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Carnobacterium maltaromaticum Infections in Feral *Oncorhynchus* spp. (Family Salmonidae) in Michigan

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Members of the genus *Oncorhynchus* were introduced from the Pacific Northwest to the Laurentian Great Lakes basin and now constitute one of its most commercially and ecologically valuable fisheries. Recently, infections by a group of Gram-positive atypical lactobacilli belonging to the genus *Carnobacterium* have been detected in feral and captive *Oncorhynchus* spp. broodstock, some of which were associated with mortalities. Out of 1564 rainbow and steelhead trout (*O. mykiss*), coho salmon (*O. kisutch*), and Chinook salmon (*O. tshawytscha*) that were bacteriologically examined, 57 *Carnobacterium* spp. isolates were recovered from the kidneys, spleen, swimbladder, and/or external ulcerations of 51 infected fish. Phenotypic and biochemical characterization, as well as partial 16S rDNA sequencing and phylogenetic analyses of 30 representative isolates identified 29 as *Carnobacterium maltaromaticum* and 1 as *C. divergens*, though some phenotypic and genotypic heterogeneity was observed. Infections with *C. maltaromaticum* were associated with signitures typical of pseudokidney disease, but on occasion were also observed in fish displaying the gross and histopathological changes characteristic of nephrocalcinosis. While *C. maltaromaticum* infections were found to be widespread in both feral and farmed spawning populations of *Oncorhynchus* spp. residing within the Great Lakes basin, infection prevalence varied significantly according to fish species and strain, gender, and across time, but not by sampling location according to logistic regression analysis. The findings of this study further underscore the presence of phenotypic variations among *Carnobacterium maltaromaticum* strains that necessitate genotypic analysis to achieve definitive identification.

Keywords: *Carnobacterium*, *Oncorhynchus*, Great Lakes, bacteria, nephrocalcinosis

Since the initial descriptions of *Lactobacillus* spp. causing disease in fish (Rucker *et al*., 1953) and the subsequent creation of the genus *Carnobacterium* in 1987 by Collins *et al*. (1987), numerous disease epizootics associated with *Carnobacterium* spp. infection in fish have been reported (Hiu *et al*., 1984; Michel *et al*., 1986; Baya *et al*., 1991; Toranzo *et al*., 1993). The genus *Carnobacterium* is currently comprised of ten species, with *Carnobacterium maltaromaticum* being the species most commonly associated with infections in fish (Herman *et al.*, 1985; Starliper *et al*., 1992). The majority of disease outbreaks associated with *C. maltaromaticum*, often referred to as pseudokidney disease, have been reported in captive adult rainbow trout (Ross and Toth, 1974; Cone, 1982; Herman *et al*., 1985; Starliper *et al*., 1992; Toranzo *et al*., 1993); however, infections have seldom been reported in other fish species. Originally, identification of *Carnobacterum* spp. relied upon cultural and biochemical characterization (i.e., Montel *et al*., 1991) but this has proven unreliable when used exclusively due to inter- and intra-species phenotypic variability (Hammes and Hertel, 2006).

To this end, the present study was undertaken in order to determine the prevalence of *Carnobacterium* infections in feral *Oncorhynchus* spp. stocks returning to spawn at multiple gamete collecting facilities in Michigan and in captive spawning rainbow trout broodstock that are used to rejuvenate the recreational fishery in Michigan. An additional objective of this study was to compare the phenotypic and genotypic characteristics amongst isolates recovered from these important fish stocks.

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Material and Methods

Fish and sampling

During the fall spawning seasons (September-November) of 2005-2008, returning Chinook and coho salmon spawners were collected, euthanized, and artificially spawned by personnel of the Michigan Department of Natural Resources (MDNR) from three gamete collecting stations in Michigan; the Little Manistee River Weir (LMRW, Manistee County) and Platte River Weir (PRW, Benzie County) located within the Lake Michigan watershed, and the Swan River Weir (SRW, Presque Isle County) located in the Lake Huron watershed (Fig. 1). During the spring spawning seasons of 2005-2008, steelhead trout were also collected, euthanized, and artificially spawned at the LMRW. In addition, captive propagated rainbow trout (Eagle Lake strain) were sampled from a Michigan State Fish Hatchery (Emmet County, Lake Michigan watershed) during routine fall health inspections (2005-2008), as well as during a mortality episode in March of 2006. A total of 90 Eagle Lake strain rainbow trout, 205 steelhead trout, 846 Michigan strain Chinook salmon, 243 Michigan Strain coho

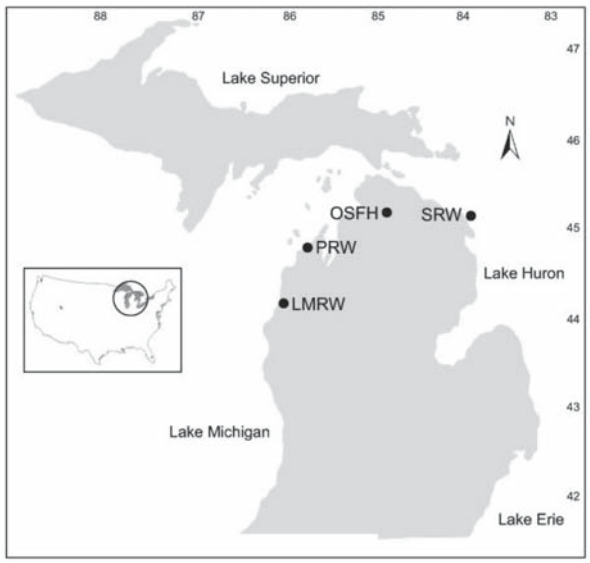

Fig. 1. Locations within Michigan where spawning *Oncorhynchus* spp. were collected over the course of this study. LMRW, Little Manistee River Weir; PRW, Platte River Weir; OSFH, Michigan State Fish Hatchery; SRW, Swan River Weir.

salmon, and 180 Hinchenbrook strain coho salmon were examined (Table 1) and were comprised of apparently healthy individuals, as well as fish exhibiting some overt clinical signs of disease.

Upon completion of gamete collection, fish were rinsed with clean water, subjected to thorough onsite external examination, surface disinfected with 70% ethanol, and then examined internally. Peritoneal cavities of fish were carefully opened using individual sterile forceps/scissors for each fish necropsy and tissue collection. Upon incision of the entire length of the renal capsule, kidney samples were taken for bacterial isolation. In addition to sampling for *C. maltaromaticum,* fish were also analyzed for other pathogenic bacteria, viruses, and parasites according to the American Fishery Society Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens (AFS-FHS, 2007) and the World Animal Health Organization (OIE) Aquatic Manual (OIE, 2006). In this report, clinical and histopathological changes are described only for fish infected solely with *Carnobacterium* spp.

