GUT MICROBES
2022, VOL. 14, NO. 1, e1997293 (17 pages)
https://doi.org/10.1080/19490976.2021.1997293

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RESEARCH PAPER

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Classification of *Parabacteroides distasonis* and other *Bacteroidetes* using O-antigen virulence gene: *RfbA*-Typing and hypothesis for pathogenic vs. probiotic strain differentiation

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ABSTRACT

Parabacteroides distasonis (*Pdis*) is the type species for the new *Parabacteroides* genus, and a gut commensal of the *Bacteroidetes* phylum. Emerging reports (primarily based on reference strain/ATCC-8503) concerningly propose that long-known opportunistic pathogen *Pdis* is a probiotic. We posit there is an urgent need to characterize the pathogenicity of *Pdis* strain-strain variability. Unfortunately, no methods/insights exist to classify *Bacteroidetes* for this purpose. Herein, we developed a virulence gene-based classification system for *Pdis* and *Bacteroidetes* to facilitate pathogenic-vs-probiotic characterization. We used DNA *in silico* methods to develop a system based on the virulence (lipopolysaccharide/bacterial wall) '*rfbA* O-antigen-synthesis gene'. We then performed phylogenetic analysis of *rfbA* from fourteen *Pdis* complete genomes (21 genes), other *Parabacteroides, Bacteroidetes*, and *Enterobacteriaceae*; and proposed a PCR-based Restriction-Fragment Length Polymorphism method. Cluster analysis revealed that *Pdis* can be classified into four lineages (based on gene gaps/insertions) which we designated *rfbA*-Types I, II, III, and IV. In context, we found 14 additional *rfbA*-types (I–XVIII) interspersed with numerous *Bacteroidetes* and pathogenic *Enterobacteriaceae* forming three major "*rfbA*-superclusters." For laboratory *rfbA*-Typing implementation, we developed a PCR-primer strategy to amplify *Pdis rfbA* genes (100%-specificity) to conduct MboII-RFLP and sub-classify *Pdis*. *In-silico* primers for other *Bacteroidetes* are proposed/discussed. Comparative analysis of lipopolysaccharide/lipid-A gene *lpxK* confirmed *rfbA* as highly discriminant. In conclusion, *rfbA*-Typing classifies *Bacteroidetes/Pdis* into unique clusters/superclusters given *rfbA* copy/sequence variability. Analysis revealed that most pathogenic *Pdis* strains are single-copy *rfbA*-Type I . The relevance of the *rfbA* strain variability in disease might depend on their hypothetical modulatory interactions with other O-antigens/lipopolysaccharides and TLR4 lipopolysaccharide-receptors in human/animal cells.

ARTICLE HISTORY
Received 25 June 2021
Revised 4 October 2021
Accepted 18 October 2021

KEYWORDS
Genomic based classification; Bacteroidetes; *Escherichia coli*; *Salmonella*; O-antigen serotyping; RFLP; MboII enzyme; superclusters; Alistipes; Prevotella; Bacteroides

Introduction

Parabacteroides distasonis (*Pdis*), a gram-negative bacterium of the intestinal tract,[1] is the type strain for the genus *Parabacteroides*, for which there is emerging controversy regarding the role that they play in human and animal health.[2] Although *Pdis* has been recognized as an intestinal commensal since the mid-1930s,[3] there is a recent increase in reports describing contradictory pathogenic (detrimental)[4–9] and probiotic (beneficial)[10–17] effects on human and animal health. There is need to identify and characterize factors that could account for reported differences in potential *Pdis* strain pathogenicity, especially because there is an emerging interest in using *Pdis* as a human probiotic, which poses a major risk to public health. To date, such contradictions indicate that the differences in the health effects could be due to strain differences. Unfortunately, there are no classification or cataloging systems to help typify *Pdis* into lineages using a virulence meaningful approach.

Extrapolating from prior research on *E. coli* and *Salmonella*,[18–20] strain dependent mechanisms linked to bacterial surface markers, such as the O-antigen, could be used to help guide research and help propose studies to determine the causes

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Supplemental data for this article can be accessed on the publisher's website.

that lead to the varied effects observed for *Pdis* on human and animal health. The O-antigen is a key virulence molecule of lipopolysaccharides (LPS) constitutively expressed on the cell wall surface of gram-negative bacteria. Lipopolysaccharide is a well understood virulence factor for gram-negative bacteria, consisting of lipid A, an oligosaccharide, and the O-antigen polysaccharide. The O-antigen is the immunogenic component of LPS, and as such can influence the host–bacterium relationship in several ways; potential mechanisms include resisting host complement and phagocytic engulfment, molecular mimicry, and colonization ability.[21] Additionally, variation in the amount/type of monosaccharides in O-antigens provide major LPS structural diversity[21] and virulence potential across bacteria (*e.g. E. coli* O157).

Historically, strains of gram-negative bacteria like *E. coli, Salmonella*, and *Shigella* have been classified based on O-antigen structure, using antigen and antibody agglutination reactions, in a laboratory method known as O-serotyping.[22] While O-serotyping is a well-known and standardized practice, this method is still deemed highly variable across laboratories.[23,24] Established viable alternatives to O-serotyping have been demonstrated for several *Enterobacteriaceae*. In an attempt to streamline the classification schemes to typify *Enterobacteriaceae*, newer techniques have examined the use of DNA sequences of genes involved in the synthesis and processing of the O-antigen.[25,26]

Genes that encode the enzymes for synthesis of O-antigens are classically clustered in a region known as the *rfb* cluster. Within the cluster, the *rfbA* gene encodes the leading enzyme glucose-1-phosphate thymidylyltransferase to produce the O-antigen. This enzyme catalyzes the formation of dTDP-glucose from dTTP and glucose 1-phosphate as the first reaction in the O-antigen synthesis pathway.[27] Since this is a critical step, we proposed the analysis of this gene to help elucidate strain differences in *Pdis*. This is especially important because the *rfbA* gene was recently identified in two strains of *Pdis*, namely, CavFT-hAR46[28] and CavFT-hAR56 (this study), which were isolated from gut wall cavitating microlesions of two different patients with severe Crohn's disease.[28]

As there are no studies on the actual molecular structures of the O-antigen in *Pdis* to make a proven connection to pathogenesis, here we propose a framework strategy to characterize the variations in length and phylogeny of the *rfbA* gene, since such modifications influence the virulence of the O-antigen products as it is known in *E. coli*,[29,30] and since the presence and length of O-antigen in LPS play an important role in bacterial pathogenesis.[31,32] Thus, *rfbA* gene variations may help categorize *Pdis* strains for future functional pathogenic vs. probiotic characterization studies. The objective of this study was to develop a classification and cataloging system for *Pdis*, applicable to other *Bacteroidetes*, based on *rfbA* according to variations in gene copy number and polymorphisms. Herein, we use DNA sequence and *in silico* methods to develop a classification system for *Pdis* based on the *rfbA* gene and discovered *Pdis*-specific *rfbA*-types (I–IV), *rfbA*-specific primers for *Pdis* (reverse and forward), three major superclusters when the *Pdis* data was contextualized with sequences of numerous *Bacteroidetes* and *Enterobacteriaceae*, and we assessed the discriminatory ability of this system compared to lipid A (of LPS) biosynthesis genes.

Materials and methods

Sequence data used

We performed *in silico* analyses of *rfbA* and *lpxK* gene sequences from bacterial genomes available in NCBI and Pathosystems Resource Integration Center (PATRIC) for *P. distasonis*, other *Parabacteroides* spp.; *Bacteroidetes (Bacteroides, Alistipes, Prevotella)*; and *Enterobacteriaceae* (*Escherichia, Klebsiella, Salmonella, Shigella)*.

Phylogenetic analyses

Parabacteroides distasonis rfbA gene sequences previously collected from NCBI were compiled in CLC Viewer 8.0 (commercially available) and used to construct two alignments and phylogenetic trees: one alignment and tree for the *Pdis rfbA* nucleotide sequences and the second for the translated amino acid sequences. Additionally, a third alignment and tree including *rfbA* gene sequences from *Pdis*, other

Parabacteroides spp., *Bacteroides* spp., *Alistipes* spp., *Prevotella* spp., *Escherichia* spp., *Klebsiella* spp., *Salmonella* spp., and *Shigella* spp., were constructed to provide evolutionary context and for observation of clustering patterns. Results were used to determine phylogenetic relatedness and *rfbA* gene variance within *Pdis* strains as well as between *Pdis* and other bacterial species. A separate phylogenetic analysis of *lpxK* gene nucleotide and amino acid sequences was performed, the results of which were used to determine the comparative discriminatory ability of the *rfbA* gene to aid in strain characterization.

