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Large-Scale Analysis of *Flavobacterium psychrophilum* Multilocus Sequence Typing Genotypes Recovered from North American Salmonids Indicates that both Newly Identified and Recurrent Clonal Complexes Are Associated with Disease

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ABSTRACT *Flavobacterium psychrophilum*, the etiological agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), causes significant economic losses in salmonid aquaculture, particularly in rainbow trout (*Oncorhynchus mykiss*). Prior studies have used multilocus sequence typing (MLST) to examine genetic heterogeneity within *F. psychrophilum*. At present, however, its population structure in North America is incompletely understood, as only 107 isolates have been genotyped. Herein, MLST was used to investigate the genetic diversity of an additional 314 North American *F. psychrophilum* isolates that were recovered from ten fish host species from 20 U.S. states and 1 Canadian province over nearly four decades. These isolates were placed into 66 sequence types (STs), 47 of which were novel, increasing the number of clonal complexes (CCs) in North America from 7 to 12. Newly identified CCs were diverse in terms of host association, distribution, and association with disease. The largest *F. psychrophilum* CC identified was CC-ST10, within which 10 novel genotypes were discovered, most of which came from *O. mykiss* experiencing BCWD. This discovery, among others, provides evidence for the hypothesis that ST10 (i.e., the founding ST of CC-ST10) originated in North America. Furthermore, ST275 (in CC-ST10) was recovered from wild/feral adult steelhead and marks the first recovery of CC-ST10 from wild/feral fish in North America. Analyses also revealed that at the allele level, the diversification of *F. psychrophilum* in North America is driven three times more frequently by recombination than random nucleic acid mutation, possibly indicating how new phenotypes emerge within this species.

IMPORTANCE *Flavobacterium psychrophilum* is the causative agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), both of which cause

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substantial losses in farmed fish populations worldwide. To better prevent and control BCWD and RTFS outbreaks, we sought to characterize the genetic diversity of several hundred *F. psychrophilum* isolates that were recovered from diseased fish across North America. Results highlighted multiple *F. psychrophilum* genetic strains that appear to play an important role in disease events in North American aquaculture facilities and suggest that the practice of trading fish eggs has led to the continental and transcontinental spread of this bacterium. The knowledge generated herein will be invaluable toward guiding the development of future disease prevention techniques.

KEYWORDS bacterial coldwater disease, fish disease, *Flavobacteriaceae*, *Flavobacterium psychrophilum*, genetic diversity, MLST, rainbow trout fry syndrome, recombination

Flavobacterium psychrophilum (family *Flavobacteriaceae*; phylum *Bacteroidetes* [1]) is a Gram-negative psychrophilic bacterium (2). It is the etiological agent of bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), both of which principally impact salmonid species (family Salmonidae [2]). These diseases cause significant mortality in farmed/hatchery-reared salmonids and can result in substantial economic losses (2, 3). Since its initial description in 1948 in Washington (4), this bacterium has been reported from at least five continents (including Asia, Europe, North America, Australia, and South America), giving it a nearly worldwide distribution, particularly where salmonids are indigenous or introduced (5).

For decades, the degree of intraspecific genetic diversity within *F. psychrophilum* was unclear (6); however, advances in molecular typing techniques, including multilocus sequence typing (MLST), subsequently revealed heterogeneity within this species (7–13). Due to the discriminatory power and reproducibility of MLST (14), the MLST scheme introduced by Nicolas et al. (7) for *F. psychrophilum* is now being used worldwide. In this initial study, 50 *F. psychrophilum* isolates from Europe ($n = 27$), North America ($n = 10$), Asia ($n = 7$), Australia ($n = 3$), and South America ($n = 3$) were examined (7), revealing the presence of 30 genotypes, some of which were closely related or correlated to a geographic location and/or host species. Since then, this genotyping technique has been applied to >1,000 *F. psychrophilum* isolates from different parts of the world, yielding nearly 200 distinct genotypes that are distributed similarly to the original MLST report (8–13). Genotypes and basic information on all the genotyped isolates published thus far are available via a PubMLST database (15), which is an invaluable resource for the epidemiological studies of *F. psychrophilum* (https://pubmlst.org/fpsychrophilum/). The relatedness of isolates is defined as sharing identical alleles at six of seven loci with at least one other member of the group. Based on this definition, the largest group of related sequences is clonal complex-sequence type 10 (CC-ST10), which encompasses hundreds of isolates and dozens of sequence types (STs), with most isolated from farm-raised rainbow trout (*Oncorhynchus mykiss*) from multiple continents (8, 10–12). The association of CCs with host species has also been noted for other CCs, including CC-ST123 (12 isolates in six STs), which has been isolated predominantly from Atlantic salmon (*Salmo salar*) in Norway (12), and CC-ST52 and CC-ST48/CC-ST56, which were recovered exclusively from nonsalmonid species (9). Interestingly, experimental challenge data provided evidence that CC-ST52 and CC-ST48/CC-ST56 also differ in their pathogenicity (16).

Despite the economic losses *F. psychrophilum* causes in North American salmonid populations, only ten isolates had been MLST genotyped as of 2016 (7). Recently, Van Vliet et al. (13) characterized an additional 95 *F. psychrophilum* isolates from nine U.S. states, finding 34 *F. psychrophilum* STs, 28 of which were previously undescribed. These STs varied spatially, by host species, and in association with mortality events similarly to those recovered outside North America (13). This targeted study focused on isolates from three *Oncorhynchus* spp. (i.e., *O. mykiss*, *O. kisutch*, and *O. tshawytscha*), more than half of which were recovered from Michigan.

To better understand the genetic diversity of *F. psychrophilum* in North America, we used MLST to genotype 314 *F. psychrophilum* isolates recovered from ten fish species, collected from 20 U.S. states and 1 Canadian province. Within this collection were historical, cryopreserved *F. psychrophilum* isolates that had been recovered over a period of nearly four decades. This unique collection of isolates allowed evaluation of the genotypic relationships not only among North American isolates but also in comparison to those in other parts of the world. An additional objective of this study was to evaluate the role of random nucleic acid mutation and/or recombination in the genetic diversity discovered using MLST.

RESULTS

Identification of novel *F. psychrophilum* MLST genotypes in North America. A total of 314 *F. psychrophilum* isolates recovered from 20 U.S. states and 1 Canadian province over nearly four decades (1981 to 2018) were genotyped in this study (Table 1, see Fig. 4; see also Table S1 in the supplemental material). Among the typed isolates, 119 represented 47 novel MLST genotypes. Of these 47 genotypes, 11 formed four novel MLST clonal complexes (Fig. 1), the majority of which exhibited apparent host specificity (Table 1; see also Table S1 in the supplemental material). Four of the remaining thirty-six novel MLST genotypes clustered with several previously described North American MLST genotypes to form three additional novel MLST CCs (Fig. 1).

