

Distribution of Different Species of the *Bacteroides fragilis* Group in Individuals with Japanese Cedar Pollinosis[∇]

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We investigated associations of species of the *Bacteroides fragilis* group with Japanese cedar pollinosis (JCPsis). Cell numbers of *Bacteroides fragilis* and *Bacteroides intestinalis* were significantly higher in JCPsis subjects than in non-JCPsis subjects before the pollen season. They correlated positively with both symptom scores and JCPsis-specific immunoglobulin E levels.

Japanese cedar pollinosis (JCPsis), an immunoglobulin E (IgE)-mediated type I allergy caused by exposure to Japanese cedar (*Cryptomeria japonica*) pollen (JCP), represents a public health issue affecting over 16% of the Japanese population (4). In clinical studies evaluating the effects of a probiotic strain, *Bifidobacterium longum* BB536, on JCPsis, we found that administration of *B. longum* BB536 significantly alleviated some subjective symptoms and affected blood markers in individuals with JCPsis (20–22). Furthermore, we observed fluctuations in the *Bacteroides fragilis* group among individuals with JCPsis in the pollen season, with administration of *B. longum* BB536 suppressing these fluctuations (12, 13). The genus *Bacteroides* is known as one of the predominant intestinal bacteria in humans. The *Bacteroides fragilis* group has been suggested to be associated with allergic disease in several clinical studies (5, 8, 16). However, the taxonomy of the genus *Bacteroides* has undergone significant changes in the past few years (15), owing to the redefinition of the genus *Bacteroides* and the application of molecular biological techniques leading to the identification of several novel species (1–3, 6, 9). Little has been determined regarding the distributions of these bacteria in human fecal microbiota.

In the present study, we designed 14 specific primer pairs to detect species in the *Bacteroides fragilis* group that have been isolated from and identified in human feces and investigated distributions of each species for individuals with JCPsis and those without JCPsis by real-time PCR, to evaluate possible associations with JCPsis.

Clinical study. Samples came from a clinical study reported by Xiao et al. (21) evaluating the effects of *B. longum* BB536 on clinical symptoms of JCPsis and blood parameters. Briefly, a total of 44 adults with JCPsis were randomized to ingest either *B. longum* BB536 powder (BB536 group; 13 men and 9 women; mean age, 36.0 ± 7.3 years) or placebo powder (placebo group; 13 men and 9 women; mean age, 36.5 ± 8.1 years), in a randomized, double-blinded design during the pollen season (20

January to 21 April). Fourteen healthy adults who were JCP specific, IgE negative, and without prior history of spring allergic rhinitis (healthy group, 11 men and 3 women; mean age, 33.4 ± 7.6 years) were administered placebo powder during the same intervention period in an identical manner to JCPsis subjects. Participants were instructed to collect specimens in a plastic tube, cool the bag immediately to <10°C, and deliver the sample within 12 h. Collected specimens were stored at –80°C until analysis.

Design and specificity of primer pairs. DNA extraction from fecal samples was performed as described previously (13). Fourteen 16S rRNA gene-targeted species-specific primers (Table 1) were designed and checked for specificity according to previous reports (10, 14). The amplification program consisted of 94°C for 10 s, followed by 35 cycles of 94°C for 5 s and 60°C for 30 s. Melting curves were obtained by heating from 60°C to 95°C in increments of 0.2°C/s, with continuous fluorescence collection. DNA extracts from the type strains listed in Table 1 were used as standards for the determination of the cell number of each species. The specificity of each primer pair was then tested using DNA extracts from all strains listed in Table 1, with the addition of *Parabacteroides distasonis* JCM 5825^T, *Parabacteroides merdae* JCM 9497^T, *Prevotella intermedia* JCM 12248^T, and *Porphyromonas gingivalis* JCM 8525, 12257^T. Each specific primer yielded positive PCR results for the corresponding target bacterium and negative PCR results for nontarget microorganisms.

Distribution of each species of the *Bacteroides fragilis* group in fecal samples. Analyses were conducted on fecal samples collected from individuals that completed the study before (20 January, before the sample intake) and after (21 April) the pollen season. We observed some different distributions of the *Bacteroides fragilis* group for the JCPsis group compared with the healthy group before the pollen season (Table 2). In particular, cell numbers of *Bacteroides fragilis* and *Bacteroides intestinalis* were significantly higher in the JCPsis group than in the non-JCPsis group (Table 2).

