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Phage Specificity of the Freshwater Fish Pathogen *Flavobacterium columnare*▽

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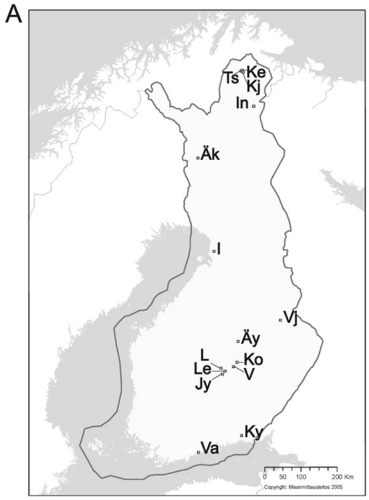
Flavobacteria and their phages were isolated from Finnish freshwaters and fish farms. Emphasis was placed on finding phages infecting the fish pathogen *Flavobacterium columnare* for use as phage therapy agents. The host ranges of the flavobacterial phages varied, phages infecting *F. columnare* being more host specific than the other phages.

Species of the genus *Flavobacterium* are widely distributed in nature, and they have been found in diverse habitats (3, 19, 30, 32, 36, 37, 38, 39). In general, flavobacteria are nonpathogenic, but some species are opportunistic pathogens (2). Columnaris, a disease caused by the fish pathogen *Flavobacterium columnare*, can cause up to 100% mortality among salmonid fingerlings (28). Antibiotic treatment must be applied to prevent mass mortalities at fish farms. Despite effective treatment, columnaris occurs repeatedly during the summer (18). Therefore, the number of antibiotic treatments and the amount of antibiotics used can be extremely high. Increased resistance of environmental bacteria to antibiotics in fish farms and their surroundings has been reported (10, 24, 26, 31), and antibiotic-resistant fish-pathogenic flavobacteria have also emerged (7). To avoid risks related to antibiotic use, enrichment of bacteriophages could be used as an ecological method of decreasing the number of *F. columnare* infections. To our knowledge, this is the first study on European *F. columnare* phages.

In this study, a total of 53 flavobacterial isolates were received during the warm-water period (May to August) in 2008 and 2009 from water samples that included both open freshwater environments (rivers and lakes not connected to fish farming) and three inland land-based fish farms rearing mainly salmonid fingerlings in Finland (Fig. 1; Table 1). One bacterium (B67) was isolated from diseased fish in 2007. Water samples were cultured on Shieh agar (25) and 1/5× Luria



Location	Abbrevation	Longitude	Latitude
Lake Konnevesi	Ko	E 26° 35.644'	N 62° 31.446'
Lake Jyväsjärvi	Jy	E 25° 44.339'	N 62° 13.779'
River Vantaanjoki	Va	E 24° 58.072'	N 60° 15.948'
River Kevojoki	Ke	E 26° 59.429'	N 69° 45.337'
River Tsarsjoki	Ts	E 26° 59.447'	N 69° 45.430'
Lake Kevojärvi	Kj	E 26° 59.608'	N 69° 45.430'
Lake Valtimojärvi	Vj	E 28° 51.000'	N 63° 40.688'
River Kymijoki	Ky	E 26° 49.926'	N 60° 41.391'
Lake Leppävesi	Le	E 25° 54.633'	N 62° 16.064'
Lake Äkässaivo	Äk	E 24° 8.640'	N 67° 41.840'
River Äyskoski	Äy	E 26° 40.756'	N 63° 0.665'
Lake Inari	In	E 27° 53.097'	N 68° 47.783'
Fishery L [1)]	L		
Fishery V [1)]	V		
Fishery I [2)]	I		

[1)] Central Finland
[2)] Northern Finland

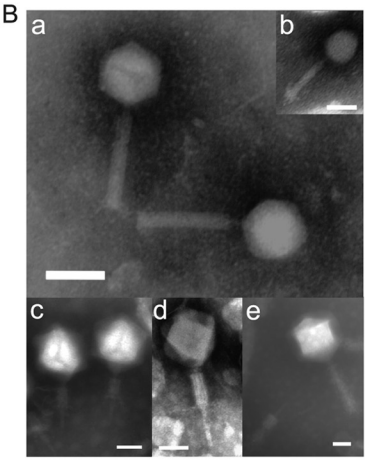


FIG. 1. (A) Sites in Finland where flavobacteria and their phages were isolated. Sampling sites are marked on the free map obtained from the National Land Survey of Finland (Maanmittauslaitos, 2005). The sites and their abbreviations and coordinates are listed on the right. (B) Electron micrographs of purified and negatively stained tailed *Flavobacterium* phages. (a) FCV-1; (b) FCL-2; (c) FJy-3; (d) FKo-2; (e) FKy-1. Bar, 50 nm.