Parabacteroides distasonis sequence cluster analysis

Results from the *Pdis rfbA* gene phylogeny were then used to perform sequence cluster analysis.[33] Gene copy analysis was first performed by recording the number of unique *rfbA* genes present in each represented *Pdis* strain. *Pdis* cluster analysis was then performed using the *rfbA* gene alignment data. Initially, aligned sequences were organized based on gross structure (i.e. matching patterns of nucleotide insertions and/or deletions). A sequence cluster, hereafter referred to as *rfbA*-Type, we defined as a set of *rfbA* sequences with an identical pattern of insertions and deletions. For each *rfbA*-Type, a cluster representative [CR] strain was chosen based on having the fewest *rfbA* copy number variations (CNVs) of its cluster or unique status (*e.g.* ATCC 8503 is the reference strain for the entire *Pdis* species and therefore will be the [CR] strain within its *rfbA*-Type). Next, within each *rfbA*-Type, individual *rfbA* gene sequences were analyzed for nucleotide (Nt) homology in three areas: (i) Nt percent homology with its *rfbA*-Type [CR] strain, (ii) Nt percent homology with the *Pdis* reference strain ATCC 8503 *rfbA* gene sequence, and (iii) *rfbA*-Type inter-cluster consensus sequence percent homology. Lastly, *Pdis* strains of each *rfbA*-Type were assigned subtypes (A-F) based on descending percent homology to their [CR] strain (*e.g.* highest percent homology was designated subtype A, second highest – subtype B, third highest – subtype C, fourth highest – subtype D, etc.). The collected *Pdis rfbA* gene sequences were then translated in CLC Viewer 8.0 to amino acid sequences in the +1, +2, and +3 reading frames. For each *rfbA*-Type, nucleotide and corresponding amino acid sequences were assessed for trends in conservation levels, both within and across reading frames. Additionally, notable amino acid mutations and their corresponding nucleotide polymorphisms within *rfbA*-Types were recorded.

Bacteroidetes/Enterobacteriaceae sequence cluster analysis

Gene copy analysis was also performed on the results from the *Pdis, Bacteroidetes*, and *Enterobacteriaceae rfbA* gene phylogeny, followed by observation for clustering patterns through which the previously designed *Pdis rfbA*-Typing system could be extrapolated to other species and genera. Expanded *rfbA*-Types were assigned based on bootstrap values and phylogenetic tree morphology (see statistics below), with each apparent cluster receiving a different *rfbA*-Type designation.

Primer design for amplification of rfbA gene

Primer design was conducted by identifying left and right flanking regions of the *rfbA* gene alignment which were whole (i.e. no gaps or deletions) throughout all *rfbA* sequences. Then, from the corresponding regions of the *rfbA* gene alignment consensus sequence, a left flank of 52 base pairs and right flank of 49 base pairs were selected and processed with Primer3Plus[34] to identify optimal primers. Primer sequences were then entered into Basic Local Alignment Search Tool (BLAST[35]) to confirm accuracy in identifying *Pdis* strains. These methods were then used to design primers for other *Bacteroidetes* genera (*Parabacteroides, Bacteroides, Alistipes, Prevotella*).

In silico rfbA-RFLP analysis

The collected *rfbA* genes were uploaded into DNASTAR (commercially available) and processed in two separate agarose gel simulations using the previously validated restriction enzyme MboII.[36,37] The first gel was simulated with the full-length *rfbA* gene PCR amplicon, and the following simulation used end-truncated *rfbA* gene PCR amplicons after

application of the previously designed primers. The digestion patterns were then used to design the *Pdis* RFLP typing system, a complementary classification scheme to the *rfbA* typing system derived from sequence cluster analysis.

Statistics

Sequences were aligned using CLC Viewer 8.0. Then, alignments were used to construct neighbor-joining phylogenetic trees using Jukes-Cantor to account for the nucleotide (Euclidean) distances across sequences.[38] Branch reproducibility was quantified using bootstrapping for 1000 replicates (bootstrap values are shown in trees).[39] Branch morphology and bootstrap values greater than 90 were used as a guide to designate a branch as a distinct *rfbA*-Type cluster. To assess the reproducibility of cluster assemblage, strain allocation within hierarchical clusters for phylogenetic trees generated with only *Pdis* sequences (21 *rfbA* gene sequences, from 14 strains) were compared to the allocation of the same strains within clusters generated in a phylogenetic tress containing a total of 89 *rfbA* genes from 49 other species, a total of 8 genera, using Fisher's exact test.[40] Significance was held at $p < .05$. For *Pdis* sequence cluster analyses, percent homologies for ***i, ii***, and ***iii*** were calculated using the Expasy SIM Alignment Tool (https://web.expasy.org/sim/),[41] and the statistical significance of homologies for ***ii*** was assessed using Kruskal–Wallis one-way analysis of variance (ANOVA) in conjunction with Dunn's test.[42,43]

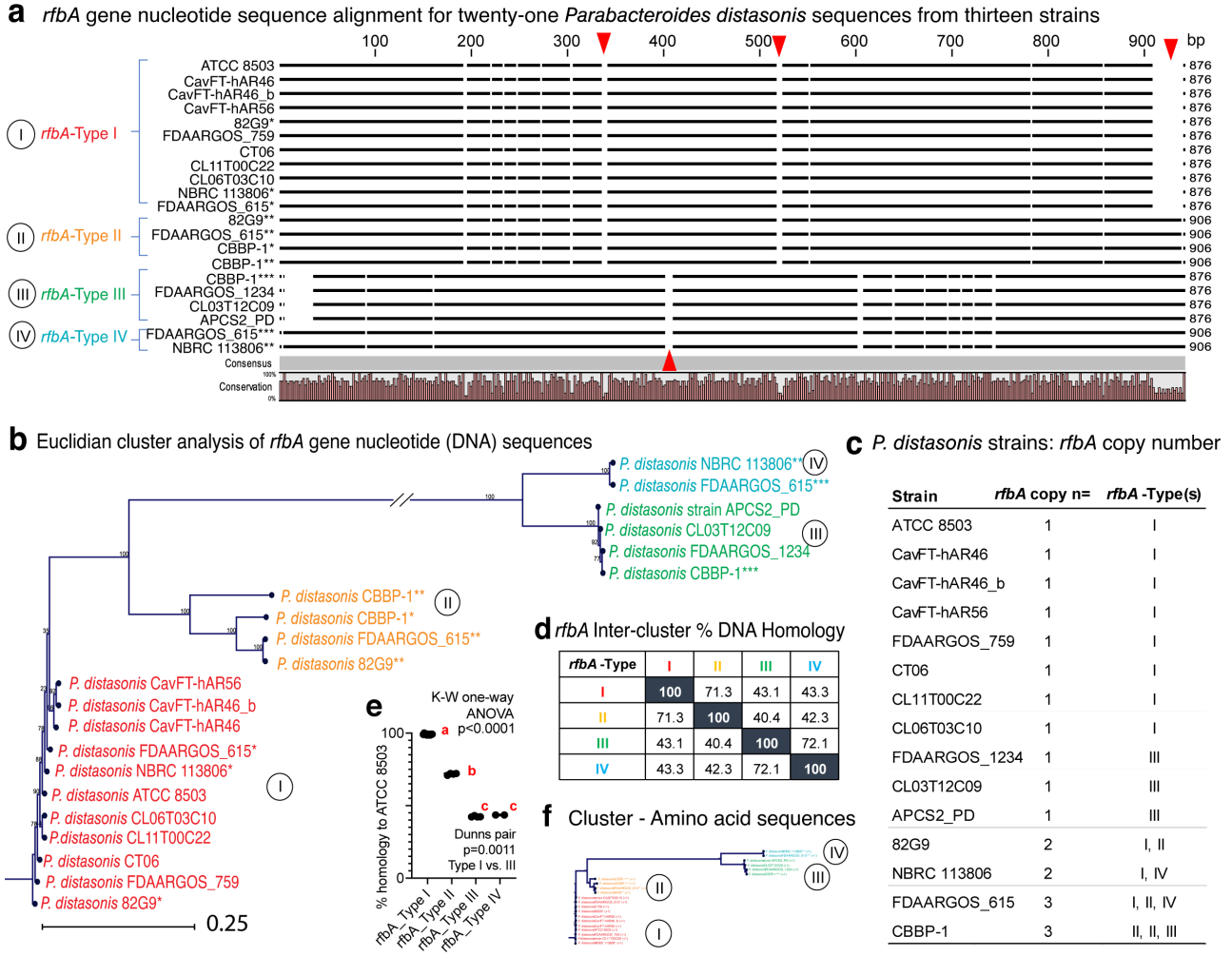
Results

Copy number and the phylogeny of rfbA nucleotide sequence in Parabacteroides distasonis