Ten of the remaining thirty-two novel MLST genotypes were placed into a single North American CC (i.e., CC-ST10) (Table 1, Fig. 1, and Table S1). Of these, nine were recovered exclusively from *O. mykiss* (most of which were diseased), whereas the remaining genotype (i.e., ST306) was recovered from both rainbow trout (*O. mykiss*) and coho salmon (Table 1; Table S1). Additionally, ST275 was the only genotype of CC-ST10 that was recovered from both captive and wild/feral steelhead trout (*O. mykiss*) (Table 1; Table S1). Nineteen of the remaining twenty-two novel genotypes were not placed into any previously or newly identified MLST CC (i.e., singletons) (Fig. 1) and thus were considered to be singleton genotypes, all of which were recovered from a single fish host species (Table 1; Table S1).

When the genotypes of this study were combined with all previously reported genotypes from other regions of the world (https://pubmlst.org/fpsychrophilum/), the remaining three novel MLST genotypes recovered in this study were placed into two additional CCs whose members were recovered from Europe (i.e., Denmark, Finland, Norway, and Switzerland). In particular, ST301 grouped within preexisting CC-ST191 as a single locus variant (SLV) of ST191, whereas ST277 and ST283 clustered with former singleton ST232 to form CC-ST232 (Fig. 2).

Identification of previously recognized *F. psychrophilum* MLST genotypes. The remaining 195 *F. psychrophilum* isolates of this study represented 19 previously reported MLST genotypes from North America and/or abroad. Among these, three genotypes (i.e., ST11, ST256, and ST257) belonged to three CCs that are currently present only in North America. Of the remaining 16 previously described genotypes, 7 belonged to one of three CCs that are present in both North America and/or Europe, Asia, and South America (i.e., CC-ST9, CC-ST10, and CC-ST31).

When the genotypes of this study were combined with all previously reported genotypes from other regions of the world, two genotypes that were recovered in this study (i.e., ST267 and ST70) belonged to two CCs whose members are from Europe or both Europe and South America. For example, ST267 belonged to CC-ST191 and was recovered from apparently healthy, wild/feral adult steelhead trout (*O. mykiss*), whereas ST70 belonged to CC-ST124, which was recovered from an Atlantic salmon (Table 1, Fig. 2, and Table S1). The remaining seven MLST genotypes were identified as singletons, five of which were recovered from a single fish host species, whereas the other two were recovered from multiple host species.

STs and geographic origin. Due to the very different number of isolates recovered from each state, no statistical comparisons of STs in association with their geographical origin were conducted. Nevertheless, several observations were apparent. The three

TABLE 1 Summary information of 314 North American *F. psychrophilum* isolates that were genotyped via MLST in this study, including the location of isolation, host species, and ST designation

ST (CC)[a]	No. of isolates	U.S. state or Canadian province of isolation[b]	Host species[c]	Wild/feral or captive
ST9 (CC-ST9)	8	BC (Canada), OR, UT, WA	COS, RBT	Unknown
ST13 (CC-ST9)	2	WA	COS	Unknown
ST10 (CC-ST10)	121	CA, CO, ID, MD, MI, MT, NC, NM, OR, PA, SD, UT, WA	CHS, COS, RBT, WST	Captive, unknown
ST78 (CC-ST10)	25	CO, ID, NC, NM, UT, WA	RBT	Captive, unknown
ST84 (CC-ST10)	1	NC	RBT	Captive
ST85 (CC-ST10)	4	CA, WA, VT	RBT	Captive, unknown
ST86 (CC-ST10)	4	UT, WA	RBT	Captive
ST275 (CC-ST10)	17	MI, NC, PA, VA, WA	RBT	Captive, wild/feral
ST294 (CC-ST10)	1	WA	RBT	Unknown
ST300 (CC-ST10)	11	ID, MT	RBT	Captive
ST303 (CC-ST10)	2	ID	RBT	Captive
ST304 (CC-ST10)	1	ID	RBT	Captive
ST305 (CC-ST10)	1	ID	RBT	Captive
ST306 (CC-ST10)	2	ID, OR	COS, RBT	Unknown
ST316 (CC-ST10)	1	WA	RBT	Captive
ST317 (CC-ST10)	1	ID	RBT	Captive
ST11 (CC-ST11)	2	OR	ATS, RBT	Unknown
ST308 (CC-ST29)	1	OR	CHS	Unknown
ST28 (CC-ST31)	1	OR	CUT	Unknown
ST70 (CC-ST124)	1	WA	ATS	Unknown
ST267 (CC-ST191)	2	MI	RBT	Wild/feral
ST301 (CC-ST191)	7	ID, PA, WV	RBT	Captive
ST277 (CC-ST232)	2	MI	ATS	Wild/feral
ST283 (CC-ST232)	1	MI	ATS	Wild/feral
ST256 (CC-ST256)	5	MI	CHS, RBT	Wild/feral
ST257 (CC-ST276)	2	MI	RBT	Wild/feral
ST276 (CC-ST276**)**	11	PA	RBT	Captive
ST279 (CC-ST281)	5	PA	RBT	Wild/feral
ST281 (CC-ST281)	2	PA	RBT	Captive, wild/feral
ST331 (CC-ST281)	1	PA	RBT	Wild/feral
ST332 (CC-ST281)	3	PA	RBT	Wild/feral
ST280 (CC-ST287)	1	WI	SPL	Captive
ST287 (CC-ST287)	1	MI	COS	Captive
ST288 (CC-ST288)	3	MI	CHS	Wild/feral
ST289 (CC-ST288)	1	MI	CHS	Wild/feral
ST296 (CC-ST296)	2	ID, WA	RBT, SOC	Unknown
ST299 (CC-ST296)	1	WA	RBT	Captive
ST291 (CC-ST310)	5	ID, NC	RBT	Captive
ST310 (CC-ST310)	2	ID	RBT	Captive
ST311 (CC-ST310)	2	ID	RBT	Captive
ST27	1	CA	RBT	Captive
ST30	5	OR	COS	Unknown
ST74	1	OR	COS	Unknown
ST76	1	WA	CHS	Unknown
ST253	6	MI	BNT, CHS	Captive
ST255	1	MI	RBT	Captive
ST258	6	IN, MI	COS, RBT	Captive, wild/feral
ST278	1	MI	LAT	Captive
ST282	1	MI	ATS	Wild/Feral
ST284	1	PA	BNT	Captive
ST285	3	MN	RBT	Captive
ST286	1	MI	RBT	Captive
ST290	1	MI	CHS	Wild/feral
ST292	6	NC	RBT	Captive
ST293	1	NC	RBT	Captive
ST295	1	WA	CHS	Unknown
ST297	1	OR	COS	Unknown
ST298	1	ID	RBT	Captive
ST302	1	ID	RBT	Captive
ST307	1	OR	RBT	Unknown
ST309	1	ID	RBT	Captive
ST312	1	ID	RBT	Captive

(Continued on next page)

TABLE 1 (Continued)

ST (CC)[a]	No. of isolates	U.S. state or Canadian province of isolation[b]	Host species[c]	Wild/feral or captive
ST313	2	MT	RBT	Captive
ST314	1	NC	RBT	Captive
ST315	1	NC	RBT	Captive
ST320	1	AK	COS	Captive

[a]Sequence types (STs) in bold are found in North America and abroad, whereas STs in italics were newly discovered in this study. The information presented is in order by ST.

