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Genomic characterization of *Tenacibaculum maritimum* O-antigen gene cluster and development of a multiplex PCR-based serotyping scheme

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Abstract

Tenacibaculum maritimum is a devastating bacterial pathogen affecting a large variety of marine fish species. It is responsible for significant economic losses in aquaculture farms worldwide. Different typing methods have been proposed to analyse bacterial diversity and population structure. Serological heterogeneity has been observed and up to four different serotypes have been described so far. However, the underlying molecular factors remain unknown. By combining conventional serotyping and genome-wide association study, we identified the genomic loci likely involved in the O-antigen biosynthesis. This finding allowed the development of a robust multiplex PCR-based serotyping scheme able to detect subgroups within each serotype and therefore performs better than conventional serotyping. This scheme was successfully applied to a large number of isolates from worldwide origin and retrieved from a large variety of fish species. No obvious correlations were observed between the mPCR-based serotype and the host species or the geographic origin of the isolates. Strikingly, the distribution of mPCR-based serotypes does not follow the core genome phylogeny. Nevertheless, this simple and cost-effective mPCR-based serotyping method could be useful for different applications such as population structure analysis, disease surveillance, vaccine formulation and efficacy follow-up.

KEYWORDS
aquaculture, fish diseases, molecular serotyping, o-antigen gene cluster, tenacibaculosis, Tenacibaculum maritimum

1 | INTRODUCTION

The global fish production is estimated to have reached about 179 million tons in 2018. Aquaculture accounted for 52% of fish for human consumption and is currently the fastest growing food-producing sector, playing an increasingly critical role in global food security (FAO, 2020). However, the fast development of aquaculture has been associated with a dramatic increase in infectious disease outbreaks, which

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can be catastrophic for the industry, causing large economic losses and impacting the environment and animal welfare (Lafferty et al., 2015; Stentiford et al., 2017).

Tenacibaculum species are Gram negative, filamentous and exclusively marine bacteria belonging to the family Flavobacteriaceae (phylum Bacteroidetes) (Bernardet, 2011). This genus encompasses several species pathogenic for marine fish, responsible for a condition referred to as tenacibaculosis (also known as marine flexibacteriosis) (see Avendaño-Herrera et al. [2006], Fernández-Álvarez & Santos [2018], and Nowlan et al. [2020] for recent reviews). *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) (Wakabayashi et al., 1986) was the first species to be described and constitutes the best-known pathogen within the genus (Avendaño-Herrera et al., 2006). It has originally been isolated in 1977 in Japan during mortality events on marine red seabream (*Pagrus major*) and black sea bream (*Acanthopagrus schlegelii*) (Masumura & Wakabayashi, 1977; Wakabayashi et al., 1984). Since then, it has been retrieved from a large variety of marine fish species worldwide including: Atlantic salmon (*Salmo salar*) in Australia (van Gelderen, 2007) and in Chile (Apablaza et al., 2017), rainbow trout (*Oncorhynchus mykiss*) in Australia (van Gelderen, 2007) and in Chile (Valdes et al., 2021), Chinook salmon (*Oncorhynchus tshawytscha*) in Canada (Ostland et al., 1999), turbot (*Scophthalmus maximus*), sole (*Solea solea*) and blackspot seabream (*Pagellus bogaraveo*) in Spain (Castro et al., 2007), batfish (*Platax orbicularis*) in French Polynesia (Lopez et al., 2022) and sea bass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*) in Europe (Muniesa et al., 2020). Fish often display gross external lesions including eroded mouth, skin ulcers or necrosis, frayed fins and tail rot (Avendaño-Herrera et al., 2006; Nowlan et al., 2020). So far, a unique vaccine[1] is commercially available for a single fish species (turbot) and a single serotype (i.e. O2). Hence, in order to cope with the disease, the treatment broadly relies on disinfectants (e.g. formol and hydrogen peroxide) and a variety of antibiotics (e.g. amoxycillin, trimethoprim, oxytetracycline, enrofloxacin, flumequine, furazolidone and florfenicol) (Avendaño-Herrera et al., 2005).