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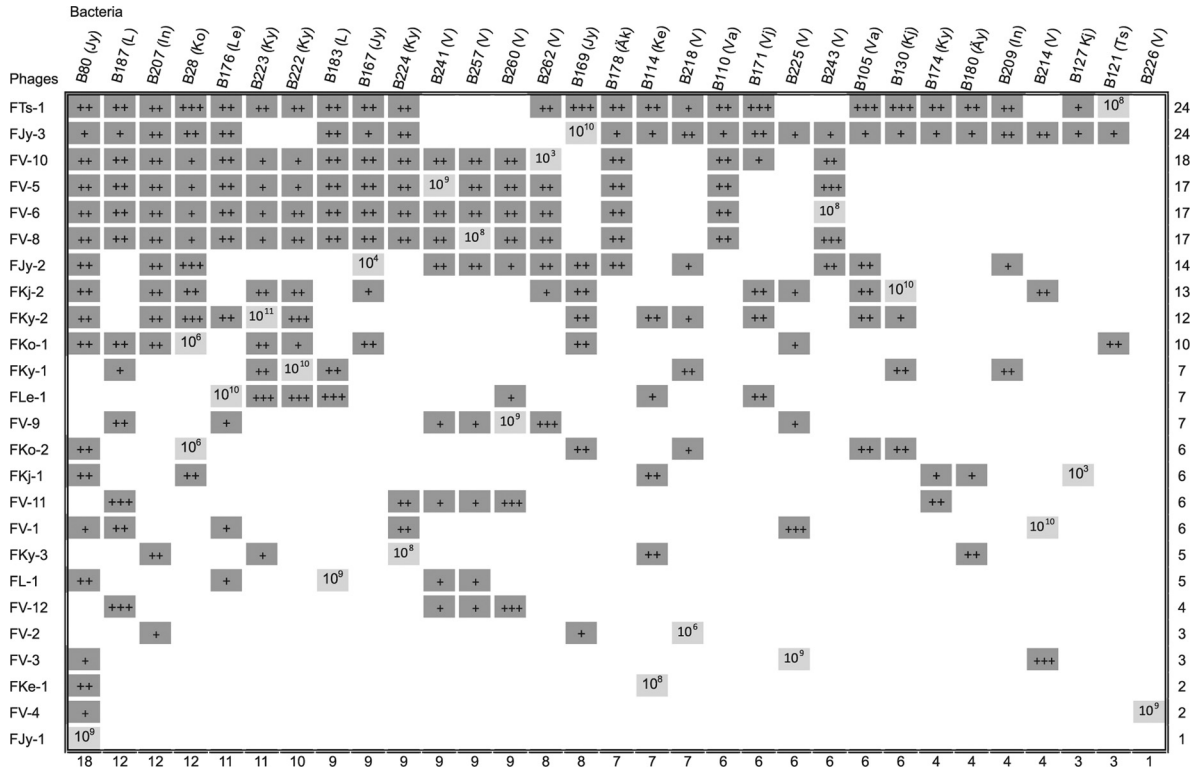

FIG. 2. Host ranges of the phages infecting different *Flavobacterium* sp. strains. Dark gray squares indicate infection; white indicates no infection, and light gray squares mark the strain which was originally used for isolation of the phage. The numbers at right and bottom are the total number of different host ranges of the phage and susceptibility of bacteria to phages, respectively. The approximate titer of each phage on the bacteria is marked with plus signs (+, $\leq 10^5$; ++, 10^6 to 10^8; and +++, $\geq 10^9$ PFU/ml). The titer (PFU/ml) of the phage on the isolation strain is marked on the light gray squares. In parentheses after the name of the bacterial strain is the abbreviation of the place of isolation, which is also included in the phage nomenclature (Fig. 1). *F. columnare* phages infected only one specific *F. columnare* RISA group, and they are listed in Table 2.