[b]Isolates were collected from a total of 20 U.S. states and 1 Canadian province. AK, Alaska; BC, British Columbia; CA, California; CO, Colorado; ID, Idaho; IN, Indiana; MD, Maryland; MI, Michigan; MN, Minnesota; MT, Montana; NC, North Carolina; NM, New Mexico; OR, Oregon; PA, Pennsylvania; SD, South Dakota; UT, Utah; VA, Virginia; VT, Vermont; WA, Washington; WI, Wisconsin; WV, West Virginia.

[c]ATS, Atlantic salmon (*Salmo salar*); BNT, brown trout (*S. trutta*); CHS, Chinook salmon (*Oncorhynchus tshawytscha*); COS, coho salmon (*O. kisutch*); CUT, cutthroat trout (*O. clarkii*); LAT, lake trout (*Salvelinus namaycush*); RBT, rainbow/steelhead trout (*O. mykiss*); SOC, sockeye salmon (*O. nerka*); SPL, splake (*S. namaycush* × *S. fontinalis*); WST, white sturgeon (*Acipenser transmontanus*).

most abundant STs (i.e., ST10, ST78, and ST275), all of which belong to CC-ST10, also had the widest geographic distribution in our data set (Table 1, Fig. 1, and Table S1). In specific, ST10, ST78, and ST275 were detected in 13, 9, and 5 states, respectively (Table 1, Fig. 1, and Table S1). The next most abundant STs (e.g., ST9, ST85, ST86, ST258, ST291, ST296, ST300, ST301, and ST306) were recovered from two to four states, whereas the remaining 54 STs were recovered exclusively from one state (Table 1, Fig. 1, and Table S1).

In the state of Idaho, where 104/314 study isolates originated, 61/104 isolates were identified as ST10 and were detected in nine distinct locations (Table 1; Table S1). The remaining 43 isolates fell into 16 additional STs, the bulk of which (12/16 STs) were recovered from a single location (Table 1; Table S1). Most isolates recovered from Idaho (91/104 isolates) belonged to CC-ST10 (Table 1; Table S1) and were isolated over a

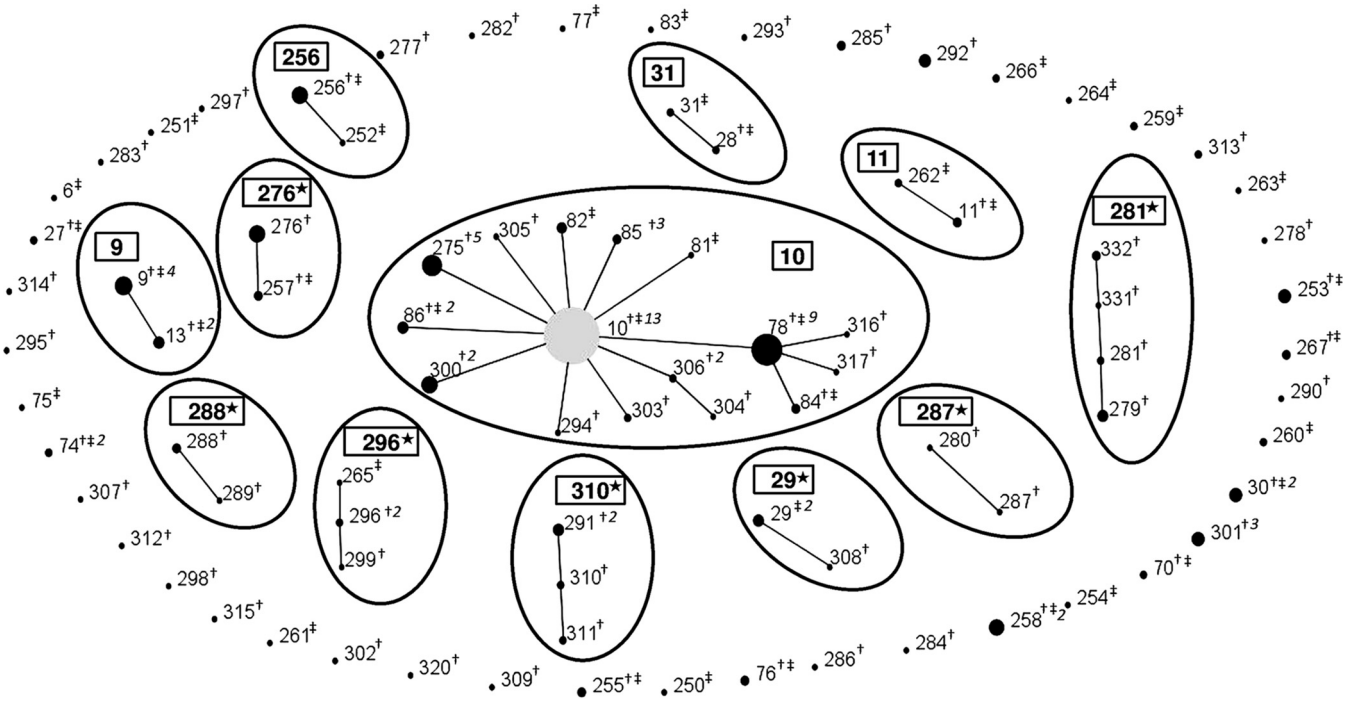

FIG 1 eBURST diagram of the 314 *F. psychrophilum* isolates genotyped in this study combined with all previously typed North American isolates ($n = 107$) in the *F. psychrophilum* MLST database. †, a sequence type (ST) that contains isolates typed in this study; ‡, an ST containing previously genotyped isolates. Italicized numbers accompanying STs indicate the number of states that ST was found in (if no number, ST was recovered from one state only). Clonal complexes (CC) are encircled and the founding ST for a CC is depicted as a number within a rectangle. Founding STs labeled with a star (★) indicate a newly described CC as a result of this study. The founding ST, or founder, is the ST with the highest number of single-locus variants (SLVs). In instances where multiple STs have the same numbers of SLVs, the founder is named after the ST with the most isolates. If both STs have the same numbers of isolates, the CC is named for the earliest found ST.