From 15 possible *Parabacteroides* species (as complete genomes) available to date,[2] *rfbA* sequences were only available for 13 species (*P. distasonis, P. johnsonii, P. merdae, P. golsteinii, P. acidifaciens, P. faecis, P. bouchesdurhonensis, P. chartae, P. massiliensis, P. provencensis, P. timonensis, P. pacaensis, P. gordonii*). Sequences for the *rfbA* gene in *P. chongii* and *P. chinchilla* were not available. DNA sequences were analyzed using phylogenetic and hierarchical analysis to illustrate the genetic distances of 21 *rfbA* gene sequences identified in 14 *Pdis* genomes. Of interest, illustrating the gene diversity and conservancy within *Pdis*, we found that the *rfbA* gene can be present in *Pdis* genomes as single, double, or triple copies, with distinct or similar gene homologies within each genome, and that different genes have unique reproducible patterns of gaps and insertions which enable the designation of *rfbA*-Types (Figure 1a). Phylogenetic analysis revealed distinct grouping of *Pdis* strains into four main clusters. Given the presence of multiple *rfbA* gene copies, some *rfbA* sequences from strains FDAARGOS 615, 82G9, NBRC 113806, and CBBP-1 were present in more than one cluster (Figure 1b). Of note, data indicate that when a *Pdis* genome has >1 *rfbA* copy, the copies are of different sequence type, except for CBBP-1, which has three *rfbA* copies only matching two sequence types (Figure 1c). The role of such diversity in health remains unknown.

Classification system based on rfbA-typing for Parabacteroides distasonis

We subsequently examined the structural differences between *rfbA* gene sequences. Based on *rfbA* sequence structural variation, our analysis revealed that *Pdis* could be classified into lineages based on the presence of gaps and insertions in the gene sequence, of which we identified Types I, II, III, IV, and Subtypes A, B, C, D, E, and F (Table 1), among the 21 *rfbA* genes derived from fourteen complete *Pdis* genomes. The *rfbA*-Type I cluster is composed of eleven *Pdis* strains, including the species reference strain ATCC 8503. *rfbA*-Types II, III, and IV contain four, four, and two strains, respectively, and have the strains ATCC 82G9, FDAARGOS 1234, and NBRC 113806 as representatives for future analysis. As a measure of inter-cluster distances, Figure 1d illustrates that some clustered sequences have a very low % of DNA homology. Relative to the *Pdis* reference strain ATCC 8503, individual strand % homologies were highest in *rfbA*-Type I strains and lowest in *rfbA*-Types III and IV strains (Figure 1e, K-W, $p < .0001$). Such differences across the *rfbA* gene could explain differences in O-antigen related virulence across strains, especially if strains have major gene sequence differences as the ones observed



Strain	rfbA copy n=	rfbA -Type(s)
ATCC 8503	1	I
CavFT-hAR46	1	I
CavFT-hAR46_b	1	I
CavFT-hAR56	1	I
FDAARGOS_759	1	I
CT06	1	I
CL11T00C22	1	I
CL06T03C10	1	I
FDAARGOS_1234	1	III
CL03T12C09	1	III
APCS2_PD	1	III
82G9	2	I, II
NBRC 113806	2	I, IV
FDAARGOS_615	3	I, II, IV
CBBP-1	3	II, II, III

rfbA -Type	I	II	III	IV
I	100	71.3	43.1	43.3
II	71.3	100	40.4	42.3
III	43.1	40.4	100	72.1
IV	43.3	42.3	72.1	100

Figure 1. ***Parabacteroides distasonis rfbA* gene variation analysis of (DNA) nucleotide sequences.** (**A**) Spatial distributions of gaps and insertions in the gene were used to designate the *rfbA* gene into four unique *rfbA*-Types (I, II, III, IV). (**B**) Phylogenetic analysis illustrating the clustering of *rfbA* nucleotide sequences. (**C**) List of *P. distasonis* strains and copy numbers). (**D**) Percent homologies between consensus sequences of *rfbA*-Types I–IV. (**E**) Analysis of individual sequence homology (by *rfbA*-Type) to ATCC 8503. (**F**) Phylogenetic analysis illustrating the clustering of *rfbA* amino acid sequences. Asterisks (*, **, ***) denote first, second and third copy of the *rfbA* gene in each strain. Detailed homology distances within each cluster are shown in Table 1.

when comparing *rfbA*-types I and IV, which can be as low as 43% using the ATCC 8503 *rfbA* gene sequence as a referent. Inferred amino acid sequence analysis demonstrated, across all *Pdis rfbA* genes, that the clustering structure observed for the DNA remained unaltered when using the amino acid sequence data (Figure 1f).

Conservation of amino acid sequences predicted from rfbA genes over decades and geography

After observing the variance in *rfbA* DNA sequence homology, analysis of the respective amino acid sequence conservation levels was performed to understand which gene polymorphisms may ultimately affect the final *rfbA* protein product. The +1-reading frame, relative to the +2 and +3 reading frames, showed the highest conservation levels in an alignment of all twenty-one *rfbA* amino acid sequences from *Pdis* genomes. Aside from *rfbA*-Type IV, which is comprised of only two, 100% homologous *rfbA* gene sequences, amino acid conservation of the *rfbA*-Type I cluster (+1-reading frame) was the highest among all *rfbA* types with only three sites of amino acid mutations across the 292 amino acid-long sequences (with 27 sites of nucleotide polymorphisms present in the *rfbA*-Type I gene cluster) (Figure 2a-b).

Table 1. *rfbA*-Type and subtype classifications based on sequence homology and *in-silico* RFLP.

rfbA-Type	Strains	% Homology to [CR]	% Homology to ATCC 8503	*rfbA*-Subtype	RFLP-Type
rfbA-Type I	ATCC 8503 [CR]	100	100	A	1
	CL11T00C22	99.4	99.4	B	1
	82G9*	99.2	99.2	C	2
	FDAARGOS_759	99.2	99.2	C	2
	CT06	99.2	99.2	C	2
	NBRC 113806*	99.2	99.2	C	1
	CL06T03C10	99.1	99.1	D	1
	FDAARGOS_615*	99	99	E	1
	CavFT-hAR46	98.6	98.6	F	1
	CavFT-hAR46_b	98.6	98.6	F	1
	CavFT-hAR56	98.6	98.6	F	1
rfbA-Type II	82G9** [CR]	100	72.1	A	3
	FDAARGOS_615**	100	72.1	A	3
	CBBP-1*	91.5	71.7	B	4
	CBBP-1**	79.7	70.9	C	5
rfbA-Type III	FDAARGOS_1234 [CR]	100	42.2	A	6
	CBBP-1***	99.9	43.1	B	6
	APCS2_PD	99.3	42.3	C	6
	CL03T12C09	99.2	42.2	D	7
rfbA-Type IV	NBRC 113806** [CR]	100	43.5	A	8
	FDAARGOS_615***	100	43.5	A	8

Within the *rfbA*-Type I amino acid sequence cluster, one of the three mutations, pos. 127 A -> V, is uniquely present in the CavFT-haR46 /46_b/56 strains. Two other mutations, pos. 185 G -> D, and G -> S, are uniquely present in the FDAARGOS 615 and CL06T03C10 strains, respectively. Of interest, the Type I *rfbA* sequences belonging to the CavFT-hAR46 and CavFT-hAR56 vs. ATCC 8503 strains are 98.6% homologous at the nucleotide level despite these two USA isolates being isolated by different institutions from patients over 85 years apart.[28,44] The initial presumed designation as 'pathogenic' or 'probiotic' and the geographical distribution of the reference strains used is listed in Table 2. Taken together, the concurrent presence of high DNA and protein sequence homology may indicate which polymorphic sites are most relevant in altering the pathogenic potential of *Pdis*.

