centrifuged at 13,040×*g* for 2 min at 4 °C. After centrifugation, the supernatants were carefully discarded, and the pellets were slowly resuspended in 1 mL B-PER. Subsequent procedures for preparing lysed and bead-disrupted bacterial samples and for the sandwich ELISA were as described above. To prepare the standard curve required for the absolute quantification of bacterial counts, the B-PER-lysed and bead-disrupted bacterial cells were serially diluted and used as samples in the sandwich ELISA.

Western blot analysis

Bacterial cells were harvested and collected by centrifugation at 10,000×*g* for 5 min at room temperature. Electrophoresis of the bacterial protein (2 µg) was performed using a NuPAGE electrophoresis system (Life Technologies) with a 4–12% discontinuous Bis–Tris gel and MES buffer. SeeBlue Plus2 prestained standard (Thermo Fisher Scientific) was used as the molecular weight size marker. Separated proteins were transferred to polyvinylidene difluoride membranes (EMD Millipore Corporation). The transferred membranes were blocked with 4% skim milk (Nacalai Tesque) diluted in PBS-T overnight at 4 °C. After the transferred membranes were washed with PBS-T, established mAbs (25–50 ng/mL) and HRP-conjugated anti-mouse IgG (Southern Biotech) were suspended in Can Get Signal Immunoreaction Enhancer Solution (TOYOBO) and reacted with the membranes for 1 h at room temperature. The transferred membranes were then washed with PBS-T and reacted with Chemi-Lumi One Super (Nacalai Tesque), and images were captured with an ImageQuant LAS 4000 instrument (Fujifilm).

Flow cytometry analysis

Bacterial cells were harvested, suspended in Farmer's fixative (ethanol: acetic acid = 7:3), and fixed at room temperature for 30 min (2×10^8 CFU/mL). After fixation, the bacterial cell suspension was centrifuged at 13,040 × *g* for 2 min at 4 °C. Bacterial cells (100 µL/tube) and established mAbs (1 µg/tube) were suspended in 1% BSA-PBS-T and incubated on ice for 1 h. Cells were washed twice with 1% BSA-PBS-T and incubated with fluorescein isothiocyanate-labeled anti-mouse IgG (clone Poly40553; BioLegend) on ice at 4 °C for 30 min in the dark. The stained bacterial cells were washed twice with 1% BSA-PBS-T. The stained samples were analyzed using a MACSQuant analyzer (Miltenyi Biotech), and data were analyzed with FlowJo 9.9 software (Tree Star).

Immunoprecipitation and proteome analysis

Bacteria were harvested and collected by centrifugation at 10,000 × *g* for 5 min at room temperature. Bacterial cells were suspended at a concentration of 20 mg/mL in B-PER bacterial protein extraction reagent (Thermo Fisher Scientific) containing 1 mM EDTA (~ 200 µL) and incubated for 15 min at room temperature. The suspensions were then centrifuged at 16,000×*g* for 20 min at room temperature, and the supernatant mixed with Protein G Sepharose 4 Fast Flow (Cytiva) in the absence (mock) or presence of mAb before being incubated on a rotary shaker for 1 h at 4 °C. The suspension was then centrifuged at 12,000×*g* for 20 min at room temperature and the pellet collected for subsequent electrophoresis using a NuPAGE electrophoresis system (Life Technologies) with a 4–12% discontinuous Bis–Tris gel and MES buffer. SeeBlue Plus2 prestained standard (Thermo Fisher Scientific) was used as the molecular weight size marker. Total protein was stained with a Pierce Silver Stain kit (Thermo Fisher Scientific). The gel band was cut out and subjected to in-gel tryptic digestion, essentially as described previously[36]. Briefly, gel pieces were destained and washed, and, after dithiothreitol reduction and iodoacetamide alkylation, the proteins were digested with porcine trypsin (modified sequencing grade; Promega) overnight at 37 °C. The resulting tryptic peptides were extracted from the gel pieces consecutively with 30% acetonitrile containing 0.3% trifluoroacetic acid and then 100% acetonitrile. The extracts were evaporated

in a vacuum centrifuge to remove organic solvent, then desalted and concentrated with reverse-phase C18 StageTips[36,37].