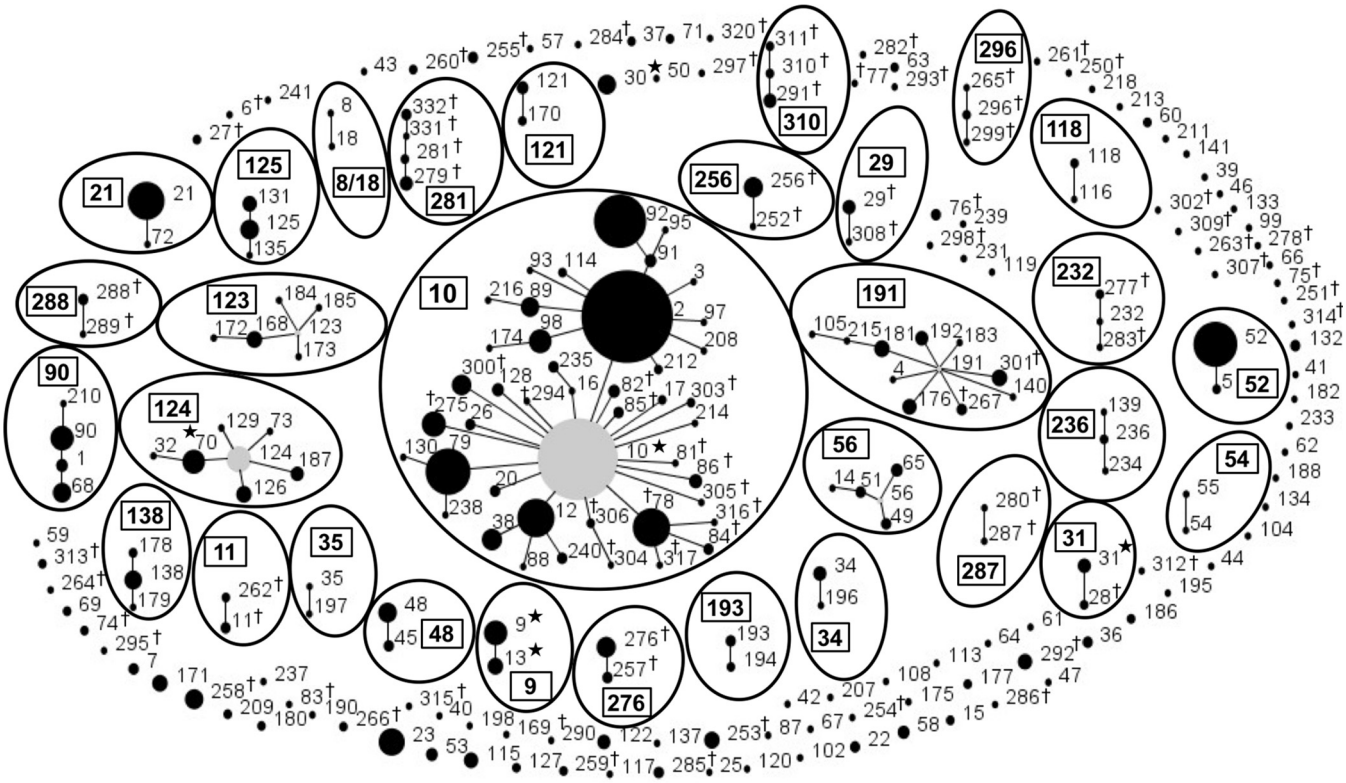

FIG 2 eBURST diagram of all 1,411 *F. psychrophilum* isolates worldwide. This includes the 1,097 isolates from the *F. psychrophilum* MLST database and the 314 newly typed isolates from this study. †, sequence type (ST) is present in North America; ★, ST was found in North America and abroad. The founding ST, or founder, is the ST with the highest number of single-locus variants (SLVs; depicted as gray circles). In instances where multiple STs have the same numbers of SLVs, the founder is named after the ST with the most isolates. If both STs have the same numbers of isolates, the clonal complex (CC) is named for the earliest found ST. The founding ST of a CC is enclosed within a box. The size of each circle is proportional to how many isolates belong to that ST.

period from 1990 to 2016. In the state of Utah, most isolates also belonged to CC-ST10 and were identified as ST10 (37/43 isolates), ST86 ($n = 3$ isolates), and ST78 ($n = 2$ isolates). Of note, ST10 was recovered from ten distinct locations in Utah from 2004 to 2015 (Table 1; Table S1). The remaining isolate from Utah was identified as ST9 (Table 1; Table S1). In Michigan (38 isolates), 17 STs were identified among seven distinct locations between the years of 2010 and 2018 (Table 1; Table S1). Of these, most of the isolates were identified as ST253 ($n = 6$ isolates), ST256 ($n = 5$ isolates), or ST258 ($n = 5$ isolates), whereas the remaining 14 STs contained ≤4 isolates (Table 1; Table S1). In contrast to the isolates from Idaho and Utah, nearly half of the isolates recovered from Michigan (16/38) were identified as singletons (Table 1; Table S1), whereas the remaining 22 isolates belonged to one of seven CCs (Table 1; Table S1). The geographical origins and genotypes for the remaining isolates are presented in Table 1 and Table S1.

***F. psychrophilum* genetic analyses.** The mean genetic diversity (H) of all typed *F. psychrophilum* isolates recovered in North America ($n = 421$) to date was calculated to be 0.68 ± 0.04. When H was calculated for the isolates recovered from the three most abundant host species (i.e., *O. mykiss*, *O. kisutch*, and *O. tshawytscha*), H values were 0.48 ± 0.06, 0.74 ± 0.05, and 0.90 ± 0.01, respectively. When all remaining host species were combined, the mean genetic diversity was 0.85 ± 0.02. The linkage disequilibrium (I_A^S) value for all *F. psychrophilum* isolates recovered in North America was estimated to be 0.66 and differed significantly from 0 ($P < 0.001$), indicating linkage disequilibrium (i.e., nonrandom association between allele types).