Compared to the pre-pollen season, totals of nine, six, and two species of the *Bacteroides fragilis* group were increased significantly after the pollen season in the placebo, BB536 and healthy

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TABLE 1. PCR primers for detection of each species in the *Bacteroides fragilis* group

Target species	Primer	Sequence (5' to 3')	Product size (bp)	Strain(s) for validation
<i>Bacteroides caccae</i>	BaCAC-F BaCAC-R	GGGCATCAGTTTGTGTTGCTT GAACGCATCCCCATCTCATA	180	JCM 9498 ^T
<i>Bacteroides coprocola</i>	BaCOP-F BaCOP-R	TATGGTGAGATTGCATGATGG ATGAACGTCAGTTACAGTTTAGCAA	575	JCM 12979 ^T , 12980
<i>Bacteroides coprophilus</i>	BaCPP-F BaCPP-R	GGGTTGTAAACTTCTTTTGTGC GCCTCAACCGTACTCAAGGT	241	JCM 13816, 13818 ^T
<i>Bacteroides dorei</i>	BaDOR-F BaDOR-R	GGA AACGGTT CAGCTAGCAATA AGTCTTGT CAGAGTCTCAGCATC	147	JCM 13471 ^T , 13472
<i>Bacteroides eggerthii</i>	BaEGG-F BaEGG-R	ATAGTTTTTCCGCATGGTTTC GGCTGGTTCAGACTCTCGTC	214	JCM 12986 ^T
<i>Bacteroides finegoldii</i>	BaFIN-F BaFIN-R	CCGGATGGCATAGGATTGTC CGTAGGAGTTTGACCGTGT	173	JCM 13345 ^T , 13346
<i>Bacteroides fragilis</i>	BaFRA-F BaFRA-R	TGATTCCGCATGGTTTCATT CGACCCATAGAGCCTTCATC	243	JCM 11017, 11019 ^T
<i>Bacteroides intestinalis</i>	BaINT-F BaINT-R	AGCATGACCTAGCAATAGGTTG ACGCATCCCCATCGATTAT	169	JCM 13265 ^T , 13266
<i>Bacteroides ovatus</i>	BaOVA-F BaOVA-R	CCGGATAGCATA CGAACATC CGTAGGAGTTTGACCGTGT	173	JCM 5824 ^T
<i>Bacteroides plebeius</i>	BaPLE-F BaPLE-R	ATCATTAAAGATTTATCGGTGTACG ACTTTCACAGCTGACTTAACGAC	404	JCM 12973 ^T , 12974
<i>Bacteroides stercoris</i>	BaSTE-F BaSTE-R	AAAGCTTGCTTTGATGGATG ACATACAAAAAGCCACACGTC	406	JCM 9496 ^T
<i>Bacteroides thetaiotaomicron</i>	BaTHE-F BaTHE-R	ATCAGACCGCATGGTCTTAT CAACCCATAGGGCAGTCATC	244	JCM 5827 ^T
<i>Bacteroides uniformis</i>	BaUNI-F BaUNI-R	TACCCGATGGCATAGTTCTT GGACCGTGTCTCAGTTCCAA	164	JCM 5828 ^T , 13286
<i>Bacteroides vulgatus</i>	BaVUL-F BaVUL-R	GCAGATGAATTACGGTGAAAGC GTCAGAGTCTCAGCGGAAC	154	JCM 5826 ^T

groups, respectively (Table 3). Among these, when taking notice of those species that increased among the JCPsis subjects, it was found that *Bacteroides caccae*, *Bacteroides vulgatus*, *Bacteroides*

fragilis, and *Bacteroides intestinalis* were significantly increased only in the placebo group.

Comparing cell numbers after pollen season, significant in-

TABLE 2. Cell numbers of each species of the *Bacteroides fragilis* group in fecal samples before pollen season