Liquid chromatography–tandem mass spectrometry was performed with an UltiMate 3000 Nano LC system (Thermo Scientific) and an HTC-PAL autosampler (CTC Analytics) coupled to a Q Exactive mass spectrometer (Thermo Scientific). Forty-five-minute gradients from 5 to 30% Buffer B were used. The Q Exactive instrument was operated as described previously[38]. Raw mass spectrometry data were processed with MaxQuant (1.6.14.0) using the Andromeda search engine and UniProt *Phocaeicola vulgatus* (JCM5826), *Faecalibacterium duncaniae* (JCM31915), and *Segatella copri* (JCM13464). The following parameter settings were used in the search: fully tryptic peptides with a minimum of seven amino acids, maximum two missed tryptic cleavage sites, and dynamic modifications allowed on the protein N-terminus (42.0106 Da/acetyl) and methionine (15.9949 Da/oxidation). For peptide spectrum match (PSM) validation, a forward/reverse concatenated database was used. The probability thresholds for all PSM/protein-level false discovery rates were set at confidence thresholds of 0.01.

Preparation of recombinant protein

Recombinant protein was prepared as described previously[39,40]. Briefly, candidates were cloned into pET16b (Invitrogen) at *Nde*I and *BamH*I cloning sites using ligation high ver.2 (TOYOBO). To obtain recombinant His-tagged proteins, the pET16b vector was transfected into *E. coli* strain BL21 containing DE3 (NEB) and protein production was induced in accordance with the manufacturer's instructions. The culture pellet was sonicated in Buffer A (10 mM Tris–HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol) and the recombinant protein was purified on an NGC chromatography system (Bio-Rad) with a HisTrap HP column (Cytiva). The purified protein was loaded onto a PD-10 column (Cytiva) for exchange into PBS and its concentration was measured using a BCA protein assay kit (Life Technologies). The purity of the protein was confirmed with a NuPAGE electrophoresis system (Life Technologies) followed by staining with Coomassie Brilliant Blue.

16S rRNA gene amplicon sequencing analysis

DNA was extracted from human fecal samples in guanidine thiocyanate solution using the bead-beating method and an automatic nucleic acid extraction system (Gene Prep Star PI-80X; Kurabo Industries), as described previously[41] with slight modification. Briefly, a human fecal sample was placed in a 2-mL vial (Wakenbtech) containing 0.5 mL lysis buffer (No. 10; Kurabo Industries) and 0.5 g of 0.1-mm glass beads (BioSpec Products). The mixture was mechanically disrupted by bead beating by using a Cell Destroyer PS1000 (Bio Medical Science) at 4260 rpm for 50 s at room temperature. After centrifugation at 13,000×*g* for 5 min at room temperature, DNA was extracted from a 0.2-mL sample of the supernatant using a Gene Prep Star PI-80X device (Kurabo Industries).

The *16S* rRNA gene amplicon in human fecal DNA was sequenced as described previously[41]. The V3–V4 region of the *16S* rRNA gene was amplified from fecal DNA samples using the following primers: forward, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGCGACAGCCTACGGGNGGCWGCAG-3′; and reverse, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′[42]. A DNA library was prepared with a Nextera kit Set A (Illumina, San Diego, CA, USA), and *16S* rRNA gene sequencing was performed using MiSeq (Illumina) in accordance with the manufacturer's instructions. The sequencing results were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) software package[43] and QIIME Analysis Automating Script (Auto-q; https://doi.org/10.5281/zenodo.1439555), as described previously[44]. Open-reference operational taxonomic unit (OTU) picking and taxonomy classification were performed based on sequence similarity (>97%) using UCLUST software[45] with the SILVA v128 reference sequence[46].

Statistical analysis

Statistical significance was evaluated by one-way ANOVA for comparison of multiple groups and the Mann–Whitney U test for two groups using Prism 10 (GraphPad Software).

Data availability

The sequencing data generated in this study are available in the DDBJ Sequence Read Archive under the accession numbers PRJDB10529 and PRJDB10530.

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Author contributions

The author's responsibilities were as follows: K.Y. and J.K. conceived and designed the study, performed data analysis, and wrote the manuscript; K.Y., E.N., M.F., Y.T., and A.M. performed the experiments and discussed the results; J.A. and N.T. contributed to data acquisition of proteome analysis; M.M. and K.H. contributed to data acquisition of *16S* rRNA gene amplicon sequencing analysis and discussed the results; all authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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