As multiple isolates belong to the same ST, sampling bias may alter the overall mean genetic diversity among the North American *F. psychrophilum* isolates (12). As such, mean genetic diversity was calculated between the 86 currently identified *F. psychrophilum* STs in North America and found to be 0.92 ± 0.02. The I_A^S value was closer to 0

TABLE 2 Allelic variations among 15 North American *F. psychrophilum* MLST clonal complexes

CC	ST of clonal ancestor	ST of SLV	Variant locus of SLV	Clonal ancestor allele type	SLV allele type	No. of nucleotide differences	Other CC(s) containing SLV	Other singletons containing SLV	Amino acid change[a]
CC-ST9	9	13	*tuf*	5	8	5	None	ST258	Syn
CC-ST10	10	78	*tuf*	2	41	5	None	None	Syn
CC-ST10	10	81	*tuf*	2	43	6	None	None	Syn
CC-ST10	10	82	*tuf*	2	44	1	None	None	P-S
CC-ST10	10	85	*tuf*	2	46	8	None	None	Syn
CC-ST10	10	86	*fumC*	2	3	2	CC-ST10; CC-ST11; CC-ST191; CC-ST287; CC-ST296; CC-ST276	ST254; ST261; ST264; ST266; ST282; ST292; ST297; ST307; ST313	Syn
CC-ST10	10	275	*tuf*	2	62	1	None	None	Syn
CC-ST10	10	294	*trpB*	2	45	1	None	None	Syn
CC-ST10	10	300	*dnaK*	2	34	1	None	None	Syn
CC-ST10	10	303	*gyrB*	8	81	4	None	None	Syn
CC-ST10	10	305	*gyrB*	8	13	3	CC-ST124; CC-ST310	ST253	Syn
CC-ST10	10	306	*murG*	2	1	7	CC-ST124; CC-ST310	ST253; ST286	P-H; E-K; I-V
CC-ST11	11	262	*murG*	7	49	1	CC-ST281	ST264; ST313	T-A
CC-ST29	29	308	*trpB*	8	15	1	None	None	Syn
CC-ST31	31	28	*gyrB*	19	16	9	None	None	Syn
CC-ST124	124	70	*trpB*	2	1	3	CC-ST10; CC-ST256; CC-ST276	ST6; ST83; ST253; ST286	Syn
CC-ST191	191	267	*gyrB*	3	73	10	None	None	Syn
CC-ST191	191	301	*trpB*	4	46	1	None	None	Syn
CC-ST232	232	277	*gyrB*	62	75	3	None	None	Syn
CC-ST256	256	252	*murG*	25	16	2	None	ST250	H-P
CC-ST276	276	257	*murG*	25	46	6	None	None	K-E
CC-ST281	281	279	*tuf*	63	5	9	CC-ST9; CC-ST11	ST266; ST313	Syn
CC-ST281	281	331	*atpA*	3	75	4	None	None	Syn
CC-ST287	287	280	*dnaK*	15	22	2	CC-ST10; CC-ST191	None	Syn
CC-ST288	288	289	*trpB*	43	44	2	None	None	Syn
CC-ST296	296	265	*atpA*	68	62	1	None	None	Syn
CC-ST296	296	299	*gyrB*	47	79	4	None	None	Syn
CC-ST310	310	291	*fumC*	1	2	1	CC-ST10; CC-ST124; CC-ST232; CC-ST256; CC-ST281; CC-ST310	ST27; ST74; ST77; ST83; ST320	Syn
CC-ST310	310	311	*trpB*	11	2	1	CC-ST10; CC-ST124	ST27; ST278; ST285; ST292; ST309	Syn

[a]Clonal ancestor amino acid is given first followed by the amino acid of the single locus variant. Syn is a synonymous change.

($I_A^S = 0.26$) among the 86 representative STs; however, it still differed significantly from 0 ($P < 0.001$), indicating linkage disequilibrium.

The 12 North American CCs presented in Fig. 1 were combined with the 3 CCs in Fig. 2 containing STs from North America (i.e., CC-ST124, CC-ST191, and CC-ST232), and 29 SLVs were discovered among the fifteen founding STs (Table 2). The CC with the highest number of SLVs was CC-ST10 ($n = 11$), whereas the other 14 CCs had one to two SLVs. The *tuf* locus accounted for the highest number of SLVs ($n = 7$), whereas the other six loci had ≤6 SLVs (Table 2).

Among the 29 SLVs, 19 were the result of multiple nucleotide changes and thus were likely the result of recombination. Additionally, another three SLVs were likely the result of recombination, because even though their variant allele type differed from their ancestral allele type by only one nucleotide, that variant allele type was also present in another CC (Table 2). The remaining seven SLVs differed from their ancestral allele type by a single nucleotide and were not found elsewhere in the data set, thereby suggesting those variant allele types likely arose from a random nucleic acid mutation (Table 2). If 22/29 SLVs resulted from recombination and the remaining 7/29 SLVs resulted from random nucleic acid mutation, then the seven North American *F. psychrophilum* loci are estimated to be three times more likely to diversify through recombination than by random nucleic acid mutation.

The potential for amino acid shifts resulting from these mutational events was also analyzed, revealing that 24 mutations were synonymous and 5 were nonsynonymous. Of note is *murG*1 (i.e., locus *murG* designated with allele type 1), which produced three distinct amino acid changes (i.e., proline to histidine, glutamic acid

TABLE 3 Summary of nucleotide sequence diversity among the 86 North American sequence types

Locus	Fragment size (bp)	No. of alleles	S[a]	π[b]	*dN*/*dS*[c]	R_{min}[d]
trpB	789	24	13	0.0042	0.0079	4
gyrB	1,077	41	22	0.0068	0.0020	7
dnaK	873	20	29	0.0057	0.0586	3
fumC	750	8	5	0.0029	0.0422	2
murG	681	32	16	0.0066	0.2751	6
tuf	795	36	26	0.0074	0.0027	6
atpA	834	38	28	0.0125	0.0162	10
Average					0.0578	
Sum			139			38
Concatenation	5,799		139	0.0055		44

[a]Number of segregating sites.
[b]Average pairwise nucleotide diversity per site.
[c]Ratio of nonsynonymous to synonymous mutations.
[d]Hudson and Kaplan's lower bound for the number of recombination events.

to lysine, and isoleucine to valine) resulting from seven nucleotide polymorphisms (Table 2). All other differences involved one amino acid change. In fact, of the five nonsynonymous substitutions, four were due to variations at the *murG* locus, and these four variations were the only ones found at this locus among the 15 CCs (Table 2).