Context of rfbA-type classification in P. distasonis vs. Bacteroidetes and Enterobacteriaceae

To better understand the newly developed *P. distasonis rfbA*-Typing framework, we examined its context among the larger set of gram-negative bacteria; the closely related *Bacteroidetes* and more distantly related *Enterobacteriaceae* (total n = 49 additional species; 8 genera). Genetic mapping of the twenty-one *Pdis rfbA* genes amongst *rfbA* genes from 12 additional *Parabacteroides* spp., *Bacteroides* spp., *Alistipes* spp., *Prevotella* spp., *Escherichia* spp., *Klebsiella* spp., *Salmonella* spp., and *Shigella* spp. illustrates consistent phylogeny of the four original *Pdis rfbA*-Types compared to their isolated cluster analysis in Figure 1b, and all four *Pdis rfbA*-Types are unique to the species (2x4 Fisher's exact $p = 1.0$; *i.e.* the clusters were identical with or without other bacteria). Gene copy analysis of the phylogeny reveals the presence of multiple unique *rfbA* gene copies in *A. shahii, A. onderdonkii*, and *A. finegoldii; E. coli* O1:H42, K-12, O2:H6, and *E. fergusonii; S. flexneri*; and *K. aerogenes*. While *P. distasonis* strains exhibit anywhere from 1 to 3 copies of the *rfbA* gene in their genome, the twelve other *Parabacteroides* species have only one *rfbA* copy per genome (Figure 3, highlighted in green).

Cluster analysis further revealed formation of three '*rfbA*-superclusters'; Supercluster 1 solely contains the *Enterobacteriaceae Escherichia, Klebsiella, Salmonella*, and *Shigella*; Supercluster 2 predominantly consists of *Pdis* (*rfbA*-Types I and II) as well as a representative from each included genus except for *Shigella*; Supercluster 3 contains at least one representative from each included genus, the majority of *Parabacteroides* spp., and the *Pdis rfbA*-Types III and IV (Figure 3). Within each supercluster, new *rfbA*-Types, determined by bootstrap values and branch morphology, were assigned to

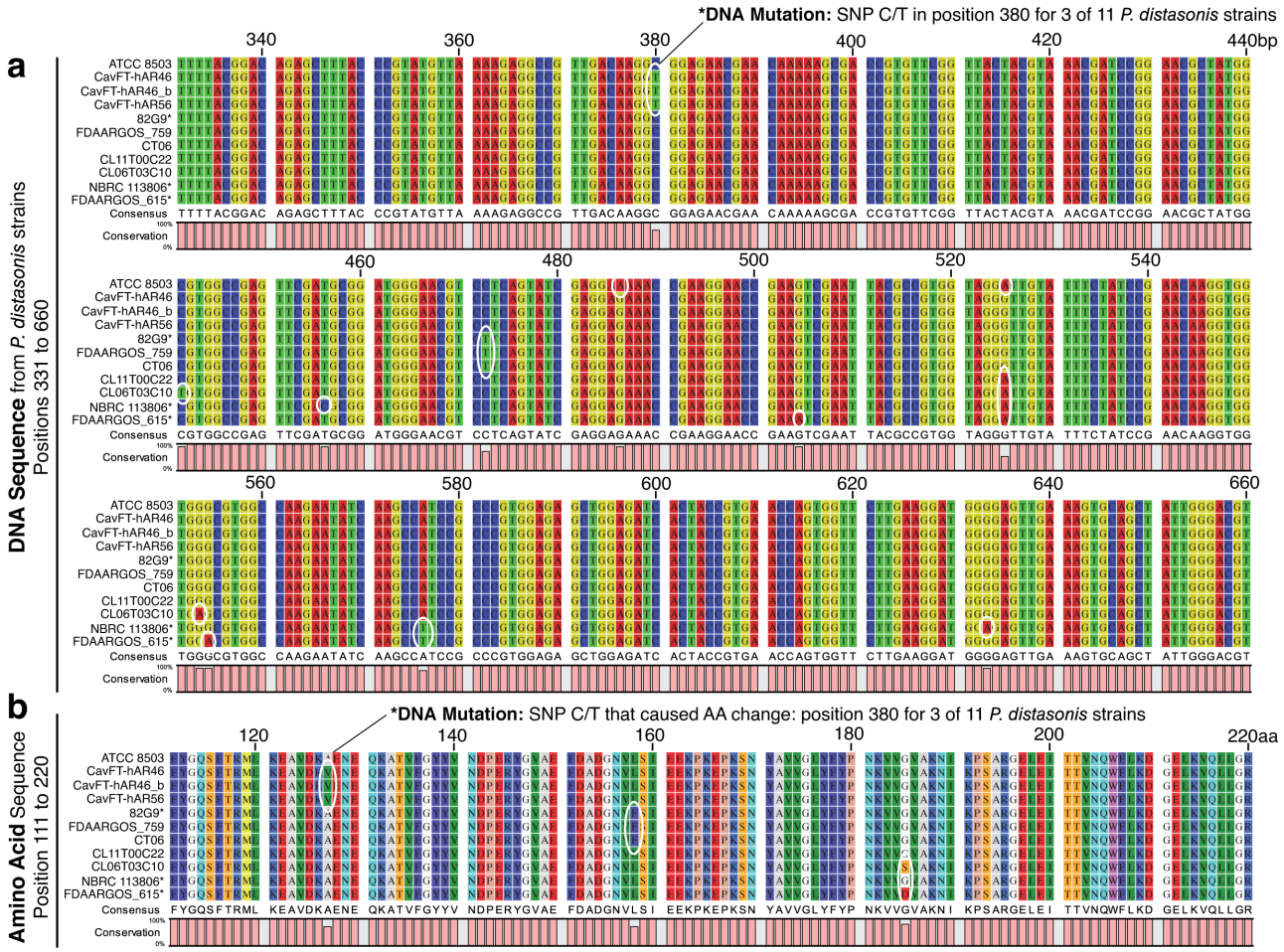


Figure 2. DNA and amino acid sequence mutations in the *rfbA*-Type I gene cluster. Out of 27 identified polymorphic sites (11 shown) in the *rfbA*-Type I DNA sequence cluster, only three sites produced changes in the *rfbA*-Type I amino acid sequences (one example shown next to asterisk (*)). (**A**) DNA single nucleotide polymorphisms (SNPs) in region corresponding to amino acid mutations. (**B**) Mutated amino acid region of the *rfbA*-Type I cluster (translated in the +1-reading frame).

extrapolate the *rfbA*-Typing system beyond *P. distasonis*. Results from expanded *rfbA*-Typing show up to eighteen total unique *rfbA*-Types (*Pdis rfbA*-Types I–IV plus fourteen newly assigned *rfbA*-Types) discernable from the eighty-nine-sequence phylogeny, with one *rfbA*-Type in Supercluster 1, eight *rfbA*-Types in Supercluster 2, and nine *rfbA*-Types comprising Supercluster 3. The clustering pattern of new *rfbA*-Types appears to be predominantly restricted to species of the same genera (*rfbA*-Types V, VI, IX, XI, XII, XIV, and XVII) with the most notable exception being the *rfbA*-Types consisting of a mix of *Enterobacteriaceae* genera (*rfbA*-Types X, XV, XVI, and XVIII). Species within *rfbA*-Types VII and VIII appear to be relatively more variable and could indicate the need for additional related species and/or *rfbA* gene sequences to illustrate more well-defined clusters.

Design of primers for amplification of 'end-truncated Pdis rfbA gene' in laboratory isolates

We designed *Pdis rfbA* gene consensus-sequence based primers to facilitate identification of the *rfbA* gene in future *Pdis* isolates, regardless of *rfbA*-Type (Figure 4a-b). The forward primer 5′-CCGCTTGTATCCGATCACT-3′ and reverse primer 5′-AAATACTGGCCGTACTGATTCTT-3′ were identified using Primer3Plus and verified with BLAST to identify *Pdis* strains with 100% specificity (see Supplementary Excel File). Use of

Table 2. Designation of *Parabacteroides distasonis* into presumed pathogenic and probiotic strains based on available information in NCBI and the literature.