Species	Mean cell no./g ± SD (% prevalence) ^a		
	Pollinosis (n = 44)	Healthy (n = 14)	Total (n = 58)
<i>Bacteroides caccae</i>	8.95 ± 0.85 (40.9)	8.61 ± 0.79 (71.4)	8.83 ± 0.83 (48.3)
<i>Bacteroides coprocola</i>	8.64 ± 0.55 (20.5)	9.03 ± 0.31 (28.6)	8.76 ± 0.51 (22.4)
<i>Bacteroides coprophilus</i>	7.93 ± 0.64 (11.4)	ND ^b (0)	7.93 ± 0.64 (8.6)
<i>Bacteroides dorei</i>	8.83 ± 0.77 (84.1)	8.49 ± 0.57 (92.9)	8.74 ± 0.73 (86.2)
<i>Bacteroides eggerthii</i>	8.17 ± 0.89 (38.6)	7.94 ± 0.63 (21.4)	8.14 ± 0.85 (34.5)
<i>Bacteroides finegoldii</i>	7.62 ± 0.51 (25.0)	8.05 ± 0.30 (14.3)	7.68 ± 0.50 (22.4)
<i>Bacteroides fragilis</i>	8.50 ± 0.86 (75.0)*	8.19 ± 0.37 (35.7)	8.46 ± 0.81 (65.5)
<i>Bacteroides intestinalis</i>	8.65 ± 0.81 (36.4)*	8.54 ± 0.00 (7.1)	8.64 ± 0.79 (29.3)
<i>Bacteroides ovatus</i>	8.28 ± 0.48 (61.4)	8.25 ± 0.50 (50.0)	8.27 ± 0.48 (58.6)
<i>Bacteroides plebeius</i>	8.17 ± 0.78 (29.6)	7.87 ± 0.81 (28.6)	8.10 ± 0.77 (29.3)
<i>Bacteroides stercoris</i>	8.89 ± 0.51 (15.9)	ND (0)	8.89 ± 0.51 (12.1)
<i>Bacteroides thetaiotaomicron</i>	9.12 ± 0.74 (45.5)	8.64 ± 0.24 (57.1)	8.99 ± 0.67 (48.3)
<i>Bacteroides uniformis</i>	9.04 ± 0.93 (79.6)	8.94 ± 0.68 (100)	9.01 ± 0.86 (84.5)
<i>Bacteroides vulgatus</i>	9.50 ± 1.09 (68.2)	9.40 ± 0.56 (71.4)	9.48 ± 0.98 (69.0)
Total (14 species)	9.88 ± 0.94 (100)	9.68 ± 0.56 (100)	9.83 ± 0.87 (100)

^a Cell numbers (mean ± standard deviation) were determined as log₁₀ cells per gram (wet weight) of feces among individuals over detection limits, with prevalence given as a percentage in parentheses. Detection limits for each bacterial species by real-time PCR were 10⁶/g (wet weight) of feces. Statistical analyses were performed using SPSS version 14.0 statistical software (SPSS, Japan). Intergroup differences were analyzed using the Mann-Whitney U test on cell numbers after logarithmic transformation, by substituting data with log 10⁶ values for individuals under the detection limits. *, P < 0.05 for significant intergroup difference compared with the healthy group.

^b ND, not detected (<log 10⁶ cells/g).

TABLE 3. Cell numbers for species of the *Bacteroides fragilis* group in fecal samples before and after pollen season