When the seven loci used in the MLST scheme for *F. psychrophilum* were concatenated, a single stretch of DNA that was 5,799 bp long was produced. Among the 86 STs present in North America, 139 single nucleotide polymorphisms were identified. These polymorphic positions represented 2.40% (i.e., 139/5,799 bp) of the total length of the concatenated sequence (Table 3). Pairwise comparisons among loci yielded a nucleotide diversity ranging from 0.29% at *fumC* to 1.25% at *atpA*, and the number of unique allele types varied from 8 at *fumC* to 41 at *gyrB* (Table 3). The ratio of nonsynonymous to synonymous mutations ranged from 0.002 at *gyrB* to 0.275 at *murG*, which indicates that nonsynonymous mutations are rare and these loci are under purifying selection (ratio of nonsynonymous to synonymous evolutionary changes [*dN*/*dS*], <1). In addition, putative recombination was detected inside every locus (minimum number of recombination events [R_{min}], >0), with *fumC* having the lowest value ($R_{min} = 2$) and *atpA* having the highest ($R_{min} = 10$) (Table 3). The cumulative minimum number of recombination events among the seven loci was 38, whereas R_{min} for the concatenated sequences was 44, consistent with additional recombination between the loci as well (Table 3). A pairwise homoplasy index (PHI) test conducted on the concatenated sequences revealed statistically significant evidence ($P < 0.0001$) of recombination between the 86 representative STs.

DISCUSSION

The results presented herein reveal substantial genetic diversity within *F. psychrophilum* recovered from North America. Combining data from this study with those of Nicolas et al. (7) and Van Vliet et al. (13) revealed that the mean gene diversity of MLST-typed *F. psychrophilum* in North America is higher than in all other studied continents (i.e., 0.68 in North America versus 0.48 to 0.66 elsewhere). In this context, a recent study provided evidence that the main lineage of *F. psychrophilum* causing disease in rainbow trout (i.e., CC-ST10) may have originated in North America, from where at least one successful genotype (e.g., ST10) was distributed to other regions of the world through the international trade of fish (17). If this *F. psychrophilum* lineage did indeed originate in North America, it would have had more time to diversify in this region, perhaps resulting in the high apparent genetic diversity discovered in this study. Nevertheless, the observed diversity in this study may be attributable, at least in part, to sampling scheme and/or the life history of some of the sampled fish host populations (i.e., recovery of *F. psychrophilum* from a wild/feral fish appears more likely

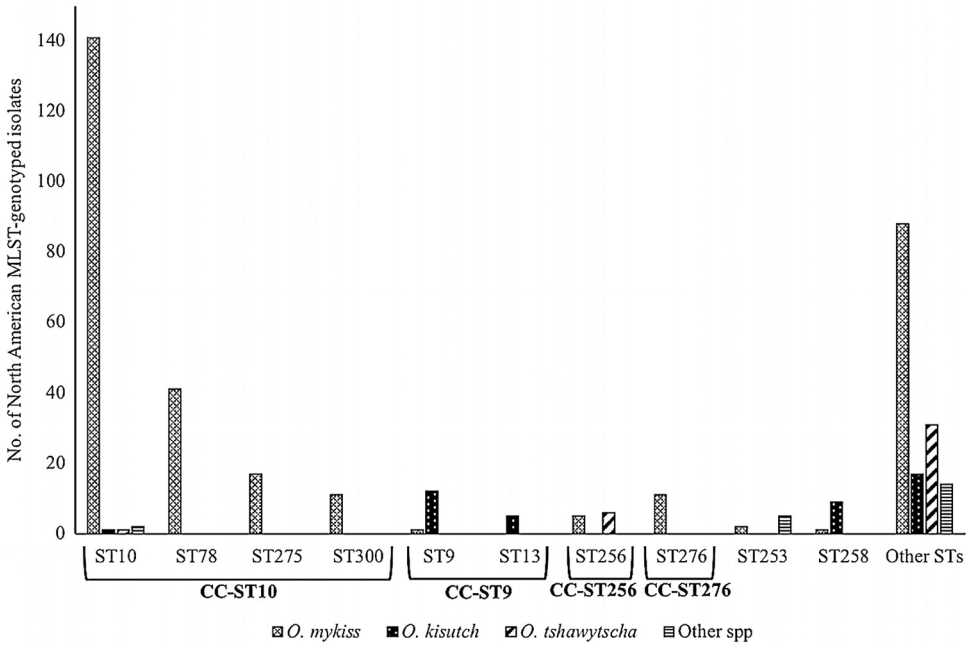

FIG 3 Frequency and host of origin among 10 North American *F. psychrophilum* sequence types (STs). STs are displayed along the *x* axis with four clonal complexes (CCs) and two singleton STs shown. All 421 currently typed North American *F. psychrophilum* isolates are presented here.

to lead to the identification of a new genotype when compared to isolates originating from captive fish) (Table 1; see also Table S1 in the supplemental material). For example, 30 *F. psychrophilum* isolates from the current study were recovered from wild/feral fish and were placed into 15 STs (i.e., an average of two isolates for every ST), whereas the 243 isolates recovered from captive fish were placed into 40 STs (i.e., >6 isolates for every ST). A similar diversity trend is also apparent in the studies of Siekoula-Nguedia et al. (8), Fujiwara-Nagata et al. (9), Strepparava et al. (10), Avendaño-Herrera et al. (11), and Van Vliet et al. (13).

In addition to revealing substantial genetic diversity, MLST genotyping also revealed some important aspects of *F. psychrophilum* distribution in general, and within North America specifically. Of the 31 currently described *F. psychrophilum* CCs (Fig. 2), nearly one-third (seven novel, two previously described) have, to date, been exclusively found in the United States, where they differ in their geographic distributions (Table 1, Fig. 1, and Table S1). This is consistent with pathogen dissemination when fish and their gametes are transported to new locations for aquaculture and stock enhancement purposes in North America (18). For example, the discovery of CC-ST29 in Oregon and Michigan is likely not coincidental, as Chinook salmon were introduced into the Great Lakes Basin from Oregon in the late 1960s (19). Likewise, it was evident that most North American CCs that originated from aquaculture facilities and hatcheries were found in multiple states, whereas those confined to one state were commonly associated with free-roaming adult fish, underscoring the potential of pathogen transmission through trade.

This study revealed the diverse host ranges that some of these U.S.-specific CCs display, whereby some were isolated from a single fish species and others from multiple fish species. This may be an indication that some North American CCs are adapted to a particular host species, whereas others are nonspecific "generalists." For example, CC-ST10, whose genotypes were recovered on numerous occasions during the course of our research, was predominantly recovered from farmed rainbow trout experiencing BCWD, which is consistent with all previous reports on this CC from other regions of the world (8, 10, 12, 13). In this study, however, ST10 and ST306 (in CC-ST10) were occasionally recovered from fish species other than *O. mykiss* (Table 1, Fig. 3, and Table

S1), and it remains to be determined if these genotypes have a broader host range or if the hosts these isolates were recovered from were in proximity to *F. psychrophilum*-infected *O. mykiss*. The prevalence of CC-ST10 in North America and throughout the world makes strains with these genotypes good candidates for BCWD vaccine development. Within North America, CC-ST10 was the most widespread among currently recognized CCs, being detected in 16 of the 20 states in the Northwest, West, Southwest, Midwest, Southeast, and Northeast regions of the United States that were examined in this study. This finding is consistent with other studies that have suggested that ST10, the predicted CC founder strain (12), along with other CC-ST10 STs, have been disseminated around the world as a result of the international salmonid egg trade, where they are also now widespread (13, 17).