Bacterial isolation

Tissue samples for bacterial isolation were collected from the kidneys using sterile disposable 10 μl inoculating loops by running the loop along the entire length of the kidney and then streaking harvested tissue directly onto trypticase soy agar (TSA; Remel, USA). Primary cultures were incubated at 22°C for up to 72 h as per the guidelines of the American Fisheries Society-Fish Health Section (AFS-FHS Bluebook, 2007), bacterial growth recorded, and individual colonies representative of present colony morphologies sub-cultured onto TSA and incubated for 24 h at 22°C for biochemical and/or molecular analyses. Recovered isolates were maintained at -80°C in trypticase soy broth (TSB; Remel) supplemented with 20% glycerol.

Table 1. Fish species/strain, site and period of collection, fish status, and number of specimens collected at each sampling event throughout the course of this four year study

Fish species	Strain	Collection site	Date of sampling	Fish status	Sample number
O. mykiss	Eagle Lake	OSFH	Oct. 2005	Captive brood-stock, spawning	20
			Nov. 2006		20
			Nov. 2007		20
			Oct. 2008		20
			Mar. 2006	Captive brood-stock, mortality event	10
O. mykiss	Steelhead	LMRW	Apr. 2005	Feral returning spawners	24
			Apr. 2006		60
			Apr. 2007		60
			Apr. 2008		60
O. tshawytscha	Michigan	LMRW	Sept. 2005	Feral returning spawners	60
			Oct. 2005		366
			Sept. 2006		60
			Sept. 2007		60
			Oct. 2008		60
O. tshawytscha	Michigan	SRW	Oct. 2005	Feral returning spawners	60
			Sept. 2006		60
			Oct. 2007		60
			Oct. 2008		60
O. kisutch	Michigan	PRW	Oct. 2005	Feral returning spawners	60
			Oct. 2006		60
			Oct. 2007		60
			Oct. 2008		60
O. kisutch	Hinchenbrook	PRW	Nov. 2005	Feral returning spawners	60
			Oct. 2006		60
			Oct. 2007		60

Phenotypic characterization of isolates

Recovered bacterial isolates were initially characterized using colonial and cellular morphology, Gram reaction of >24 h old sub-cultures, cytochrome oxidase (Pathotec test strips, Remel) and catalase production (3% H_2O_2, Remel), motility, H_2S, and indole production (Sulfur Indole Motility Medium, Remel), esculin hydrolysis, ability to grow on MacConkey agar, nitrate reduction (Indole-Nitrite medium; 20 g pancreatic digest casein, 2 g disodium phosphate, 1 g glucose, 1 g agar, 1 g potassium nitrate, into 1 L distilled H_2O), production of arginine dihydrolase (Remel), glucose utilization (final concentration of 1% glucose in phenol red broth base), mixed acid fermentation (methyl red test), 2,3-butanediol production from glucose (Voges-Proskauer test; MRVP medium, Remel), and the triple sugar iron reaction (TSI, Remel). Upon presumptive assignation to the genus *Carnobacterium*, representative isolates (n=30) from sampling events were speciated according to the scheme of Holt *et al*. (2000) using results from motility and Voges-Proskauer assays, as well as the ability of isolates to utilize amygdalin, inulin, mannitol, α-methyl-D-glucosidase, and xylose (final concentrations of 1% in respective phenol red broth base). Isolates were further characterized using tests for lysine and ornithine decarboxylase (Remel), *o*-nitrophenyl-b-D-galactopyranoside (ONPG, Remel), citrate utilization (Simmons citrate, Remel), gelatinase (3 g beef extract, 5 g peptone, 120 g gelatin, 2 g agar into 1 L distilled H_2O), and ability to utilize lactose, trehalose, arabinose, maltose, salicin, sorbitol, sucrose, mannose, cellobiose, galactose, and rhamnose (1% concentration, phenol red broth base). Results of all biochemical assays were read up to 7 days post-inoculation, with the following exceptions; methyl red, Voges-Proskauer, TSI, ONPG, indole production, and Simmons citrate, which were read at 48 h. The *C. maltaromaticum* ATCC 27865 type culture was also included in these analyses for control/comparative purposes.

16S rRNA gene (16S rDNA) amplification

Twenty-nine representative isolates identified as *C. maltaromaticum* and one identified as *C. divergens* according to phenotypic and biochemical characterizations, along with *C. maltaromaticum* strain 27865, were subjected to further identification via molecular analyses. First, single colonies from approximately 24 h-old cultures on TSA were picked, inoculated into trypticase soy broth (TSB; Remel), and incubated at 22°C for 24 h. Bacteria were then harvested from broth cultures by centrifugation for 10 min at 7,500 rpm, broth decanted, pellet resuspended in sterile 0.9% saline, vortexed for 10 sec, centrifuged for 10 min at 7,500 rpm, and the pellets used for DNA extraction. DNA extractions were performed using a Qiagen DNeasy Tissue Extraction kit (QIAGEN Sciences, USA) according to the manufacturer's protocol for Gram-positive bacteria. Quantification of extracted DNA was performed using the Quant-iT™ DS DNA assay kit in conjunction with a Qubit® flourometer (Invitrogen, USA). Partial amplification of the 16S rRNA gene was conducted via the polymerase chain reaction (PCR) using the universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1387R [Marchesi *et al.* (1998); 5′-GGG CGG WGT GTA CAA GGC-3′; numbering is based on 16S rRNA gene of *Escherichia coli*, Brosius *et al.* (1978)]. The 50 ml PCR reaction for each sample contained a final concentration of 200 nM for each primer, 25 µl of 2× Go-Taq Green master mix (Promega, USA), and 20 ng of DNA template, with DNase-free water comprising the remainder of the reaction mixture. DNA amplification was carried out in an Mastercycler® Radient Thermalcycler (Eppendorf, USA) with an initial denaturation step at 95°C for 5 min, followed by 32 cycles of amplification, which included denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and elongation at 72°C for 60 sec. A final extension step was performed at 72°C for 7 min. Amplicons were combined with SYBR® Green gel stain (Cambrex Bio Science Rockland, Inc., USA), run on a 1.5% agarose gel at 50 V for 30 min, and then visualized under UV exposure. A 1-kb ladder (Roche Applied Science, USA) was used as a molecular marker.