Strain	*rfbA* copy number	*rfbA*-Type	Presumed pathogenic/ probiotic	Year	Isolation country	Isolation source	Remarks	References
ATCC 8503	1	I	Pathogenic	1933	USA	Human feces	Isolated from distal human gut microbiota and used as reference genome in this study	[44]
CavFT-hAR46	1	I	Pathogenic	2019	USA	Human intramural gut wall	Isolated from a gut wall cavitating micro-lesion in a patient with Crohn's disease	[28]
CavFT-hAR46_b	1	I	Pathogenic	2019	USA	Human intramural gut wall	Isolated from a gut wall cavitating micro-lesion in a patient with Crohn's disease	Same isolate as[28] Re-sequenced, Not Published
CavFT-hAR56	1	I	Pathogenic	2019	USA	Human intramural gut wall	Isolated from a gut wall cavitating micro-lesion in a patient with Crohn's disease	[28]
FDAARGOS_759	1	I	Not specified	-	-	Human feces	Used as reference genomes in NCBI	[45]
CL11T00C22	1	I	Not specified	2009	USA	Human feces	Isolated from feces of healthy adult	[46]
CL06T03C10	1	I	Not specified	2009	USA	Human feces	Isolated from feces of healthy adult	[46]
CL06T03C09	1	III	Not specified	2009	USA	Human feces	Isolated from feces of healthy adult	[46]
FDAARGOS_1234	1	III	Not specified	-	-	-	-	Not Published
APCS2/PD	1	III	Not specified	2017	Ireland	Human feces	Laboratory host for propagation of bacteriophage PDS1	Not Published
82G9	2	I, III	Pathogenic	-	Japan	Human feces	Isolated from human feces	Not Published
NBRC 113806	2	I, IV	Not specified	-	-	Human feces	-	Not Published
FDAARGOS_615	3	I, II, IV	Pathogenic	-	-	Human feces	Clinical isolate	Not Published
CBBP-1	3	II, III, IV	Probiotic	-	-	Feces	-	[47]

these primers should generate an expected PCR product of about 850 bp in length (Figure 4c). To examine the potential to identify one general primer that could encompass all species at the genus level, we conducted *in-silico* primer design and analysis for the major cluster of *Bacteroidetes* (*Parabacteroides, Bacteroides, Alistipes*, and *Prevotella*) shown in Figure 3. A sample of selected primers for the genera and their BLAST performances are listed in the Supplementary Excel File. Of note, the development of primers to cover most species within each genus was more challenging to design due to a wide array of gaps and insertions across multiple species owed to *rfbA* sequence variability (Supplementary Figure 1).

Enhanced discrimination ability of Parabacteroides distasonis using MboII-RFLP rfbA subtyping

To further aid in *rfbA* gene-based identification methods of *Pdis* isolates, we designed a lab-accessible application of the *rfbA*-typing system based on RFLP. Results from *in silico rfbA*-RFLP of both full-length genes and end-truncated PCR amplicons demonstrate eight unique patterns by MboII restriction digest. (Figure 4d-e). The *rfbA*-Type I sequences demonstrated two unique digestion patterns, and *rfbA*-Type II, III, and IV demonstrated three, two, and one unique digestion patterns, respectively. RFLP *in silico* analysis revealed that the PCR amplicons produced with our designed primers yielded similar patterns of classification compared to the complete gene sequences derived from the complete *Pdis* genomes.

Effective discrimination of strains by rfbA compared to the lipid A phosphorylation gene lpxK

Lipopolysaccharides (LPS) are composite molecules consisting of a lipid (Lipid A) and a polysaccharide composed of the O-antigen, and two (outer and inner) oligosaccharide cores linked by covalent bonds. Thus far, we have herein examined the phylogeny of the *rfbA* gene. To quantify the discriminatory ability of the *rfbA* gene in differentiating *Pdis* strains, we compared a potential classification scheme based on the sequence homologies of the same strains using the *lpxK* gene which encodes a lipid A phosphorylation enzyme. The *lpx* genes have been implicated in toll-like receptor 4 (TLR4) mediated pathology of other gram-negative

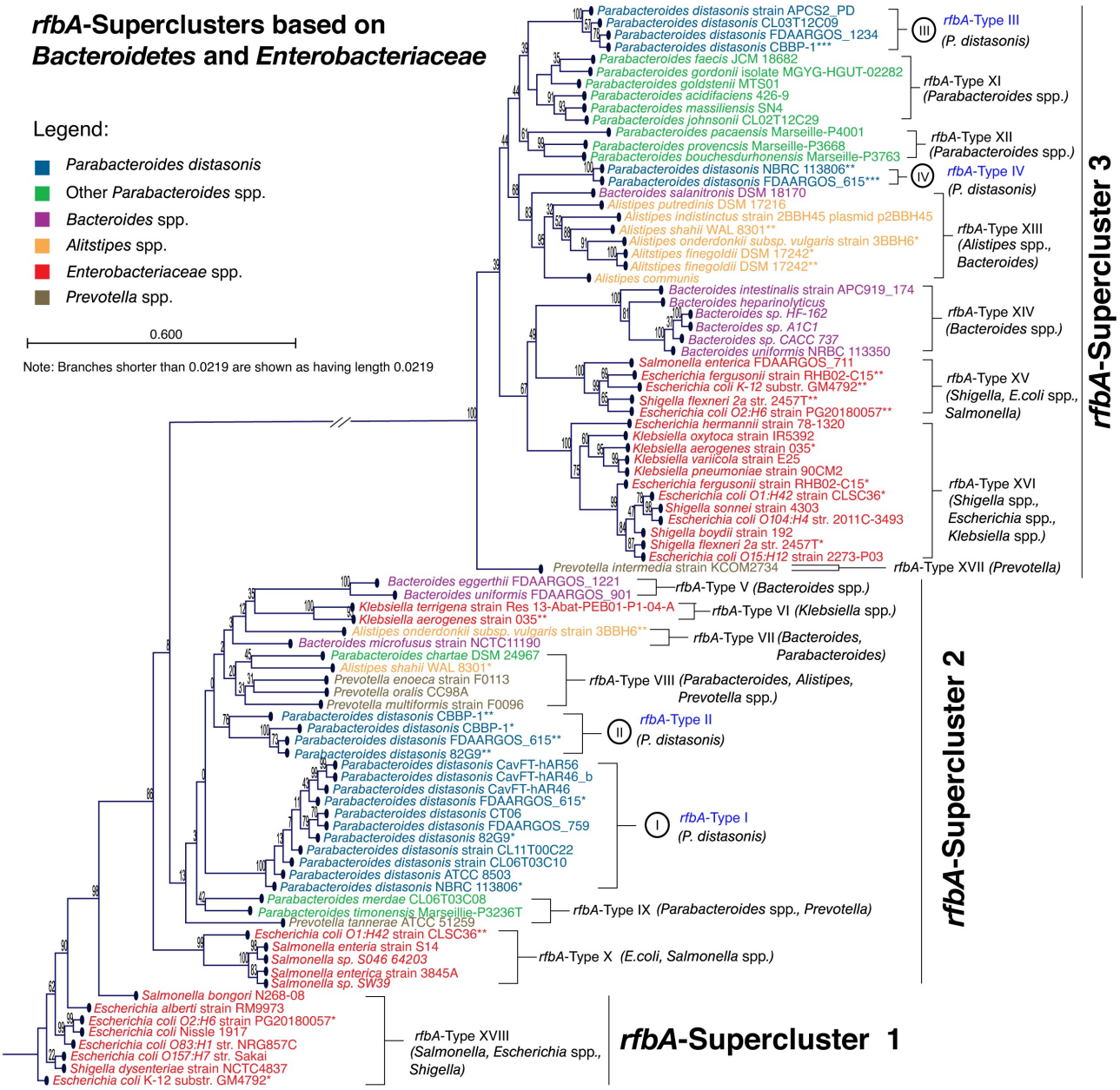


Figure 3. ***RfbA*-Superclusters and contextualization of *rfbA*-Typing system for the *P. distasonis* with respect to other *Bacteroidetes* and pathogenic *Enterobacteriaceae*.** Asterisks (*, **, ***) denote first, second, and third copy of the *rfbA* gene in each species and/or strain.

bacteria.[48,49] Analysis shows that *i)* the *lpxK* gene is present as a single copy in all the *Pdis* genomes used in this study, and that *ii)* all strains have high sequence homology and conservation at nucleotide (98.65%) and amino acid level (98.1%), making the *lpxK* gene suboptimal as a classification system (Figure 5). This comparative analysis reassures that the classification and cataloging proposed for *Pdis* strains based on the O-antigen *rfbA* gene is highly discriminant and useful compared to the lipid A *lpxK* gene. As an immediate mechanistic application of this *rfbA*-Typing system, we examined a potential interaction of O-antigen/LPS molecules (or membrane fractions) of various *P. distasonis* strains with that of other O-antigen/LPS molecules of known pathogens, for example *E. coli* or other *Enterobacteriaceae*. To facilitate the understanding of such hypothetical interactions, Figure 6 illustrates how variability in *rfbA*/LPS geno/phenotypes could modulate proinflammatory/apoptotic pathways.