Species	Time relative to pollen season	Mean cell no./g \pm SD (% prevalence) ^a		
		Placebo (n = 13)	BB536 (n = 20)	Healthy (n = 14)
<i>Bacteroides caccae</i>	Before	8.84 \pm 0.06 (30.8)	8.73 \pm 0.81 (35.0)	8.61 \pm 0.79 (71.4)
	After	8.73 \pm 0.90 (61.5)*	8.47 \pm 0.99 (45.0)	8.54 \pm 0.46 (64.3)
<i>Bacteroides coprocola</i>	Before	8.69 \pm 0.46 (23.1)	8.72 \pm 0.38 (15.0)	9.03 \pm 0.31 (28.6)
	After	9.89 \pm 0.00 (7.7)	9.77 \pm 0.26 (15.0)	8.96 \pm 0.54 (28.6)
<i>Bacteroides coprophilus</i>	Before	7.70 \pm 0.33 (23.1)	8.99 \pm 0.00 (5.0)	ND ^b (0)
	After	7.95 \pm 0.00 (7.7)	9.28 \pm 1.72 (10.0)	7.72 \pm 0.20 (14.3)
<i>Bacteroides dorei</i>	Before	8.98 \pm 0.89 (92.3)	8.82 \pm 0.62 (75.0)	8.49 \pm 0.57 (92.9)
	After	9.52 \pm 0.94 (100)*	9.16 \pm 1.15 (95.0)**	8.74 \pm 0.67 (92.9)
<i>Bacteroides eggerthii</i>	Before	7.89 \pm 0.58 (46.2)	8.15 \pm 0.90 (30.0)	7.94 \pm 0.63 (21.4)
	After	8.97 \pm 0.77 (53.9)*	8.64 \pm 0.76 (50.0)*	9.26 \pm 0.56 (21.4)
<i>Bacteroides fingoldii</i>	Before	7.76 \pm 0.30 (30.8)	7.53 \pm 0.24 (15.0)	8.05 \pm 0.30 (14.3)
	After	8.70 \pm 0.37 (30.8)	8.52 \pm 0.22 (15.0)	8.41 \pm 0.11 (21.4)
<i>Bacteroides fragilis</i>	Before	8.80 \pm 0.78 (92.3)†	8.30 \pm 0.51 (70.0)	8.19 \pm 0.37 (35.7)
	After	9.43 \pm 0.78 (92.3)*†‡	9.12 \pm 0.65 (50.0)	8.36 \pm 0.79 (42.9)
<i>Bacteroides intestinalis</i>	Before	8.13 \pm 0.41 (23.1)	8.60 \pm 0.83 (45.0)	8.54 \pm 0.00 (7.1)
	After	8.87 \pm 1.45 (69.2)*†	9.37 \pm 0.93 (35.0)	7.70 \pm 0.10 (14.3)
<i>Bacteroides ovatus</i>	Before	8.24 \pm 0.51 (84.6)	8.41 \pm 0.47 (60.0)	8.25 \pm 0.50 (50.0)
	After	8.88 \pm 0.55 (92.3)*	8.78 \pm 0.71 (70.0)*	8.61 \pm 0.64 (71.4)**
<i>Bacteroides plebeius</i>	Before	8.42 \pm 0.65 (23.1)	8.04 \pm 0.58 (35.0)	7.87 \pm 0.81 (28.6)
	After	9.50 \pm 1.60 (30.8)	9.97 \pm 1.76 (40.0)*	8.86 \pm 0.51 (28.6)
<i>Bacteroides stercoris</i>	Before	9.04 \pm 0.42 (23.1)	8.05 \pm 0.00 (5.0)	ND (0)
	After	9.04 \pm 0.23 (15.4)	8.85 \pm 0.64 (20.0)	ND (0)
<i>Bacteroides thetaiotaomicron</i>	Before	9.24 \pm 0.97 (46.2)	8.88 \pm 0.21 (40.0)	8.64 \pm 0.24 (57.1)
	After	9.50 \pm 0.62 (69.2)*	9.53 \pm 0.61 (55.0)*	9.04 \pm 0.48 (64.3)
<i>Bacteroides uniformis</i>	Before	9.04 \pm 0.88 (76.9)	9.03 \pm 0.69 (75.0)	8.94 \pm 0.68 (100)
	After	10.43 \pm 0.59 (84.6)**	9.76 \pm 0.98 (85.0)**	9.46 \pm 0.71 (100)**
<i>Bacteroides vulgatus</i>	Before	9.16 \pm 1.22 (53.9)	9.37 \pm 0.77 (65.0)	9.40 \pm 0.56 (71.4)
	After	9.36 \pm 1.42 (76.9)*	9.22 \pm 1.11 (85.0)	9.26 \pm 0.47 (71.4)
Total (14 species)	Before	9.85 \pm 0.88 (100)	9.66 \pm 0.81 (100)	9.68 \pm 0.56 (100)
	After	10.93 \pm 0.50 (100)**†	10.45 \pm 0.94 (100)**	9.93 \pm 0.55 (100)

^a Cell numbers were determined as log 10 cells per gram (wet weight) of feces, with prevalence given as a percentage in parentheses. Detection limits for each bacterial species by real-time PCR were 10⁶/g (wet weight) of feces. Statistical analyses were performed with SPSS version 14.0 statistical software (SPSS, Japan). Intra- and intergroup differences were analyzed using the Wilcoxon signed-rank test and Kruskal-Wallis H test with Bonferroni's correction, respectively. Both statistical analyses were conducted on cell numbers after logarithmic transformation, by substituting data with log 10⁶ values for individuals under detection limits. * and **, *P* < 0.05 and *P* < 0.01, respectively, for significant intragroup difference from baseline (before); †, *P* < 0.01 for significant intergroup difference from the healthy group at each time point; ‡, *P* < 0.01 for significant intergroup difference between the placebo and BB536 groups at each time point.