16S rDNA sequencing and phylogenetic analysis

Amplicons were purified with the Wizard SV Gel and PCR Clean-up System (Promega, USA) according to the manufacturer's protocol and the resultant DNA concentration measured with a Quanti-iT DNA kit (Invitrogen). Purified PCR products were then submitted for gene sequencing at the Genomics Technology Support Facility of Michigan State University. Contigs were assembled in the BioEdit Sequence Alignment Editor (Hall, 1999) using the contig assembly program (CAP). Generated sequences were analyzed using the BLASTN software from the National Center for Biotechnology Information, (NCBI, USA) to assess sequence similarity with those contained within the nucleotide database from NCBI. Partial 16S rDNA sequences for the type strains of all described *Carnobacterium* spp. (n=10), as well three other related Gram-positive bacteria, were retrieved using BLASTN software from NCBI. Sequences from all 44 bacterial taxa were aligned and neighbor-joining analysis was performed (Saitou and Nei, 1987) using the Molecular Evolutionary Genetics Analysis software (MEGA; Ver. 4.0), with evolutionary distances being ascertained via the Maximum Composite Likelihood method (Tamura *et al*., 2004). A total of 1,585 characters were examined in this analysis, and a bootstrap test of 1,000 replicates was conducted. Any alignment gaps and/or missing data were deleted only in pairwise sequence comparisons. The tree was rooted with *Lactobacillus sakei* as the out-group.

Histopathological analyses

Representative tissue samples exhibiting gross clinical abnormalities were preserved in 10% buffered formalin, embedded within paraffin, sectioned at 5 mm, stained with hematoxylin and eosin (H&E; Prophet *et al.*, 1992), and observed under a light microscope.

Statistical analyses

In this study, prevalence was calculated by dividing the number of fish found to be infected with a *Carnobacterium* spp. by the total number of fish sampled during that event. Differences in the presence/absence of *Carnobacterium*-derived infections according to fish species/strain, location, year, and gender were assessed using Binary Logistic Regression and significance evaluated using the Hosmer and Lemeshow chi-square test of goodness of fit and Omnibus tests of model coefficients. The Hinchenbrook Coho salmon strain, LMRW, year of 2008, and females were set as the reference categories for strain, location, year, and gender, respectively. All statistical tests were conducted in PASW Statistics 17.0 by SPSS Inc. using the Binary Logistic Regression with Stepwise Forward LR method. Unless otherwise noted, a statistical test with $P<0.05$ was regarded as statistically significant.

Results

Isolation and phenotypic analysis

All bacterial morphologies present on primary TSA cultures were sub-cultured for identification according to the AFS-

FHS Bluebook (2007). Among these, 57 isolates (two from external ulcerations, one from the spleen, four from swim-bladder fluid, and fifty from the kidneys) were recovered from a total of 51 out of 1,564 (3.3%) fish at an intensity ranging from 100 colony forming units (CFU) to over 2×10^4 CFU/g of tissue. Colonies were 0.5-2.0 mm in diameter, translucent white in color that varied between umbonate with undulate margins or low convex with entire margins. In one instance, a culture was comprised of both colonial morphologies. All 57 isolates were Gram-positive bacilli measuring ~1.0-1.5 mm by 0.5 mm that were often arranged in palisades. The isolates were non-motile, unable to produce catalase, hydrogen sulfide, or cytochrome oxidase, did not reduce nitrate, utilized glucose in the presence and absence of oxygen, produced an acid slant over an acid butt without the production of gas or hydrogen sulfide on TSI, and were unable to grow on MacConkey agar (Table 2). In addition, the cultured bacteria produced a sweet malt-like aroma. Based on these characteristics, the 57 isolates were identified as *Carnobacterium* spp.

Further phenotypic analyses were performed on 30 *Carnobacterium* spp. isolates, representing all sampling events, species and strains, and geographic locales. The *C. maltaromaticum* strain 27865 was also included (Table 2). According to the scheme of Holt *et al.* (2000), 29 of the 30 isolates (Cm1-5, 7-9, 12-32) recovered in this study, as well as the *C. maltaromaticum* type strain were identified as *C. maltaromaticum*; however, variability in acid production from inulin and mannitol, as well as use of α-methyl-D-glucosidase, was observed

Table 2. Phenotypic characteristics of *Carnobacterium* spp. isolated from spawning *Oncorhynchus* spp. of Michigan, as well as those of the *C. maltaromaticum* type strain (ATCC 27865) and other closely related bacteria; percentage +, positive reaction; -, negative reaction; (+), weak positive reaction; nr, not reported; V, variable result; A/A/-/-, Triple Sugar Iron result of an acid butt over an acid slant without the production of gas or hydrogen sulfide; ONPG, *o*-nitrophenyl-b-D-galactopyranoside.

Biochemical Test	Isolates Cm 1-5, 7-9, 12-22, 24-30, (n=28)		Isolate Cm 10 (n=1)	Isolate Cm 23 (n=1)	*C. maltaromaticum* Type Strain (ATCC 27865)	*C. maltaromaticum* of lake whitefish (Loch *et al.*, 2008)
Gram stain	+	(100%)	+	+	+	+
Motility	-	(0%)	-	-	-	-
Cytochrome oxidase	-	(0%)	-	-	-	-
Catalase	-	(0%)	-	-	-	-
H_2S production	-	(0%)	-	-	-	-
Indole production	-	(0%)	-	-	-	-
Nitrate reduction	-	(0%)	-	-	-	-
Simmons citrate	-	(0%)	-	-	-	-
MacConkey agar	-	(0%)	-	-	-	-
Triple sugar iron	A/A/-/-	(100%)	A/A/-/-	A/A/-/-	A/A/-/-	A/A/-/-
Esculin hydrolysis	+	(100%)	+	+	+	+
Gelatin hydrolysis	-	(0%)	-	-	-	nr
Methyl red	+	(100%)	+	+	+	+
ONPG	+	(75%)	+	+	+	+
Voges-Proskauer	+	(100%)	+	+	(+)	v
Arginine dihydrolase	+	(100%)	+	+	+	+
Lysine decarboxylase	-	(0%)	-	-	-	-
Ornithine decarboxylase	-	(0%)	-	-	-	-
Utilization of:						
Arabinose	-	(0%)	-	-	-	-
Amygdalin	+	(100%)	+	+	+	nr
Cellobiose	+	(100%)	+	+	+	+
Galactose	+	(79%)	+	+	(+)	+
Glucose	+	(100%)	+	+	+	+
Inulin	+	(82%)	-	+	+	v
Lactose	+	(82%)	-	(+)	+	+
Maltose	+	(100%)	-	+	+	+
Mannitol	+	(100%)	-	+	+	v
Mannose	+	(96%)	-	+	+	+
Rhamnose	-	(0%)	-	-	-	-
Salicin	+	(100%)	+	+	+	+
Sorbitol	+	(96%)	-	+	-	-
Sucrose	+	(100%)	+	+	+	+
Trehalose	+	(100%)	-	+	+	+
Xylose	-	(0%)	-	-	-	-
Methyl-α-D-Glucopyranoside	+	(93%)	-	-	+	nr