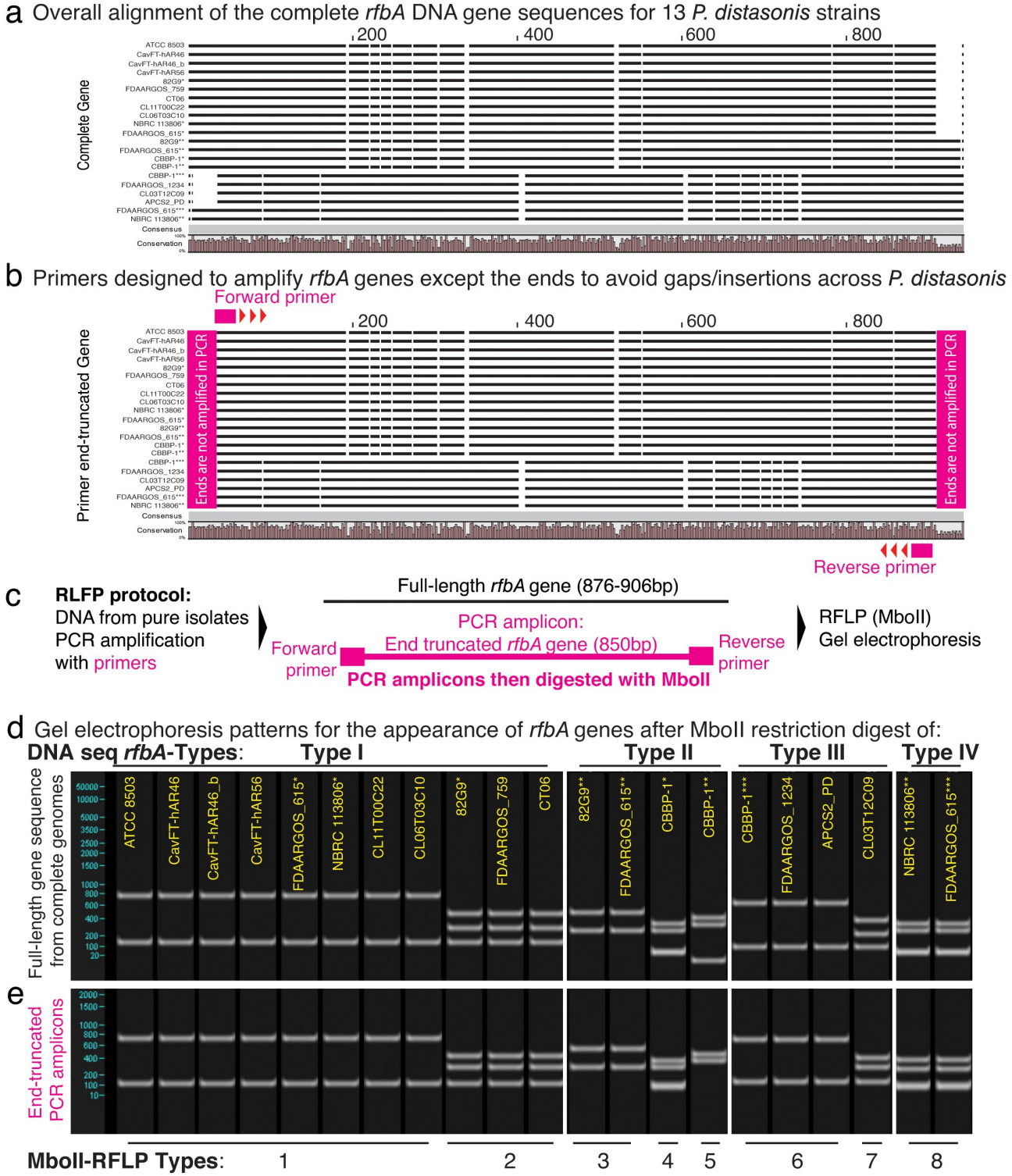


Figure 4. ***In silico*** **MboII restriction digest of full-length and end-truncated** ***P. distasonis rfbA*** **genes.** (**A**) Alignment of complete *rfbA* genes. (**B**) Alignment of primer end-truncated *rfbA* genes. (**C**) RFLP protocol. (**D**) Gel electrophoresis patterns for full-length *rfbA* genes after MboII digest. (**E**) Gel electrophoresis patterns for end-truncated *rfbA* genes after MboII digest. Gels shown in panels D and E depict *Pdis rfbA*-Types alongside newly designated RFLP Types based on restriction digest patterns. Asterisks (*, **, ***) denote first, second and third copy of the *rfbA* gene in each species and/or strain.