Bertani agar (22), and yellow- and orange-pigmented colonies were selected for analyses. The flavobacterial isolates were subjected to PCR with universal primers (UP-PCR) (for methods, see references 5, 6,, and 13), and the 16S rRNA genes of different groups were sequenced (Table 1). All *F. columnare* isolates fell into the same UP-PCR group, and thus they were further analyzed with ribosomal intergenic spacer analysis (RISA) (8, 29). According to RISA, the isolates were assigned to five groups (Table 1). Based on our data, the occurrence of *F. columnare* seems to be connected to the fish farming environment; this organism was not isolated from natural waters. However, it is likely that the initial source of *F. columnare* at the farms is nature, because there is evidence that it is also present outside fish farms (20, 21; H. Kunttu, L.-R. Sundberg, and E. T. Valtonen, unpublished data).

Previous reports describe phages infecting the genus *Flavobacterium* and their interaction with the bacterial host mostly in marine environments (4, 9, 12, 14), but the phage-host relationship of the fish pathogen *Flavobacterium psychrophilum* has also been studied (27). A total of 49 bacteriophages were isolated from water samples (Table 2). Phages were enriched using flavobacterial isolates from freshwaters, fish farms, and previously described *F. columnare* strains. Phage stocks were prepared, and selected phages were grown by infecting the host bacterium (multiplicity of infection, 5 to 10) at the proper cell density, concentrated, and purified (22). Many of the isolated phages produced low-titer lysates, and they were used only for infection tests (see below for host range studies). Phage genomic DNA was extracted (for methods, see references 1 and 23) and digested with BamHI, EcoRI, HindIII, and PstI. For the genomes that were cut, the genome size was calculated from the resulting restriction profile (Table 2). For the phages that were sequenced (FKj-2, FL-1, FCL-2, and FCV-1), approximately 3,000 bp of each genome (except FCV-1, for which 1,600 bp was used) was subjected to BLAST searching (http://blast.ncbi.nlm.nih.gov/Blast.cgi; May 2011), and no significant DNA sequence similarity was found in the database. Putative protein-coding genes were analyzed using Vector NTI 11.0.0 (Invitrogen). Best matches for FCL-2 were to a hypothetical protein of the *Vibrio* phage VP16T (score, 57.8) and to a hypothetical protein of the *Vibrio* phage VP16C (score, 55.1). For FCV-1, the best match was to a hypothetical protein, B40-8030, of the *Bacteroides* phage B40-8 (score, 53.9).

Transmission electron microscopy (TEM) was used to study phage morphology. All of the flavobacterial phages that were characterized were tailed phages of the families *Myoviridae*, *Podoviridae*, and *Siphoviridae* (Fig. 1 and Table 2). Most of these phages had an average head size of 50 to 70 nm, but some of the myovirus isolates had capsid sizes of about 100 nm or more (FJy-3, FKo-2, FKj-2, FKy-1, and FKy-3).