Table 3. Assignation of 30 further characterized *Carnobacterium* isolates recovered from *Onchorynchus* spp., as well as the *C. maltaromaticum* type strain, into biovars according to results from assays where variability amongst isolates occurred. +, positive result; -, negative result

Biochemical assay	Biovar											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Lactose	+	+	+	+	-	+	+	-	-	+	+	+
Inulin	+	+	+	+	+	+	-	+	-	+	+	+
Mannitol	+	+	+	+	+	+	+	+	-	+	+	+
Trehalose	+	+	+	+	+	+	+	+	-	+	+	+
Maltose	+	+	+	+	+	+	+	+	-	+	+	+
Sorbitol	-	+	+	+	+	-	+	+	-	+	+	+
Mannose	+	+	+	+	+	-	+	+	-	+	+	+
Galactose	+	+	-	+	+	-	+	+	+	+	-	+
ONPG	+	-	+	+	+	+	+	-	+	-	-	+
α-methyl-D-glucosidase	+	+	+	+	+	-	+	+	-	-	+	-
Number of isolates in each biovar	1*	4	4	7	4	1	5	1	1 [a]	1	1	1[b]

* *C. maltaromaticum* type culture (ATCC 27865); [a] isolate Cm 10; [b] isolate clustering most closely with *C. divergens* according to 16S rDNA sequencing; ONPG, *o*-nitrophenyl-b-D-galactopyranoside.

Table 4. Location, host species, presence or absence of clinical signs in host from which the isolate was recovered, month/year of recovery, 16S rDNA sequence length, and accession number by biovar for 30 *Carnobacterium* isolates recovered from spawning *Oncorhynchus* spp. in Michigan.

Biovar	Isolate ID	Location	Species	Clinical signs	Date	Sequence length (bp)	NCBI accession no.
I	Cm 11		ATCC Type Culture (27865)			784	GQ304909.1
II	Cm 1	SFH	Rainbow trout, Eagle Lake strain	+	Oct. 2005	736	GQ304919.1
	Cm 21	PRW	Coho Salmon, Michigan strain	+	Oct. 2006	1400	GQ304929.1
	Cm 22	PRW	Coho Salmon, Michigan strain	+	Oct. 2006	1400	GQ304930.1
	Cm 26	PRW	Coho Salmon, Hinchenbrook strain	+	Oct. 2007	1408	GQ304934.1
III	Cm 2[a]	SFH	Rainbow trout, Eagle Lake strain	+	Mar. 2006	721	GQ304918.1
	Cm 20	PRW	Coho Salmon, Michigan strain	+	Oct. 2005	1400	GQ304928.1
	Cm 30	PRW	Coho Salmon, Michigan strain	-	Oct. 2008	1410	GQ304938.1
	Cm 32	PRW	Coho Salmon, Michigan strain	-	Oct. 2008	1410	GQ304940.1
IV	Cm 3[a]	SFH	Rainbow trout, Eagle Lake strain	+	Mar. 2006	825	GQ304917.1
	Cm 4	SFH	Rainbow trout, Eagle Lake strain	+	Mar. 2006	780	GQ304916.1
	Cm 12	LMRW	Chinook Salmon	-	Oct. 2005	1400	GQ304920.1
	Cm 13	LMRW	Chinook Salmon	+	Oct. 2005	1407	GQ304921.1
	Cm 14	LMRW	Chinook Salmon	-	Sept. 2005	1400	GQ304922.1
	Cm 24	PRW	Coho Salmon, Hinchenbrook strain	+	Oct. 2006	1402	GQ304932.1
	Cm 31	PRW	Coho Salmon, Michigan strain	+	Oct. 2008	1401	GQ304939.1
V	Cm 5	SFH	Rainbow trout, Eagle Lake strain	+	Mar. 2006	779	GQ304915.1
	Cm 17	PRW	Coho Salmon, Michigan strain	-	Oct. 2005	1409	GQ304925.1
	Cm 28[b]	SRW	Chinook Salmon	+	Oct. 2008	1403	GQ304936.1
	Cm 29	PRW	Coho Salmon, Michigan strain	+	Oct. 2008	1393	GQ304937.1
VI	Cm 7[c]	LMRW	Steelhead trout	+	Apr. 2007	724	GQ304913.1
VII	Cm 8[c]	LMRW	Steelhead trout	+	Apr. 2007	786	GQ304912.1
	Cm 18	PRW	Coho Salmon, Michigan strain	+	Oct. 2005	1407	GQ304926.1
	Cm 19	PRW	Coho Salmon, Michigan strain	+	Oct. 2005	1401	GQ304927.1
	Cm 25	PRW	Coho Salmon, Michigan strain	+	Oct. 2007	1401	GQ304933.1
	Cm 27[b]	SRW	Chinook Salmon	+	Oct. 2008	1401	GQ304935.1
VIII	Cm 9	SFH	Rainbow trout, Eagle Lake strain	+	Nov. 2007	823	GQ304911.1
IX	Cm 10	LMRW	Steelhead trout	-	Apr. 2008	533	GQ304910.1
X	Cm 15	PRW	Coho Salmon, Michigan strain	-	Oct. 2005	1401	GQ304923.1
XI	Cm 16	PRW	Coho Salmon, Michigan strain	+	Oct. 2005	1399	GQ304924.1
XII	Cm 23*	PRW	Coho Salmon, Michigan strain	-	Oct. 2006	1400	GQ304931.1

[a] recovered from same fish but different organs; [b] recovered from same fish but different organs; [c] recovered from same fish but different organs; * isolate clustering most closely with *C. divergens* according to 16S rDNA sequencing.

in 6, 1, and 2 *C. maltaromaticum* isolates, respectively. On the other hand, the remaining isolate (Cm 10), was speciated as *C. divergens*. Further testing on the 30 isolates showed that they all hydrolyzed esculin, were methyl red positive, and produced arginine dihydrolase. None of the recovered 30 isolates produced indole, gelatinase, lysine decarboxylase, ornithine decarboxylase, nor were able to utilize citrate as a sole carbon source. Carbohydrate utilization studies showed that all isolates utilized amygdalin, salicin, sucrose, and cellobiose, while no isolates broke down arabinose, xylose, or rhamnose. Variability amongst isolates was observed in use of mannitol, trehalose, and maltose (29/30 +), sorbitol and mannose (28/30 +), α-methyl-D-glucosidase (26/30 +), galactose, lactose and inulin (24/30 +), and ONPG (23/30 +; Table 2).