Although >90% of the CC-ST10 strains of this study were recovered from farmed/hatchery-reared fish, one novel genotype of CC-ST10 (i.e., ST275) was recovered from apparently healthy feral steelhead trout (*O. mykiss*) at a weir in Michigan and is, to date, the only ST of CC-ST10 reported from a wild/feral host in North America (Table 1, Table S1). Furthermore, the eggs collected from this group of spawning steelhead trout were subsequently reared at a Michigan state fish hatchery that later had an outbreak of BCWD where ST275 caused mass mortality among the resultant juvenile steelhead trout (*O. mykiss*). The original source of ST275 in the hatchery facility remains to be determined, but the possibility for vertical transmission and thus circumvention of currently utilized egg disinfection strategies (i.e., iodophor disinfection) cannot be discounted. Indeed, *F. psychrophilum* has been shown to resist surface disinfection with povidone iodine in previous studies (20–22), and *F. psychrophilum* is believed to reside within the perivitelline space (23), where it can resist salmonid egg lysozyme (24). Other explanations for the occurrence of ST275 both at the weir and in the hatchery environment are possible, however, including transmission via contaminated fomites. In any case, strict biosecurity protocols are needed to prevent the spread of *F. psychrophilum*, and future research exploring additional egg and equipment disinfection protocols are needed.

Although not nearly as widespread as CC-ST10, CC-ST191 was also detected in multiple regions of the United States (Table 1; Table S1) and prior to 2016, was only reported in Europe (e.g., Denmark, Finland, France, Norway, and Switzerland) (7, 8, 10, 12, 13). The detection of this CC from the United States on multiple occasions is of interest, because the founding ST and almost all of its SLVs were exclusively detected in Europe, suggesting this CC may have been introduced from Europe to North America. Likewise, all STs within CC-ST124, which is the third largest currently described *F. psychrophilum* CC in the world and predominately recovered from Atlantic salmon, were originally only reported from Europe (7, 12). However, ST70 (in CC-ST124) was found in Chile by Avendaño-Herrera et al. (11) following the Atlantic salmon trade between Chile and Norway in the 1990s (25) and has now been documented in North American Atlantic salmon by Nilsen et al. (12) and in the present study. Whether the international trade of Atlantic salmon eggs is responsible for ST70 dissemination, however, remains to be determined.

Thirty-one North American *F. psychrophilum* singletons were also detected in this study, bringing the total number of recognized North American singletons to 44 (Fig. 1) (7, 13). Based upon current data, most *F. psychrophilum* singletons appear to be geographically limited, where they were recovered from one specific aquaculture facility or site (Table 1; Table S1) (13). Although this may not be surprising, it was striking that some singletons were found repeatedly at the same facility over the years (e.g., ST253 in 2010, 2013, and 2017) (Table 1; Table S1), emphasizing that some unique strains may find a niche and perpetuate themselves within a particular aquaculture facility.

By combining the 314 North American *F. psychrophilum* isolates presented herein and all previously typed North American *F. psychrophilum* isolates, it is evident that some North American STs have persisted longer than others. For example, some of the earliest recovered STs in North America (e.g., ST6, ST9, ST10, ST11, ST27, ST28,

ST29, ST30, ST70, ST74, ST78, ST297, ST306, ST307, ST308; 1981 to 1996) (Table 1; Table S1) (7, 13) have not been rediscovered in the last 20 years, whereas others are still reappearing decades later. Of note, ST10 appears to be capable of producing equally successful and persistent clones, such as ST78. Interestingly, ST78 has produced several of its own clones, which have been isolated from three U.S. states (i.e., Idaho, North Carolina, and Washington) where ST78 has not yet been recovered, suggesting ST78 may be more geographically widespread than is currently recognized.

In this study, the contribution of recombination and random nucleic acid mutation to the diversification among North American *F. psychrophilum* STs was best explained by recombination, whereby 22/29 SLVs were hypothesized to have resulted from recombination and the remaining 7/29 SLVs resulted from random nucleic acid mutation (i.e., a ratio of 3.14:1 at the allele level), which is similar to the 4.5:1 ratio that was reported initially by Nicolas et al. (7). This estimation relies upon a visual inspection of gene sequences to estimate the mechanism of diversification (26). To validate this estimation, additional sequence-based computational tests (e.g., PHI and R_{min}) were utilized, and both tests revealed evidence for recombination either within and/or between the MLST loci. Interestingly, all polymorphisms leading to an SLV at the locus *murG*, which encodes glycosyltransferase murein G, were nonsynonymous. The exact function of this enzyme in *F. psychrophilum* has not been examined; however, in *Escherichia coli*, *murG* is required for biosynthesis of the peptidoglycan layer (27) and therefore may confer an environmental advantage to epidemic clones of *F. psychrophilum*. The ratio of nonsynonymous to synonymous mutations within *murG*, however, is <1 (i.e., $dN/dS = 0.2751$); therefore, accumulated nonsynonymous mutations are selected against and are projected to be lost over time (12) but may create short-lived advantages during BCWD outbreaks. Although the natural mechanism(s) for recombination (e.g., transduction, transformation, and conjugation) among *F. psychrophilum* isolates remains to be determined, the intermingling of isolates naturally and during the trade and introduction of fish and eggs to different facilities, bodies of water, or states provides a means for the interaction of different *F. psychrophilum* genotypes. In fact, the recovery of multiple *F. psychrophilum* genotypes from a single infected fish has been reported on multiple occasions (9, 12, 13). In this context, Gliniewicz et al. (28) found no similarities in DNA methylation motifs for two *F. psychrophilum* strains, which may have been a result of environmental differences from which these strains were recovered, whereby different phage communities contribute to long-term diversification of different *F. psychrophilum* lineages (i.e., novel genotypes as a by-product of transduction). Even though recombination appears to be a driving force for diversity among *F. psychrophilum* isolates, the results of our analysis on linkage disequilibrium revealed statistically significant evidence for clonal populations (i.e., $\bar{I}_A^S$ value differs significantly from 0). This may not be surprising, as very few bacterial species (e.g., *Neisseria gonorrhoeae* and *Helicobacter pylori*) exhibit near linkage equilibrium (i.e., a nonclonal population structure) (29–31). Nonetheless, even in the presence of recombination, well adapted *F. psychrophilum* genotypes (i.e., CC founders) appear to be capable of producing clones (i.e., SLVs), some of which have persisted long enough to produce clones of their own (e.g., ST78 and ST306).