To improve the control of bacterial infections in fish farms, data at both the epidemiological and molecular levels are urgently needed (Bayliss et al., 2017). Indeed, different typing methods for studying the relationships between *T. maritimum* isolates have been proposed allowing to better understand the population structure, the spreading and the epidemiology of this pathogen (see Bridel et al. [2020] and reference therein). Among those, serotyping methods have been assessed and up to four serotypes have been described so far (Avendaño-Herrera et al., 2004; Avendaño-Herrera et al., 2005; Fernández-Álvarez et al., 2018; Piñeiro-Vidal et al., 2007). This serological diversity might have important consequences for the selection of appropriate candidate strain(s) for vaccine development, selective breeding for increased disease resistance, follow-up studies, epidemiological surveillance, disease control as well as for a better understanding of virulence and host resistance traits. However, conventional serotyping methods suffer from several severe disadvantages such as the choice of the appropriate scheme, the need to raise antisera from animals and the bias of human interpretation (Gorski, 2021; Sloan et al., 2017). Therefore, molecular tools such as microarray or multiplex PCR (mPCR) serotyping assays have been developed for a number of bacteria (Howell et al., 2015; Ludwig et al., 2020; Nakaue et al., 2021), including fish pathogens (Rochat et al., 2017; Torres-Corral & Santos, 2020).

Conventional serotyping is based on the immunogenicity of various surface-exposed bacterial structures (e.g. lipopolysaccharide [LPS], capsular polysaccharide, flagella). LPS is one of the most pro-inflammatory compounds of Gram-negative bacteria, representing 75% of the total bacterial surface. Essential for the structure and function of the external membrane, LPS is considered as a determining factor during host–bacteria interactions (Tan & Kagan, 2014). This amphipathic glycoconjugate is composed in three different domains: the lipid A anchored in the outer membrane, the core oligosaccharide which connects the lipid A to the O-antigen and the O-antigen itself which is the hydrophilic and immunodominant portion, facing the external environment. The latter is composed of repeated carbohydrate units and its chemical composition and structure exhibit very high levels of variation, even within a single species (Mostowy & Holt, 2018). Genes coding for the biosynthesis of the O-antigen are often located in a single genomic cluster (the O-AGC), and fall into three classes: (i) the nucleotide sugar biosynthesis genes, (ii) the sugar transferase genes and (iii) those required for O-units translocation, chain synthesis and chain length determination (see Bohl & Aihara [2018] and Whitfield et al. [2020] for recent reviews). The O-antigen of *T. maritimum* is composed of a disaccharide repeated unit that has been resolved for a unique strain; nothing is known about the mechanisms and genes involved (Vinogradov et al., 2003).

In this study, we took advantage of a set of recently published *T. maritimum* genomes (Bridel et al., 2020; Pérez-Pascual et al., 2017) to identify the O-AGC in this species. We used conventional serotyping together with a genome-wide association study to develop an mPCR-based serotyping scheme (mPCR). Our study aims to provide an efficient, cost-effective and reproducible molecular tool able to discriminate distinct gene combinations. This mPCR serotyping scheme was successfully applied to a collection of 124 *T. maritimum* isolates.

2 | MATERIALS AND METHODS

2.1 | Genome comparison

The complete or draft genome sequences (whole-genome shotgun [WGS]) used in this study are listed in Table 1. Genome comparisons were performed using the MicroScope platform v3.15.3 (Vallenet et al., 2020), which allows graphic visualization enhanced by a synchronized representation of synteny groups. Analysis of gene organization was conducted using syntons (i.e. maximal set of orthologous gene pairs displaying a conserved organization) constructed with MicroScope default parameters (i.e. orthologous gene sets were computed using BLAST-P Bidirectional Best Hit or displaying at least 30% identity on 80% of the shortest sequence and a genomic co-localization with

[1] https://www.hipra.com/portal/fr/hipra/animalhealth/products/detail-global/icthiovac-tm

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TABLE 1 *Tenacibaculum maritimum* isolates used in this study