The phenotypic variability amongst *Carnobacterium* isolates recovered from *Onchorynchus* spp. that occurred in lactose, inulin, mannitol, trehalose, maltose, sorbitol, mannose, galactose, ONPG, and α-methyl-D-glucosidase was used to dichotomize the 30 isolates, plus the *C. maltaromaticum* type culture, into 12 biovars (I-XII, Table 3). The most prevalent biovar was IV, which contained 7 isolates, followed by biovar VII (5 isolates), II, III, and V (4 isolates each), while the remaining 7 biovars contained 1 isolate each (Table 3). The *C. maltaromaticum* type strain was slightly different than the 29 *C. maltaromaticum* isolates recovered in this study and formed Biovar I. *Carnobacterium* isolates recovered from Michigan-adapted coho salmon at the Platte River weir were the most phenotypically variable, belonging to 8 biovars, followed by isolates recovered from captive rainbow trout (Eagle Lake strain), which were scattered in 5 biovars. Interestingly, all isolates recovered from Chinook salmon returning to the LMRW were identical to each other and fell in biovar IV, while in three instances, *Carnobacterium* isolates recovered from the same fish but from different organs fell into different biovars (Table 3).

Gene sequence analysis

Upon PCR amplification of partial stretches of the 16S rRNA gene from 30 representative *Carnobacterium* isolates, the resultant amplicons were sequenced. The length of the generated sequences ranged from 533 to 1,410 bp (accession numbers are given in Table 4). Twenty nine of the thirty representative *Carnobacterium* sequences recovered from *Oncorhynchus* spp. were highly similar to the reference *C. maltaromaticum* strain (ATCC 27865) sequenced in this study (99-100% similar, expectation value=0). When the same sequences were compared to the 16S rRNA gene sequences contained within the GenBank using BLASTN, 99% similarity and expectation values of 0 were observed for most *C. maltaromaticum* strains recovered from fish, as well as other sources. One of the thirty *Carnobacterium* isolates (Cm 23), which showed a phenotypic similarity to *C. maltaromaticum,* was 99% similar to strains of *C. divergens* contained within the NCBI database, with an expectation value of 0.

According to phylogenetic analyses, 29 of the isolates, along with *C. maltaromaticum* 27865 that was sequenced in our laboratory, were most similar to *C. maltaromaticum* (Cm 1-5, Cm 7-10, Cm 12-22, Cm 24-32) with a boot-strap value of 94 (Fig. 2). Once again, Cm 23 was identified as *C. divergens* (boot-strap value of 100), while isolate Cm 10, which was phenotypically closer to *C. divergens*, was identified as *C. maltaromaticum* according to phylogenetic analysis (Fig. 2). Within the *C. maltaromaticum* clade, four nodes displayed a boot-strap value ranging from 56-80, providing evidence that intra-species genotypic variability amongst the *C. maltaromaticum* isolates recovered from Michigan *Oncorhynchus* spp. does exist. Of particular interest was the node with a bootstrap value of 56 that separates Cm2 from Cm3, as these two isolates were recovered from the same fish but from different tissues (external ulceration and kidney, respectively) and the node that separates Cm1 from the Cm2-Cm3 sister group (bootstrap=71), as all three of these isolates were recovered from captive rainbow trout-eagle lake broodstock at the same fish hatchery.

When the sequences of the 29 isolates most similar to *C. maltaromaticum* were compared with *C. maltaromaticum* ATCC strain 27865 and *C. maltaromaticum* M58825 in the multiple alignment constructed in MEGA using *C. maltaromaticum* M58825 as the reference, 30 variable sites were observed at bases 58-60, 62-66, 82, 98, 133, 318, 653, 796, 828, 829, 831, 847-850, 853, 898, 918, 1054, 1154, 1470, 1471, 1473, 1474. Among the 30 variable sites, 3 were substitutions that were present in all isolates recovered from Michigan *Oncorhynchus* spp. (sites 133, 318, 1054) when compared to *C. maltaromaticum* M58825, while 12 of the substitutions were found in only 1 of the 29 Michigan isolates at that particular site. Base 98 was found to be highly variable, with *C. maltaromaticum* M58825 having a guanine compared to 25 of the Michigan isolates (86%) containing a cytosine, 3 isolates (10%) having a thymine, and 1 isolate containing a deletion at this site (3%). Additionally, when compared to *C. maltaromaticum* M58825, deletions were found at site 67 in 24/29 (83%) of the Michigan isolates, at site 71 in 5 strains (17%; Cm17, 15, 16, 25, 30), at site 75 in 8 strains (28%; Cm9, Cm5, Cm3, 12, 13, 14, 18, 21), at site 81 in 22 strains (76%; Cm17, 11, 10, 9, 8, 7, 5, 3, 2, 12, 14, 16, 18, 19, 21, 22, 25, 26, 29, 30, 31), and at site 100 in 4 strains (14%; Cm 2,12,14,27). Lastly, insertions were found at site 681 in Cm27, at site 695 in Cm30, and at site 733 in Cm17.

C. maltaromaticum prevalence and statistical analysis

Throughout the course of this study, five strains of three *Oncorhynchus* spp. originating from four sites were sampled and subjected to bacteriological analyses (1564 fish; Table 1). Among these, 2/80 (2.5%) captive-propagated and 43/1,474 (2.92%) feral members of the genus *Oncorhynchus* spp. were infected with *Carnobacterium* spp. *C. maltaromaticum* was recovered from 2/80 (2.5%) captive spawning Eagle Lake strain rainbow trout over a four year period. Additionally, a mortality event in this group of fish occurred in March of 2006 and was attributed to *C. maltaromaticum* (6/10 fish infected). Of the feral steelhead trout that were returning to spawn in the LMRW from 2005-2008, a total of 4/205 (1.95%) were infected with *C. maltaromaticum*. In feral Chinook salmon returning to the LMRW from 2005- 2008, *C. maltaromaticum* infections were observed in 14/606 (2.31%), while 1/240 (0.42%) chinook salmon returning to the SRW was infected. *C. maltaromaticum* infections in feral Michigan Strain coho salmon returning to spawn at the PRW were detected in 19/243 (7.82%) and in 5/180 (2.78%) Hinchenbrook strain coho salmon. Additionally, the Cm23 isolate, which clustered with *C. divergens*, was re-