Phages infecting *F. columnare* were isolated only from fish

TABLE 1. Bacterial strains isolated and used in the study

Bacterial strain[a]	UP-PCR or RISA group[b]	EMBL accession no.[c]	Sampling site	Source or reference	Bacterial strain[a]	UP-PCR or RISA group[b]	EMBL accession no.[c]	Sampling site	Source or reference
B67	A		Fishery L	This study	B244	ND		Fishery V	This study
B28	1	FR696328	Lake Konnevesi	This study	B245	C		Fishery V	This study
B80	2	FR696329	Lake Jyväsjärvi	This study	B247	C		Fishery V	This study
B105	3	FR696330	River Vantaanjoki	This study	B257	26	FR696355	Fishery V	This study
B110	4	FR696331	River Vantaanjoki	This study	B259	C		Fishery V	This study
B114	5	FR696332	River Kevojoki	This study	B260	ND	FR696356	Fishery V	This study
B121	6	FR696333	River Tsarsjoki	This study	B261	C		Fishery V	This study
B127	7	FR696334	Lake Kevojärvi	This study	B262	ND	FR696357	Fishery V	This study
B130	8	FR696335	Lake Kevojärvi	This study	B263	ND	FR696358	Fishery V	This study
B167	9	FR696336	Lake Jyväsjärvi	This study	B267	C		Fishery V	This study
B169	10	FR696337	Lake Jyväsjärvi	This study	B268	C		Fishery V	This study
B171	11	FR696338	Lake Valtimojärvi	This study	B269	ND		Fishery V	This study
B174	12	FR696339	River Kymijoki	This study	B270	C		Fishery V	This study
B176	13	FR696340	Lake Leppävesi	This study	B271	C		Fishery I	This study
B178	14	FR696341	Lake Äkässaivo	This study	B272	J		Fishery I	This study
B180	15	FR696342	River Äyskoski	This study	B273	C		Fishery I	This study
B183	16	FR696343	Fishery L	This study	B274	C		Fishery I	This study
B185	G	FR696344	Fishery L	This study	B275	C		Fishery I	This study
B187	17	FR696345	Fishery L	This study	Rz-A	A			29
B207	18	FR696346	Lake Inari	This study	R-B	B			29
B209	19	FR696347	Lake Inari	This study	Rz-C	C			29
B214	20	FR696348	Fishery V	This study	S-C	C			29
B218	21		Fishery V	This study	R-D	D			29
B222	22	FR696349	River Kymijoki	This study	S-D	D			29
B223	23	FR696350	River Kymijoki	This study	Rz-E	E			29
B224	24		River Kymijoki	This study	R-E	E			29
B225	24	FR696351	Fishery V	This study	S-E	E			29
B226	25	FR696352	Fishery V	This study	S-F	F			*F. columnare* type strain NCIMB 2248
B230	I		Fishery V	This study					
B234	C		Fishery V	This study					
B235	C		Fishery V	This study	Rz-G	G			29
B236	ND		Fishery V	This study	R-G	G			29
B237	C		Fishery V	This study	S-G	G			29
B241	26	FR696353	Fishery V	This study	R-H	H			29
B243	27	FR696354	Fishery V	This study					

[a] The previously studied *F. columnare* strains (genomic groups A to H and colony morphologies 1 to 4) are referred to in this study by placing the colony morphology after the genomic group: Rz, rhizoid (previously 1); R, rough (previously 2 and 3); and S, smooth (previously 4). For example Rz-C corresponds to the previous designation C1.

[b] UP-PCR group (numbers) for *Flavobacterium* sp. or RISA group (letters) for *Flavobacterium columnare*. ND, not determined.

[c] For the partial 16S rRNA sequence.

farms during disease outbreaks. These phages might survive inside the host cell during the cold-water period and start a lytic cycle when nutrients become available for the host cell and enough energy is available.

Studies on aquatic phage-host interplay have been conducted extensively in marine environments (15), but less is known about this interplay in freshwaters (15, 34, 35). In our study, the host ranges differed greatly between the phage isolates (Fig. 2). In the initial screening for susceptibility of the bacteria for phages, each bacterium was infected with each phage isolate by spotting the phage lysate on top agar containing the host bacterium. Each bacterium was then infected with each phage using a plaque assay. Some of the isolated phages infecting *Flavobacterium* species were identified as having a broad host range. These phages infected bacteria isolated from both freshwater and fish farm samples, although it has been suggested that single-host enrichments select for phages with narrow host ranges from sewage and marine environments (11, 33). Some of the phages (especially all *F. columnare* phages) were more host specific, as determined with our collection of *Flavobacterium* strains. *F. columnare* strains isolated from the same location as the phage were the only ones susceptible to that specific phage.

No infection of *F. columnare* strains by *Flavobacterium* sp. phages was observed, and vice versa. However, 11 *Flavobacterium* sp. phage lysates (FTs-1, FKo-2, FL-1, FV-1, FKy-1, FKy-2, FKy-3, FV-3, FV-4, FV-5, FV-6, and FV-8) inhibited the growth or lysed the underlying bacterial culture on all *F. columnare* strains tested but produced no individual plaques (data not shown). It could be that the phages were able to bind to the bacteria and cause death but were not able to produce progeny, or the clear spots could be an indication of bacteriocin activity. The causative agent of this strong inhibition or lysis should be studied further for the possibility of developing antimicrobial agents. One of the phages (FCL-1) was isolated from a fish suffering from columnaris. The presence of the *F. columnare* phages in the fish indicates the possibility of controlling a fish disease by enrichment of these phages. A number of successful reports on phage