In summary, MLST was used to reveal substantial diversity among 314 newly typed North American *F. psychrophilum* isolates, whereby 66 STs were identified (47 of which were novel), leading to the formation of seven novel CCs. These isolates were recovered from 20 U.S. states and 1 Canadian province over a period of nearly four decades, which is the most comprehensive collection of *F. psychrophilum* isolates to be genetically analyzed in North America to date. The results also implicated recombination as a potential driving force of *F. psychrophilum* diversification in North America, which may also be the mechanism for phenotypic changes, such as antimicrobial resistance within this species, and warrants further investigation.

FIG 4 Map of the United States and Canada. Distribution of 314 *F. psychrophilum* isolates recovered from the current North American study. These isolates were recovered from 20 U.S. states and 1 province of Canada. AK, Alaska; BC, British Columbia; CA, California; CO, Colorado; ID, Idaho; IN, Indiana; MD, Maryland; MI, Michigan; MN, Minnesota; MT, Montana; NC, North Carolina; NM, New Mexico; OR, Oregon, PA, Pennsylvania; SD, South Dakota; UT, Utah; VA, Virginia; VT, Vermont; WA, Washington; WI, Wisconsin; WV, West Virginia.

MATERIALS AND METHODS

Fish collection and isolation of *F. psychrophilum*. A total of 314 *F. psychrophilum* isolates recovered from 20 U.S. states and 1 Canadian province over nearly four decades (i.e., 1981 to 2018) were genotyped in this study (Table 1, Fig. 4; see also Table S1 in the supplemental material). The isolates were recovered from four fish genera and ten species, including rainbow/steelhead trout (*O. mykiss*; $n = 260$), coho salmon (*O. kisutch*; $n = 25$), Chinook salmon (*O. tshawytscha*; $n = 11$), cutthroat trout (*O. clarkii*; $n = 1$), sockeye salmon (*O. nerka*; $n = 1$), Atlantic salmon (*Salmo salar*; $n = 6$), brown trout (*S. trutta*; $n = 6$), lake trout (*Salvelinus namaycush*; $n = 1$), splake (*S. fontinalis* × *S. namaycush*; $n = 1$), and white sturgeon (*Acipenser transmontanus*; $n = 2$) (Table 1, Table S1). The isolates were recovered from external and internal tissues of fish either showing gross signs of BCWD or that were apparently healthy (Table S1).

Recovery of the aforementioned *F. psychrophilum* isolates was completed by multiple investigators using flavobacterial selective media, including cytophaga agar (CA) (32) and tryptone yeast extract agar (TYES) (33) with or without supplementation of 4.0 mg · liter^{-1} of neomycin sulfate, or Hsu-Shotts medium (HSU) (34). *F. psychrophilum* isolates were procured from project collaborators as actively growing cultures on solid media or as frozen cryostock and revived and subcultured on TYES agar to verify isolate purity. A single colony was then inoculated in TYES broth for the generation of glycerol-supplemented cryostock (maintained at −80°C) for further use.

Bacterial DNA extraction and *F. psychrophilum* identity confirmation. *F. psychrophilum* isolates were revived from cryostocks in TYES broth and incubated for 72 to 96 h at 15°C, at which time bacterial cells were subcultured on fresh TYES agar to verify purity. Bacterial genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacure's protocol for Gram-negative bacteria, quantified using a Qubit fluorometer (Life Technologies, Grand Island, NY), and then diluted to 20 ng/μl. Prior to MLST analyses, the identity of all isolates was confirmed as *F. psychrophilum* using the conventional PCR assay of Toyama et al. (35) as detailed previously (36).

Multilocus sequence typing. Partial sequences of seven housekeeping genes (*trpB*, *gyrB*, *dnaK*, *fumC*, *murG*, *tuf*, and *atpA*), previously selected by Nicolas et al. (7), were PCR amplified (13). The PCR product (5 μl) was electrophoresed in a 1.5% agarose gel prepared with 1× SYBR Safe DNA gel stain for 45 min at 100 V. The gel was then viewed under UV transillumination to confirm the presence of an appropriately sized band. The PCR product (1 μl) was then purified by adding 0.25 μl of ExoSAP-IT (Applied Biosystems) and 3.0 μl of 1× PCR buffer with $MgCl_2$ (Sigma-Aldrich) and incubated for 20 min at 37°C, followed by enzyme inactivation for 10 min at 95°C. The same primers employed for PCR

amplification of each of the seven housekeeping genes were used to bidirectionally sequence purified PCR products at the Genomics Technology Support Facility (Michigan State University).

MLST data analysis. Chromatograms were manually verified for quality prior to assignation of allele types and STs via an in-house script (P. Nicolas, INRA). eBURST v3 (eburst.mlst.net) (37, 38) was used to dichotomize STs into CCs or as singletons based on locus variations in their allelic profiles. STs were considered single-locus variants (SLVs) when they differed at one of the seven allele types, whereas STs that varied at two of the seven allele types were considered double-locus variants. CCs were comprised of STs on the basis of their shared SLVs and were named after the predicted founding ST (i.e., the ST with the highest number of SLVs). STs that did not form part of a CC were referred to as singletons. All isolates within the publicly accessible MLST database for *F. psychrophilum* ($n = 1,097$; https://pubmlst.org/fpsychrophilum/) (15), including all previously typed isolates from North America ($n = 107$) (7, 13, 39), along with the 314 isolates of this study, were included in the eBURST v3 (37, 38) analysis.

MLST genetic analyses. Analysis of sequence variation among the seven housekeeping genes relied on several analysis programs. DNAsp v6.10.01 (40) was used to identify the number of alleles for each locus (i.e., allele types), the number of segregating sites (S), nucleotide diversity (π), and the Hudson and Kaplan lower bound on the minimum number of recombination events (R_{min}) (41). Utilizing SLV STs from CCs and a method described by Feil et al. (26), the contributions of recombination or random nucleic acid mutations to clonal diversification was estimated. Recombination was also evaluated among the concatenated sequences of representative STs using the pairwise homoplasy index (PHI) executed in SplitsTree v4.14.6 (42). START2 (43) was used to calculate the ratio of nonsynonymous to synonymous substitutions (*dN*/*dS*) for each locus based on pairwise sequence comparisons. LIAN 3.7 (44) served to compute the extent of linkage disequilibrium (I_A^S) between the allele types and to assess its statistical significance using a Monte Carlo simulation, whereby loci were resampled 1,000 times without replacement. LIAN 3.7 was also used to measure mean genetic diversity (H) ± standard error.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02305-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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