^b ND, not detected (<log 10⁶ cells/g).

tergroup differences were found for *Bacteroides fragilis* and *Bacteroides intestinalis* between the placebo and healthy groups and significant intergroup differences were found for *Bacteroides fragilis* between the placebo and BB536 groups.

Correlation analyses. Significant positive correlations with clinical symptom scores and JCP-specific IgE levels were observed for cell numbers of *Bacteroides fragilis* and *Bacteroides intestinalis* either before or after the pollen season (Table 4). Conversely, significant negative correlations with JCP-specific IgE level were observed for cell numbers of *Bacteroides uniformis* and *Bacteroides caccae* before the pollen season.

Conclusions. We observed that cell numbers of *Bacteroides fragilis* and *Bacteroides intestinalis* were significantly higher in the JCPsis group than in the healthy group before and after the pollen season. Furthermore, significant positive correlations were found between the cell numbers of these two species with composite symptom scores and JCP-specific IgE. Our data suggest that prevalence of *Bacteroides fragilis* and *Bacteroides intestinalis* might represent risk factors for JCPsis. In addition, no significant change was observed in cell numbers of *Bacteroides fragilis* or *Bacteroides intestinalis* in the BB536 group, suggesting that intake of *B. longum* BB536 may play a role in

TABLE 4. Correlations between cell number of *Bacteroides* species and composite symptom score and JCPsis-specific IgE

Species	ρ value for ^a :			
	AUC of composite symptom scores ^b		JCP IgE	
	Before pollen season	After pollen season	Before pollen season	After pollen season
<i>Bacteroides caccae</i>	-0.151	-0.088	-0.337*	-0.110
<i>Bacteroides coprocola</i>	-0.092	-0.103	0.024	-0.024
<i>Bacteroides coprophilus</i>	0.245	0.236	0.073	-0.020
<i>Bacteroides dorei</i>	0.182	0.298*	0.172	0.349*
<i>Bacteroides eggerthii</i>	0.067	0.131	-0.005	0.070
<i>Bacteroides finegoldii</i>	0.077	0.130	0.034	-0.039
<i>Bacteroides fragilis</i>	0.345*	0.412**	0.320*	0.363*
<i>Bacteroides intestinalis</i>	0.491**	0.622**	0.301*	0.304*
<i>Bacteroides ovatus</i>	0.186	0.238	0.073	0.173
<i>Bacteroides plebeius</i>	0.000	0.030	0.130	0.133
<i>Bacteroides stercoris</i>	0.321*	0.041	0.163	0.195
<i>Bacteroides thetaiotaomicron</i>	-0.060	0.008	0.042	0.180
<i>Bacteroides uniformis</i>	-0.176	0.097	-0.420**	-0.043
<i>Bacteroides vulgatus</i>	-0.116	0.108	-0.163	0.187
Total (14 species)	0.067	0.330*	-0.042	0.419**

^a Analyses were performed using SPSS version 14.0 statistical software (SPSS, Japan). Spearman's ρ coefficients and P values were calculated on cell numbers after logarithmic transformation, by substituting data with log 10⁶ values for individuals under detection limits. * and **, P < 0.05 and P < 0.01, respectively, for significant correlation.

^b Weekly total scores for sneezing, rhinorrhea, nasal blockage, nasal itching, eye symptoms and throat symptoms recorded during the pollen season were totaled as composite scores. Areas under the curves (AUC) were then calculated using changes in score during pollen dispersion (21).

stabilizing the microbiota, which might in turn exert suppressive effects on sensitization to pollen and/or symptom development.

Increased prevalence of the *Bacteroides fragilis* group has been observed in individuals with allergic diseases or under stress conditions (5, 7, 8, 16, 17). In vitro studies have demonstrated that *Bacteroides fragilis* perturbed host immunity (11, 12, 18, 19). These lines of evidence implied an exacerbating effect of the *Bacteroides fragilis* group on allergic disorders. To the best of our knowledge, this is the first report to outline a possible association between the species of the *Bacteroides fragilis* group and allergic diseases, although we cannot deny that there might be some biases in the cell numbers of each species since they have only been determined by the quantitative PCR method. Nevertheless, the results from the intra/intergroup differences should have not been influenced greatly. Further studies are needed to confirm these results, especially for *Bacteroides intestinalis* since the prevalence was relatively low.

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