TABLE 2. Bacteriophages isolated and characterized in this study

Phage[b]	Sampling site	Isolation strain	Phage family	Approximate genome size (kbp)[c]	RISA group[d]
FJy-1	Lake Jyväsjärvi	B80	*Myoviridae*	30	
FJy-2	Lake Jyväsjärvi	B167	ND[a]	>48	
FJy-3	Lake Jyväsjärvi	B169	*Myoviridae*	L	
FKo-1	Lake Konnevesi	B28	*Myoviridae*	25–48	
FKo-2	Lake Konnevesi	B28	*Myoviridae*	20–30	
FKe-1	River Kevojoki	B114	*Myoviridae*	ND	
FTs-1	River Tsarsjoki	B121	ND	25–48	
FKj-1	Lake Kevojärvi	B127	ND	>48	
FKj-2	Lake Kevojärvi	B130	*Myoviridae*	25–48	
FKy-1	River Kymijoki	B222	*Myoviridae*	20–48	
FKy-2	River Kymijoki	B223	*Podoviridae*?	25–48	
FKy-3	River Kymijoki	B224	*Myoviridae*?	L	
FLe-1	Lake Leppävesi	B176	*Siphoviridae*?	20–30	
FL-1	Fishery L	B183	*Myoviridae*	55	
FV-1	Fishery V	B214	*Myoviridae*	28	
FV-2	Fishery V	B218	ND	25–48	
FV-3	Fishery V	B225	*Myoviridae*	ND	
FV-4	Fishery V	B226	*Podoviridae*	25–48	
FV-5	Fishery V	B241	ND	L	
FV-6	Fishery V	B243	ND	30–40	
FV-8	Fishery V	B257	ND	L	
FV-9	Fishery V	B260	*Podoviridae*?	L	
FV-10	Fishery V	B262	ND	L	
FV-11	Fishery V	B263	ND	>48	
FV-12	Fishery V	B278	*Podoviridae*?	60	
FCL-1	Fishery L	B67	*Myoviridae*/*Podoviridae*	50	A
FCL-2	Fishery L	B185	*Myoviridae*	30	G
FCL-3	Fishery L	R-G	ND	30	G
FCL-4	Fishery L	R-G	ND	ND	G
FCV-1	Fishery V	Rz-C	*Myoviridae*	50	C
FCV-2	Fishery V	B235	ND	ND	C
FCV-3	Fishery V	B236	ND	ND	C
FCV-4	Fishery V	Rz-C	ND	ND	C
FCV-5	Fishery V	Rz-C	ND	ND	C
FCV-6	Fishery V	Rz-C	ND	ND	C
FCV-7	Fishery V	Rz-C	ND	ND	C
FCV-8	Fishery V	Rz-C	ND	ND	C
FCV-9	Fishery V	B245	ND	ND	C
FCV-10	Fishery V	B247	ND	ND	C
FCV-11	Fishery V	Rz-C	ND	ND	C
FCV-12	Fishery V	Rz-C	ND	ND	C
FCV-13	Fishery V	B261	ND	ND	C
FCV-14	Fishery V	Rz-C	ND	ND	C
FCV-15	Fishery V	Rz-C	ND	ND	C
FCV-16	Fishery V	Rz-C	ND	ND	C
FCV-17	Fishery V	Rz-C	ND	ND	C
FCV-18	Fishery V	Rz-C	ND	ND	C
FCV-19	Fishery V	Rz-C	ND	ND	C
FCV-20	Fishery V	Rz-C	ND	ND	C

[a] ND, not determined.
[b] The first letter(s) of the phage name indicates the isolation host (F, *Flavobacterium* sp.; FC, *F. columnare*); subsequent letters refer to the sampling site.
[c] L, much larger than the typical tailed phage genome (50 kb).
[d] The *F. columnare* RISA group that the phage is specific to.

therapy against fish diseases have been published; in these studies, the phages were applied directly to the water (16, 17). Another possible benefit related to phages is that they could be developed for use as diagnostic tools. In the present study, we show that bacteriophages and flavobacteria are widespread in northern freshwaters. We found phages of *F. columnare* to be host specific, making them good candidates for phage therapy.

Nucleotide sequence accession numbers. All sequences have been submitted to the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) under the accession numbers given in Table 1 and FR714876 (FCL-2), FR714877 (FL-1), FR714878 (FKj-2), and FR865436 (FCV-1).

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