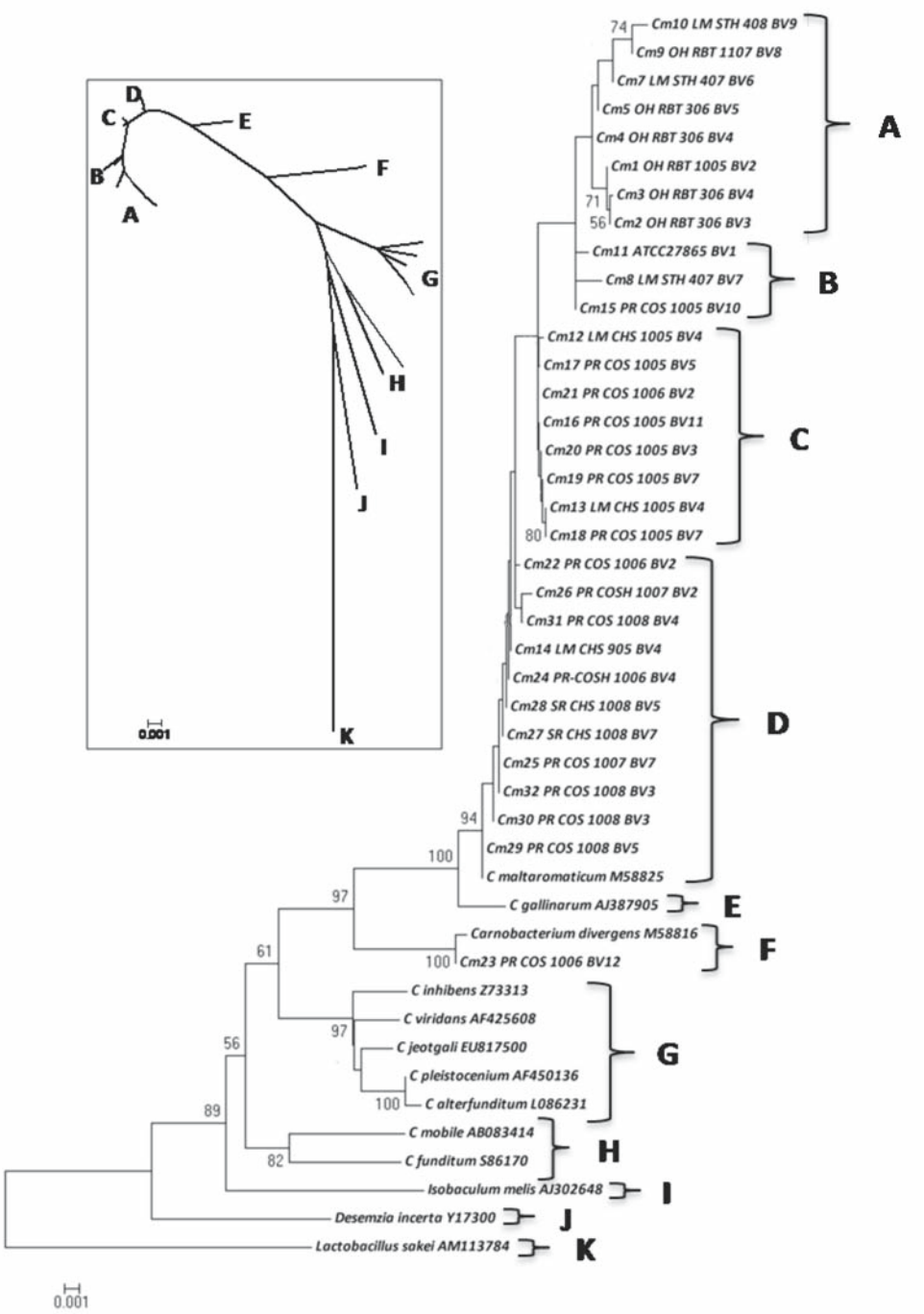

Fig. 2. A bootstrap consensus tree inferring the evolutionary history of 44 Gram-positive bacterial taxa that was constructed using the Neighbor-Joining method (MEGA 4.1) with 1,000 replicates. The inset represents an identical tree but displayed in a radial form (groups of taxa are identified by letter). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Values next to the branches represent the percentage of replicate trees in which the associated taxa clustered together; only bootstrap values $\geq$ 50 are displayed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. There were a total of 1,585 positions in the final dataset.

covered from the kidney of an infected Michigan strain PRW coho salmon. Logistic regression analyses demonstrated a statistically significant relationship between *Carnobacterium* infections and fish species/strain (p-value$<$0.001). Michigan strain coho salmon had the highest *C. maltaromaticum* infection prevalence (p=0.036) and had 3.050 (95% CI:

1.078-8.630) times the odds of being infected with *C. maltaromaticum* than did the Hinchenbrook strain of coho salmon. Eagle Lake strain rainbow trout had 0.779 (95% CI: 0.144-4.214) times the odds of being *C. maltaromaticum* infected than the Hinchenbrook strain coho salmon, while steelhead trout had 0.910 (95% CI: 0.229-3.620) times the odds of being infected. Lastly, Chinook salmon had 0.442 (95% CI: 0.155-1.262) times the odds of having a *C. maltaromaticum* infection than did the Hinchenbrook strain coho salmon.

When analyzed according to location, fish collected from PRW had the highest overall *C. maltaromaticum* infection prevalence at 5.7% (24/423), followed by fish from the hatchery (2/80, 2.5%) and LMRW (18/811, 2.2%). The lowest prevalence of *C. maltaromaticum* infections was found in fish from the SRW, at 0.42% (1/240). However, spatial differences in *C. maltaromaticum* infection prevalence were not statistically significant according to our logistic regression model (p-value =0.397). According to year of sampling, *C. maltaromaticum* infection prevalence was highest in 2005 (28/653, 4.3%), followed by 2008 (8/260, 3.1%), 2006 (5/321, 1.6%), and 2007 (4/320, 1.3%), and these differences were statistically significant (p-value=0.001). Logistic regression analyses showed that 2005 had 2.279 (95% CI: 0.965-5.383) times the odds of a *C. maltaromaticum* infection occurring in sampled *Oncorhynchus* spp. than did 2008, while 2006 (OR 0.507, 95% CI: 0.160-1.604) and 2007 (OR 0.408, 95% CI: 0.119-1.399) varied in *C. maltaromaticum* infection likelihood when compared to 2008.

Of the 782 feral male *Oncorhynchus* spp. that were sampled, 16 were *C. maltaromaticum* infected (2.05%), while 27/692 (3.90%) feral female *Oncorhynchus* spp. were found infected. Among captive rainbow trout broodstock, 0/28 males were found infected with *C. maltaromaticum*, while 2/52 female rainbow trout (3.85%) were infected. However, this does not include the mortality event in rainbow trout brood-stock that occurred in March of 2006, during which only males were sampled and 6/10 (60.00%) were found infected. In steelhead returning to spawn at the LMRW, 2/103 (1.94%) males and 2/102 females (1.96%) were infected with *C. maltaromaticum*. Of the 348 sampled males Chinook salmon returning to the LMRW, 7/348 (2.01%) were *C. maltaromaticum* infected, while 7/258 (2.71%) females were infected. *Carnobacterium maltaromaticum* incidence in Chinook salmon returning to the SRW was 0/120 (0.00%) and 1/120 (0.83%) in males and females, respectively. *C. maltaromaticum* incidence in Michigan adapted Coho salmon returning to the PRW was higher in females at 14/122 (11.48%) than in males (5/121, 4.13%), as was the case in Hinchenbrook strain coho salmon (females 3/90,