Strain	Serotype	Type	Geographic origin	Host	Year	Genome[c]
NCIMB 2154^T	O1, O2, O1/O2[a]	**1-0**	Japan	*Pagrus major*	1977	GCF_900119795.1
NBRC 15946[b]	O1	1-0	Japan	ND	ND	GCF_000509405.1
P2-27	O1	1-0	Spain	*Scophthalmus maximus*	2011	GCF_902705465.1
Aq 16–85	O1	1-0	French Polynesia	*Platax orbicularis*	2016	GCF_902705305.1
FC	O1	1-0	Chile	*Scophthalmus maximus*	1998	GCF_902705415.1
190605A03b	ND	1-0	French Polynesia	*Platax orbicularis*	2019	pubmlst.org (id=151)
Tasmania_1513	ND	1-0	Australia	*Latris lineata*	2003	pubmlst.org (id=131)
FS08(1)	O1	**1-1**	Italy	*Sparus aurata*	2006	GCF_902705395.1
NAC SLCC MFF	O1	1-1	Malta	*Dicentrarchus labrax*	ND	GCF_902705345.1
P2-48	O1	1-1	France	*Solea senegalensis*	2010	GCF_902705555.1
CVI1001048	O1	**2-0**	The Netherlands	*Solea solea*	2010	GCF_902705265.1
NCIMB 2158	O2	**2-1**	Scotland	*Solea solea*	1981	GCF_902705425.1
UCD SB2	O2	2-1	USA	*Atractoscion nobilis*	1995	GCF_902705445.1
JIP10/97	O2	2-1	France	*Scophthalmus maximus*	1997	GCF_902705285.1
JIP46/00	O2	2-1	France	*Scophthalmus maximus*	2000	GCF_902705435.1
P1-39	O2	2-1	France	*Dicentrarchus labrax*	2010	GCF_902705535.1
190115A05h	ND	2-1	French Polynesia	*Platax orbicularis*	2019	pubmlst.org (id=150)
Tasmania_0663	ND	2-1	Australia	*Salmo salar*	1996	pubmlst.org (id=117)
Tasmania_0809	ND	2-1	Australia	*Salmo salar*	2000	pubmlst.org (id=127)
Tasmania_0811	ND	2-1	Australia	*Salmo salar*	2000	pubmlst.org (id=129)
Tasmania_2854	ND	2-1	Australia	*Salmo salar*	2009	pubmlst.org (id=132)
TM-KORJJ	ND	2-1	South Korea	*Paralichthys olivaceus*	2016	GCF_004803875.1
190628A06a	ND	**3-0**	French Polynesia	*Platax orbicularis*	2019	pubmlst.org (id=153)
Baxa GBF-8601	ND	3-0	Japan	*Paralichthys olivaceus*	1986	pubmlst.org (id=46)
USC RPM522.1	ND	3-0	Spain	*Scophthalmus maximus*	1992	pubmlst.org (id=61)
TFA4	O3	**3-1**	French Polynesia	*Platax orbicularis*	2013	GCF_902705565.1
902	O3	3-1	France	*Dicentrarchus labrax*	2013	GCF_902705365.1
JIP32/91-4	O3	3-1	France	*Dicentrarchus labrax*	1991	GCF_902705385.1
P4-45	O3	3-1	France	*Dicentrarchus labrax*	2010	GCF_902705495.1
190709D08d	ND	3-1	French Polynesia	*Platax orbicularis*	2019	pubmlst.org (id=152)
Tasmania_3064	ND	3-1	Australia	*Salmo salar*	2013	pubmlst.org (id=120)
Tasmania_4635	ND	3-1	Australia	*Salmo salar*	2018	pubmlst.org (id=137)
DPIF 89/0239-1	O3	**3-2**	Australia	*Salmo salar*	1989	GCF_902705355.1
DPIF 89/3001-6.2	O3	3-2	Australia	*Latris lineata*	1989	GCF_902705315.1
Tasmania_0759	ND	3-2	Australia	*Salmo salar*	1998	pubmlst.org (id=126)
USC SE30.1	O4	**4-0**	Spain	*Oncorhynchus kisutch*	1993	GCF_902705525.1
USC SP9.1	O4	4-0	Spain	*Salmo salar*	1993	GCF_902705515.1
Tasmania_0814	ND	4-0	Australia	*Oncorhynchus mykiss*	1991	pubmlst.org (id=130)
Tasmania_4574	ND	4-0	Australia	*Salmo salar*	2017	pubmlst.org (id=135)
Tasmania_4579	ND	4-0	Australia	*Salmo salar*	2017	pubmlst.org (id=136)

Note: The representative strains are indicated in bold.

Abbreviation: ND, not determined.

[a]Different serotypes have been reported for the type strain NCIMB 2154T.

[b]Strain NBRC 15946 is not the type strain according to Pérez-Pascual et al. (2017).

[c]NCBI genome accession number or genome available in PubMLST (https://pubmlst.org/bigsdb?db=pubmlst_tenacibaculum_isolates&page=query). Isolate id is given in parentheses.