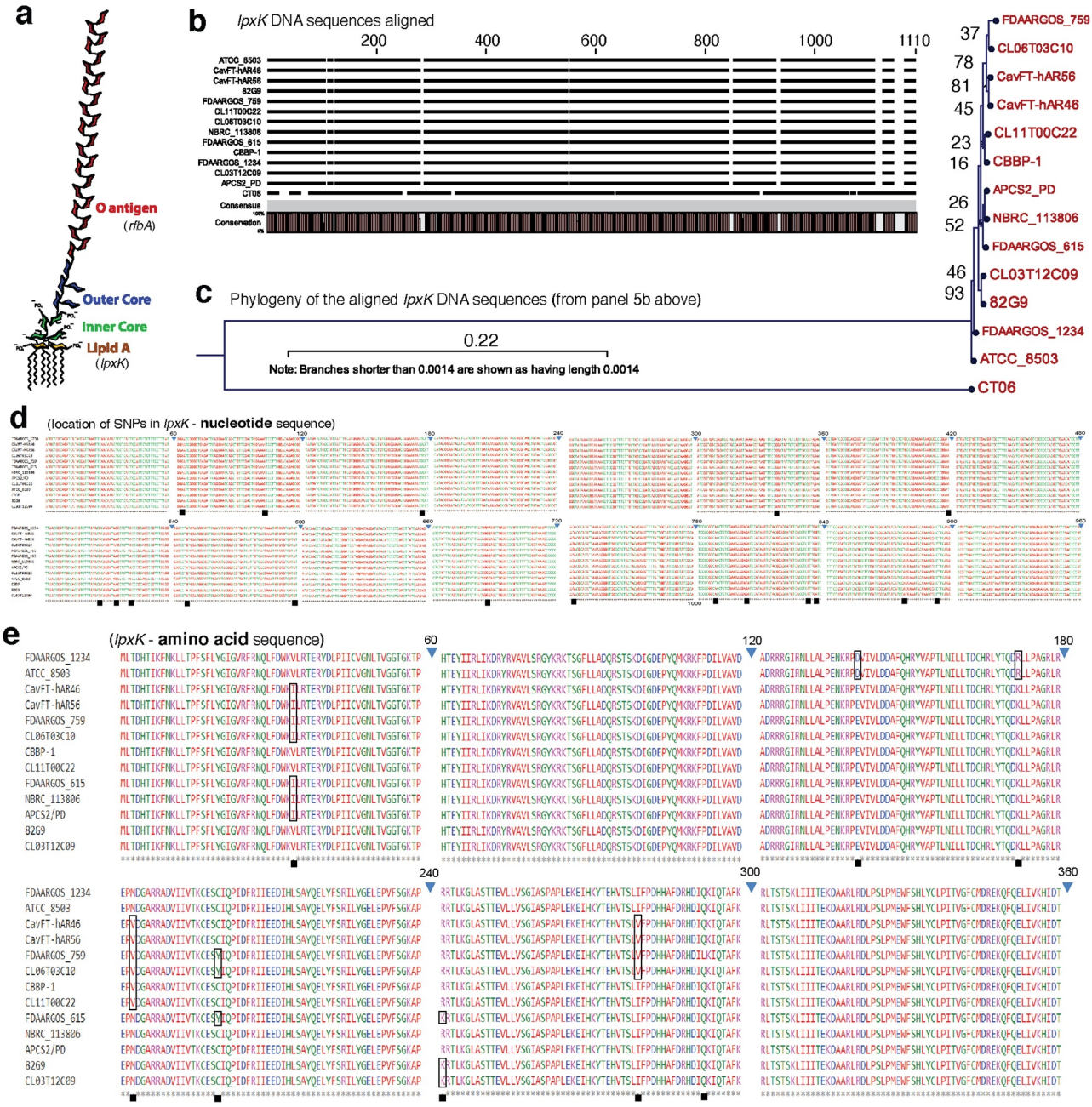


Figure 5. Overview of a lipopolysaccharide structure and limited genetic variability (discriminatory ability) of the *lpxK* enzyme gene. (A) Schematic representation of the spatial relationship between the O-antigen and the Lipid A. Public domain Mike Jones©2010. CC BY-SA 3.0. (**B**) Alignment of *P. distasonis lpxK* genes demonstrating identical structural homology with one outlier (CT06). (**C**) Phylogenetic tree of *Pdis lpxK* gene alignment further highlighting genetic similarity of sequences. (**D**) Detailed nucleotide sequences and (**E**) Amino acid sequences of the *lpxK* gene. Black squares indicate location of nucleotide or amino acid variant. For comparison, notice in Figure 1 that the genetic variability of the *rfbA* is much more pronounced (major deletions and insertions, and copy number variability) than the one shown here for *lpxK* which best enables the classification of *Pdis* using *rfbA*-Types of the O-antigen.

Nomenclature and reporting of *rfbA*-Types

To facilitate the reporting of an *rfbA* profile, we suggest the use of the following designation format: *i) P. distasonis* '*rfbA*-Type n1-I' for strains having one *rfbA* gene copy (n1) with a nucleotide sequence of the *rfbA*-Type I; *ii) P. distasonis* '*rfbA*-Type n3-I, II, II' for strains with three gene copies, one of each was either types I, II or III; and *iii*) *P. distasonis* '*rfbA*-Type n4-I,IV' for strains with four copies, with at least one Type I and one Type IV. If more details are desired,

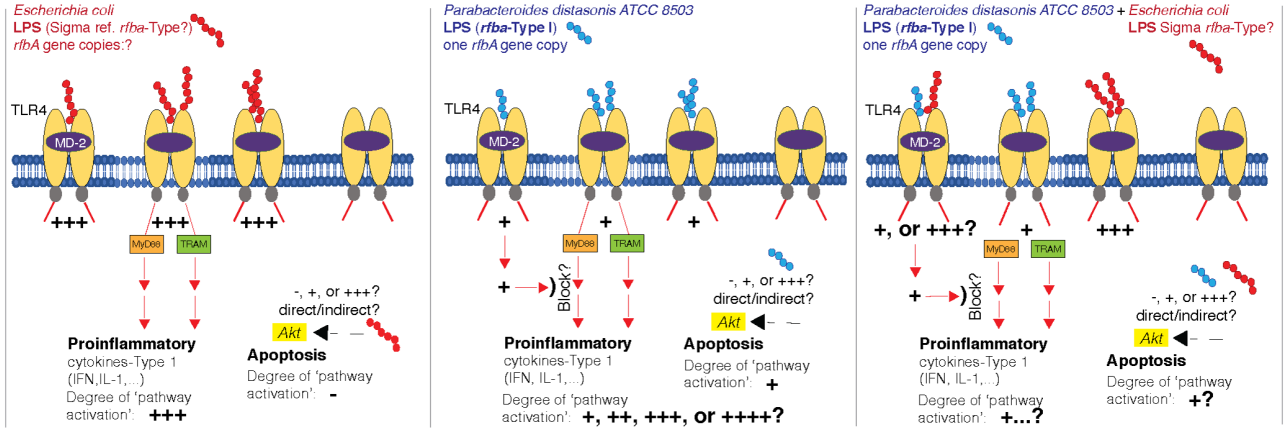


Figure 6. Interspecies mechanistic interaction across bacterial *rfbA*/lipopolysaccharide (LPS) Types on TLR4 and proinflammatory/apoptotic pathways. Graphical representation for *E. coli* LPS effects on proinflammatory cytokine production and cellular apoptosis, and hypothetical mechanisms through which *Parabacteroides distasonis rfbA*-Type variability across strains and LPS/O-antigens could modulate the induction of LPS-driven pathogenic (inflammatory) or probiotic (anti-inflammatory) effects.

the latter case could be presented as *iv*) *P. distasonis* '*rfbA*-Type n4-I,IV(2,2)' or 'Type n4-I,IV(1,3)' or 'Type n4-I,IV(3,1)' to provide the detailed counts of each unique sequence type in subscript parentheses with numerically ordered digits representing each ordered corresponding *rfbA*-Type.

Discussion

Parabacteroides distasonis has emerged in recent years for its contradictory dual potential for pathogenicity and probiotic ability, although our current knowledge of the potential for this bacterium to modulate health or cause disease is suboptimal and incomplete. Of the 14 studies cited, only 7 detailed the specific *Pdis* strain examined;[5,7,9–12,15,17] the strain being either ATCC 8503[5,7,9–12,15,17] or a strain not cataloged in NCBI.[12,15] Data available in the literature and NCBI on the presently examined 14 strains of *Pdis* indicate that 5 strains are presumed pathogenic, 1 probiotic, whereas 7 were neither presumed to be probiotic or pathogenic. Out of the presumed five pathogenic strains, two were isolated from gut wall cavitating micro-lesions in two patients with severe surgical Crohn's disease, one was associated with enhancing colitis in mice, and two were human clinical isolates. Of potential relevance to disease, *rfbA*-Type I was a common genotype to all the pathogenic strains of *Pdis*, irrespective of the number of *rfbA* gene copies in the genome (Table 2).

While this bacterium has reported associations with IBD and other diseases, its specific mechanisms are not well understood.[2] The fact that *P. distasonis* had been found in extraintestinal lesions (*e.g.*, abscesses) does not necessarily indicate that *Pdis* is a primary pathogen, but rather indicates that the dissemination of this bacterium from the gut lumen may make *Pdis* an opportunistic pro-inflammatory microorganism. To what extent this intestinal commensal promotes inflammation in the gut wall in humans and how this varies with human genetics and predisposition to IBD remains unclear, but strain isolation from pus-containing intramural microscopic lesions (CavFT-hAR46 and CavFT-hAR56) indicates that opportunistic inflammation may depend on the environment where *Pdis* is encountered.[28] Furthermore, experiments in animals with genetic deficiencies and induced colitis (peptidoglycan recognition protein *pglyrp* gene, 5% DSS colitis) have shown that *Pdis* (ATCC 8503) is a colitis-promoting species (in BALB/c mice with specific-pathogen free microbiota, with and without antibiotics), compared to mice that did not receive *Pdis*, or to mice that received *Alistipes finegoldii* (another type strain for an emerging *Bacteroidetes* genus[50]) which protected mice from colitis.[5] To contextualize the relevance of the 5% DSS model and the pathogenic effect of live *Pdis* aggravating colitis in this model, it is worth noting that the group of mice treated with *Alistipes finegoldii* (*rfbA*-Type XIII) were protected from colitis, bodyweight loss, and stool (bleeding) scores. Thus, the addition of *Pdis* (ATCC 8503) to mice exposed to DSS-colitis

exhibited significantly worse effects compared to mice not receiving any bacteria or mice receiving *Alistipes finegoldii*.