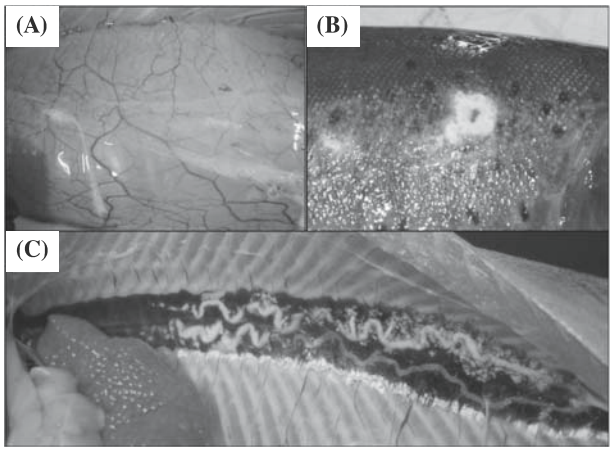

Fig. 3. (A) Opacity and thickening, along with pseudomembrane formation and hyperemia in swim-bladder vessels, of a *C. maltaromaticum* infected Michigan strain coho salmon returning to the Platte River Weir. (B) Right dorso-lateral aspect of a *C. maltaromaticum* infected captive rainbow trout showing an ulceration that penetrated through the epidermis and dermis into the underlying muscle from which the bacterium was recovered. (C) Kidney of a *C. maltaromaticum* infected rainbow trout exhibiting nephrocalcinosis of the ureters and renal tubules.

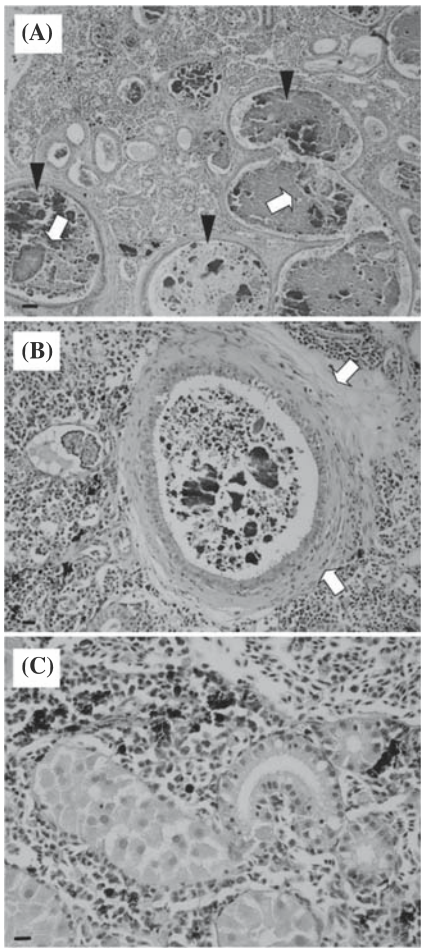

Fig. 4. (A) Hematoxylin and eosin (H&E) stained kidney section from which *C. maltaromaticum* was recovered (100× magnification; size bar in lower left corner=55 μm). Note severe dilation of renal tubules (arrowhead) and mineral deposits within the lumen (white arrow). (B) H&E stained kidney section from which *C. maltaromaticum* was recovered exhibiting fibrosis of renal tubule (white arrow; 200× magnification size bar in lower left corner=33 μm). (C) H&E stained kidney section from which *C. maltaromaticum* was recovered exhibiting renal tubular degeneration (400× magnification; size bar in lower left corner=22 μm).

3.33%; males 2/90, 2.22%). Overall, gender showed a statistically significant relationship to *C. maltaromaticum* infection prevalence (p-value-0.027), with males being 0.49 (95% CI: 0.261-0.921) times as likely to be infected as females, meaning males were approximately half as likely to be infected when compared to females.

Clinical and histopathological findings

Among *Oncorhynchus* spp. infected with *C. maltaromaticum* only, the most common clinical signs were visceral congestion and opacity/thickening of the swim-bladder walls (Fig. 3A), while external ulcerations penetrating to the musculature in the nuccal and trunk regions (Fig. 3B), bilateral exophthalmia, pseudomembrane formation (Fig. 3A), petechial hemorrhage in the liver, and splenic friability were also observed. In addition, gross signs consistent with nephrocalcinosis (i.e., renal swelling, dilation of ureters with a white granular substance, and multifocal clusters of white nodules) were observed in a portion of the captive rainbow trout broodstock infected solely with *C. maltaromaticum* (Fig. 3C). When kidneys displaying these signs were examined for tissue alterations microscopically, they again revealed changes commonly associated with nephrocalcinosis, such as severe dilation of renal tubules due to the formation of renal casts (Fig. 4A), fibrosis of mineralized tubules (Fig. 4B), and renal tubular degeneration (Fig. 4C). Additional sections of liver, kidney, swim-bladder, and spleen samples were also stained and analyzed. However, these fish were concurrently infected with additional pathogenic organisms and were thus excluded from further analysis.

While all isolates belonging to biovars II and VII were recovered from fish displaying clinical signs of disease (Table 4), no clear associations between the presence or absence of clinical signs and *Carnobacterium* biovar, genotype, or host species and strain were observed.

Discussion

Findings of this study clearly demonstrate that infections with *C. maltaromaticum* are widespread in feral and captive *Oncorhyncus* spp. stocks in Michigan. Other than our previous report on the distribution of *C. maltaromaticum* in lake whitefish and its potential role in causing pathological lesions (Loch *et al.,* 2008), reports on this infection in other fish species residing in the Great Lakes basin is lacking. An unexpected finding of this study was that while *C. maltaromaticum* infections were present in both diseased and apparently healthy captive rainbow trout as previously reported, this bacterium was even more prevalent in feral salmon stocks. Therefore, this study is considered the first to report clinical *C. maltaromaticum* infections in feral *Oncorhynchus* spp. Additional studies on the impact of this bacterium in feral fish populations are warranted.

Chinook salmon collected from the Swan River weir had the lowest detected infection prevalence, although differences were not significant according to logistic regression analysis. This goes side by side with our previous study on lake whitefish (Loch *et al*., 2008), which found that *Carnobacterium* infection prevalence in Lake Huron stocks was lower than infection prevalence observed in Lake Michigan stocks. On a year by year basis, *Carnobacterium* infection prevalence varied significantly, being highest in 2005, dropping off in 2006, and then increasing in both of the following years. Unfortunately, more than four years of data are required before any trends can be definitively resolved and the sources of these fluctuations determined.