Emphasizing the potential dichotomous role of *Pdis* in health, recent studies conducted with the tumor-prone A/J mouse line have shown a beneficial effect using the same *Pdis* strain (ATCC 8503) and freeze-dried *Pdis* membrane fractions (LPS/O-antigen). In the A/J model, *Pdis* beneficially attenuated toll-like receptor 4 signaling (TLR4; present in myeloid cells: monocytes, macrophages, dendritic cells; and nonimmune cells: endothelial cells, adipocytes) and *Akt* activation, attenuated tumorigenesis, modulated inflammatory markers and promoted intestinal barrier integrity in azoxymethane-treated mice, with and without a high-fat diet.[10,11] Additionally, another study utilizing membrane fractions from *Pdis* ATCC 8503 on BALB/c mice attenuated the severity of colitis induced with 3% DSS and prevented increases in proinflammatory cytokines, indicating that membrane components of *Pdis*, though not specifically live *Pdis* cells, could modulate intestinal inflammation.[13]

Of interest, studies in cancer cells found that *Pdis* (ATCC 8503) membrane fractions inhibited *E. coli* derived LPS-induced TLR4 activation in a dose-dependent manner.[10,11] To explain the anti-TLR4 signaling effects of *Pdis* membrane fractions when added to *E. coli* LPS, our *rfbA*/*lpxK* (LPS/O-antigen) analysis suggests the novel hypothesis that the LPS from *Pdis*, which may vary with *rfbA*-Type and copy number, may directly compete and/or displace LPS from other pathogens on the surface of LPS receptors (TLR4) on host cells (*in vitro, in vivo*). Therein, it follows that potential *Pdis*-LPS/LPS-receptor interactions could reduce and/or modulate the intensity of cell signaling as illustrated in Figure 6. Thus, it is possible that the pathogenic effects induced by live *Pdis* is through mechanisms other than its LPS/O-antigen membrane fractions. *In vivo*, the anticolitic effect of the *Pdis* membrane fractions was not observed in mice with severe combined immunodeficiency (lacking T cells and B cells), which suggests that anti-colitic effects could be due T-regulatory cell modulation.[13] If an anti-inflammatory mechanism by membrane fractions depends on this hypothesized competition of LPS, this feature could also occur in other *Pdis* strains. To date, the aforementioned studies have only examined the strain ATCC 8503 which is the reference for the genus. Based on our studies, strain-to-strain variability should be expected within this mechanism because *rfbA* structure and copy numbers vary across the *P. distasonis* species. Future studies could examine the effect of various membrane fractions across the *Bacteroidetes* phylum to determine the extent to which this feature correlates with *rfbA*-Type(s)/copy number(s), and if it is unique to all the *Pdis* strains within the *rfbA*-Type I cluster.

To help determine in the future whether the presumed pathogenicity and probiotic duality of *Pdis* is due to a stable phenotype or a fluctuation of the phenotype (pathogenic or probiotic), we proposed a classification system based on the genetic variability of the O-antigen synthesis *rfbA* gene. Our classification system, accompanied with controlling for variables that include animal genetics, models for disease induction (e.g. DSS concentration/duration (protocol REFs), azoxymethane dose), diet, microbiota, and the use of antibiotics will be needed to determine the mechanisms that may play a dual role in animal and human health. Given the potential pathogenic effects,[4–9] it is important to determine disease mechanisms before considering *Pdis* to be a probiotic species for humans.

Phylogenetics reveal that copy number and structure of the *rfbA* gene in *Pdis* can be used as a classification system (*rfbA*-Typing) of bacterial isolates for future studies. The remarkable conservation of the *rfbA*-Type I sequences in isolates that spanned over 85 years (ATCC 8503, 1933 vs. CavFT-hAR46, 2019)[28,44] indicate that some *rfbA* genes are highly conserved within *Pdis*. Of interest, the *rfbA*-Type I cluster was composed mostly of strains that contain only one gene copy. Today it remains uncertain to what extent a greater number of *rfbA* gene copies could influence virulence associated with potentially increased O-antigen production. In *E. coli*, the gene deletion has been shown to eliminate O-antigen production,[51] and different types of *rfbA* represent different types of antigens; For example, two gene

products, *rffH* and *rmlA*, encode glucose-1-phosphate thymidylyltransferase, catalyzing the same enzymatic reaction, yet they are part of different operons and function in different pathways.[29] The clinical downstream effects of *rfbA* gene variance on the *Pdis* O-antigen structure remains to be elucidated. Additionally, future studies to validate *P. distasonis* O-antigens are warranted and cannot be conducted at this time since there is currently no available literature on their physical structures.

The relationship between O-antigen structures and subsequent virulence is longstanding and well-characterized in gram negative *Enterobacteriaceae*, namely, *E. coli*,[51–53] *Shigella sonnei*[32] and *Shigella flexneri*,[31] where the presence and length of the O-antigen of the LPS play a crucial role in pathogenesis. Compared to other *Bacteroidetes* and *Enterobacteriaceae*, the recognition of at least three major superclusters, wherein *Parabacteroides* shares gene homology with that of *Enterobacteriaceae* highlights the potential virulence contribution of the *Bacteroidetes* phylum in animal and human health via the *rfbA* O-antigen synthesis gene. In context with *Bacteroidetes* and *Enterobacteriaceae* (the latter in which *rfb* genes have been well described[51,54,55]), conserved clustering of the four distinct *Pdis rfbA*-Types highlights the not only the uniqueness of the *rfbA* gene in this species, but the specificity of *rfbA*-Types I–IV to *Pdis*. Similarly, most *rfbA*-Types assigned to other *Bacteroidetes* were unique to their respective genera, but those assigned to *Enterobacteriaceae* consistently contained at least two genera per *rfbA*-Type (except *rfbA*-Type VI which consists only of two *Klebsiella spp.*). To help with the characterization of *Pdis* isolates, we propose RFLP analysis using the MboII restriction enzyme which has been validated in *E. coli* and *Shigella*,[36,37] however the analysis could be expanded in the future with different enzymes.

In conclusion, this is the first study that provides some insight on the relation of O-antigen with the pathogenesis of *Parabacteroides distasonis* and that of other *Bacteroidetes*. The novel framework applied here to *Pdis* could help differentiate strains based on virulence potential linked to LPS production. Sequences and strains comprising the *rfbA*-Type I cluster are of significant interest for further investigation, and the primers and laboratory RFLP technique we designed should facilitate this and other studies of the *rfbA* gene in *Pdis*. Herein, we showed that *rfbA* gene variability (insertions/deletions) also occurs in other major genera within the *Bacteroidetes* phylum (*Parabacteroides, Bacteroides, Alistipes*, and *Prevotella*), creating unique '*rfbA*-superclusters' that share homology with known pathogenic *Enterobacteriaceae* (*Escherichia, Klebsiella, Salmonella*, and *Shigella*), indicating the same potential use for '*rfbA*-Typing' classification of *Bacteroidetes* in general. As a novel hypothesis, data indicate that for *P. distasonis*, applicable to other *Bacteroidetes*, there could be potential interactions between the *rfbA*-LPS/membrane fractions of *Pdis* with that of other bacteria to modulate the intensity and direction of cell signaling and inflammatory pathways in immune cells. Therein, it is possible that the pathogenic effects induced by whole *Pdis* cells in some strains (e.g. *rfbA*-Type I) could be through mechanisms other than LPS/O-antigen membrane fractions and *rfbA*-Type variation.

Acknowledgments

We acknowledge the Biorepository Core of the NIH Silvio O. Conte Cleveland Digestive Disease Research Core Center (P30DK097948; PD Fabio Cominelli, M.D. Ph.D.) at Case Western Reserve University for clinical advice and provision of deidentified surgical specimens for the above-mentioned R21 project. V.S. joined A.R.-P. laboratory after completing her Ph. D. program in 2021 at CSIR-Institue of Microbial Technology and Jawaharlal Nehru University, India.

Data availability

The genome sequencing data generated for the strains CavFT-hAR46 (BioSample SAMN11642307, PMID: 31488526), CavFT-hAR46_b (this study, same isolate as CavFT-hAR46, re-sequenced), and CavFT-hAR56 (this study) are available in GenBank within the BioProject number PRJNA542869.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This project was supported by NIH grant R21 DK118373 to A. R.-P., entitled "Identification of pathogenic bacteria in Crohn's disease." Partial support earlier originated for A.R.-P. from a career development award [310472] and a student

research fellowship award [644955] from the Crohn's & Colitis Foundation. Partial support for N.C.B earlier originated from The Digestive Health Research Institute's T35, "The Case Medical Student Summer Research Program (MSSRP)" [T35DK111373, Fabio Cominelli, M.D. Ph.D, Theresa Pizarro Ph.D.] at Case Western Reserve University.

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