Some of the original studies that linked *C. maltaromaticum* to pseudokidney disease involved fish that were spawning or had recently undergone spawning (Ross and Toth, 1974; Cone, 1982; Starliper *et al*., 1992); in this context, this study found an association between *C. maltaromaticum* infection and fish gender in Michigan *Oncorhynchus* spp., with males being approximately half as likely to be infected as females according to regression analysis. A predisposition for infection with this bacterium in captive female rainbow trout broodstock has been suggested by other authors (Cone, 1982; Starliper *et al*., 1992), though rigorous statistical analysis, such as those conducted in this study, were not used. While this study further supports that female Pacific salmon may be more susceptible to *C. maltaromaticum* infection, it is of interest to note that this phenomenon was also observed in feral broodstock, which has not been reported in previous studies. Despite an apparent susceptibility of females to this infection, the March 2006 disease epizootic involving *C. maltaromaticum* in an all male rainbow trout captive broodstock highlights that pseudokidney disease can ensue if conditions are right, regardless of gender.

Bacterial isolates recovered from Michigan *Oncorhynchus* spp. displayed the phenotypic and genotypic characteristics commonly associated with *Carnobacterium* spp.; however, some distinct differences between the findings of this study and earlier publications were observed. For example, *C. maltaromaticum* ATCC strain 27865 (originally retrieved from raw milk) and the strains of this study were different in their ability to produce acid from sorbitol (Table 2). A lack of acid production from sorbitol is reported in the original description of *C. piscicola* (now *C. maltaromaticum*) by Hiu *et al.* (1984) and Austin and Austin (2007), and was also reported for the *Carnobacterium* strains recovered from lake whitefish by Loch *et al.* (2008); however, 93% of the *C. maltaromaticum* isolates recovered in this study were sorbitol positive, while Baya *et al.* (1991), Starliper *et al*. (1992), Toranzo *et al.* (1993), and Lai and Manchester (2000) reported either a positive or a weak/ delayed positive reaction for sorbitol. Thus, this character is quite variable among different strains of *C. maltaromaticum*. Other biochemical characteristics, such as acid production from mannitol and inulin, which have classically been used to dichotomize members of the genus *Carnobacterium* (Montel *et al*., 1991; Holt *et al*., 2000), showed intra-species variation in this study, as well as in the studies of Hiu *et al.* (1984), Baya *et al.* (1991), Starliper *et al.* (1992), and Loch *et al.* (2008) and continue to hamper definitive identifications that rely solely on phenotypic and biochemical schemes. The current study further substantiates this point and is epitomized by strain Cm 10 of this study, which was identified as *C. divergens* phenotypically but definitively identified as *C. maltaromaticum* through molecular and phylogenetic analysis, while the opposite was true for *C. maltaromaticum* strain Cm23, a matter that underscores the importance of genetic approaches in the definitive identification of *Carnobacterium* spp.

As displayed in Table 4, no clear association between biovars containing more than one isolate and year of isolation, host

species/strain, or location could made, though the three isolates recovered from Chinook salmon originating from the Little Manistee River Weir all belonged to biovar IV. Also of interest was that all isolates belonging to biovars II and VII were recovered only from fish with clinical signs of disease. Intra-species differences in the biochemical profiles of *Carnobacterium* spp. have been described in association with differences in strain functionality (Laursen *et al*., 2006). Thus, whether *C. maltaromaticum* strains belonging to biovars II and VII have altered pathogenicity and/or virulence warrants further investigation.

Partial sequencing of the 16S rRNA gene and subsequent phylogenetic analysis strongly supported the identification of 29 of the bacterial isolates as *C. maltaromaticum*, and 1 as *C. divergens*. Additional phylogenetic studies using Maximum Parsimony analysis with the heuristic search option in PAUP 4.0 (Phylogenetic Analysis Using Parsimony) and Bayesian analysis in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) also demonstrated that Cm23 formed a highly supported cluster with *C. divergens*, while the other representative strains were highly similar to *C. maltaromaticum* (data not shown). Despite the strong phylogenetic similarities, genotypic heterogeneity within the *C. maltaromaticum* isolates recovered in this study was also observed. While some sites in the 16S rDNA sequences of the Michigan *C. maltaromaticum* isolates were universally different from the *C. maltaromaticum* type strain, differences between Michigan isolates were also present, though no correlation to host species/strain, location, year of isolation, or biovar could be made. Although the 16S rRNA gene is highly conserved and provides a reliable marker to use in the differentiation of numerous bacteria including *Carnobacterium* spp. (Hammes and Hertel, 2006), it also known to have variable regions in some bacteria (Stackebrandt *et al*., 1991). Some studies have examined genotypic heterogeneity amongst *Carnobacterium* spp. and other closely related bacteria recovered primarily from food products using the 16S-23S rDNA intergenic spacer region (Kabadjova *et al*., 2002; Chenoll *et al*., 2003; Rachman *et al*., 2004), while Michel *et al.* (2007) examined inter- and intra-species variability in a number of lactic acid bacteria recovered from fish according to amplified rDNA gene restriction analysis and found evidence for intra-species variability in *C. maltaromaticum*. However, we are unaware of any studies that have examined the genotypic heterogeneity of *C. maltaromaticum* isolates recovered from fish using 16S rDNA sequence analysis.

The majority of carnobacterial infected fish within this study displayed disease signs that are typical of such infections and similar to those described by Austin and Austin (2007). Others isolates came from fish that did not display any signs of disease, which has also been described by others (e.g., Evelyn and McDermott, 1961; Humphrey *et al*., 1987; Starliper *et al*., 1992). However, some of the other disease signs observed in infected fish of this study, such as the gross and histopathological signs that are characteristic of nephrocalcinosis (Harrison and Richards, 1979), have not been reported in association with *C. maltaromaticum* infection before. Indeed, the definite cause of nephrocalcinosis has never been determined and is a matter of continuous debate (Dunbar and Herman, 1971; Harrison and Richards, 1979; Smart *et al*., 1979; Chen *et al*., 2001). In this study, multiple rainbow trout-eagle lake broodstock within a state hatchery showed the typical signs of nephrocalcinosis and were infected only with *C. maltaromaticum*. Other infectious etiologies for nephrocalcinosis have been previously investigated in earlier studies (Snieszko, 1961; Landolt, 1975), though no bacterial etiology was found. Based solely on findings of this study, we cannot claim that *C. maltaromaticum* infection can lead to nephrocalcinosis development or vice versa. It should also be noted that we did not observe any nephrocalcinosis in the feral broodstock infected with *Carnobacterium* spp., nor in lake whitefish infected with this bacterium (Loch *et al*., 2008), pointing to a likely etiology associated with the hatchery environment.

In conclusion, this study provides strong evidence that infections with *C. maltaromaticum* are widespread in feral *Oncorhynchus* stocks, as well as in hatchery reared rainbow trout in Michigan, and that isolates recovered from infected hosts are commonly phenotypically and genotypically heterogeneous.

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