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allowed gap set to five genes). In order to analyse the gene content of the *T. maritimum* type strain's O-AGC, similarity searches were performed with each encoding proteins: (i) using BLAST-P against the Swiss-Prot database (release 2022_01) and (ii) using HHpred with default parameters in the PDB_mmCIF70 database (release 12 October 2021). HHpred is a sensitive protein homology detection, function and structure prediction based on HMM–HMM comparison (Zimmermann et al., 2018).

For phylogenetic reconstruction, comparison of the gene content between strains was done by pairwise proteome similarity search using BLAST-P Bidirectional Best Hit and the MicroScope default parameters (i.e. >80% protein identity, >80% coverage). A set of 2034 groups of core genome proteins was retained and multiple alignments on individual groups were performed using MUSCLE using an inhouse R script. The resulting alignments were concatenated using an inhouse script and tree phylogenetic reconstruction was inferred by approximately-maximum-likelihood using Fastree v2.1.10 with default parameters (Price et al., 2010). The tree was visualized using FigTree v1.1.4 and rooted using midpoint.

2.2 | Bacterial isolates

The *T. maritimum* isolates used in this study are listed in Table 1 for those with complete or draft genome sequences and in Table S2 for those typed using mPCR. Additional information including country, year, host, MLST type (Habib et al., 2014) or MALDI-TOF profile (Bridel et al., 2020) was included when available. Bacterial isolates were grown on Marine Agar 2216 (Difco) at 27°C for 48 h. All isolates were confirmed as belonging to the species *T. maritimum* by molecular techniques (PCR and/or MALDI-TOF MS) and/or biochemistry depending on the way of identification.

2.3 | Conventional serotyping

The serological characterization of the *T. maritimum* isolates was performed using both slide agglutination and dot blot assay with unabsorbed and absorbed antisera as described previously (Avendaño-Herrera et al., 2005; Castro et al., 2007). Rabbit antisera raised against representative strains of the four serotypes described for this pathogen were used. Antisera raised against the sole (*Solea senegalensis*) isolates PC503.1 (O1) and ACC13.1(O3), the turbot (*S. maximus*) isolate PC424.1(O2) and the salmon (*S. salar*) isolate SNW20.2 (O4) were used. Only a reaction similar to that exhibited by the homologous control strain was scored as positive.

2.4 | multiplex PCR

Primers (Table 2) were designed using the Benchling software[2] according to the complete and draft genome sequences available and conservation of their sequences was verified. For mPCR optimization, reactions were performed using bacterial gDNA extracted using the genomic DNA purification kit (Macherey Nagel) according to the manufacturer's instructions. For this purpose, the type strain NCIMB 2154^T and different representative strains (i.e. strains FS080(1), CVI100104801, NCIMB 2158, 190628A06a, TFA4 and DPIF 89/0239-1) were used. mPCR reactions contained 0.25 μl DreamTaq DNA polymerase (Thermo Fisher Scientific), 5 μl of 10× DreamTaq buffer (Thermo fisher scientific), 5 μl of $MgCl_2$ (at 25 mM), 1 μl dNTPs at 10 mM, 1.5 μl of each primer at 10 μM and 1 μl (about 100 ng) of gDNA template in a 50 μl final reaction volume according to the manufacturer's recommendations. The mPCR amplification mix was heated at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 90 s and a final extension at 72°C for 5 min. The amplified products were electrophoresed in 2.0% agarose gels run in 1× TBE buffer with the GeneRuler™ DNA ladder mix (Thermo Fisher Scientific) as a molecular size marker. For typing the large collection of isolates, cells from a bacterial colony (or a few depending on the size) were resuspended in 20 μl sterile water, vortexed and heated at 100°C for at least 5 min for full lysis, then briefly centrifuged to pellet the bacterial debris; 1 μl of the supernatant was used as DNA template for the mPCR reaction.

3 | RESULTS

3.1 | Conventional serotyping assay

Conventional serotyping was performed using both slide agglutination and dot blot assay on 22 *T. maritimum* strains, whose genomes were previously sequenced (Bridel et al., 2020). These strains were retrieved from various geographic locations and host fish species. They display a significant diversity as they encompass the four serotypes previously described (Table 1). Indeed, strains were originally selected to maximize the genomic diversity and therefore expected to display such a serotypic heterogeneity. Each strain reacted strongly with a unique serum, except strain NCIMB 2154^T that was successively reported to belong to the intermediate minor serotype O1/O2 by Avendaño-Herrera et al. (2004), to serotype O2 by Avendaño-Herrera et al. (2005) and to serotype O1 by Fernández-Álvarez et al. (2018). This is likely the consequence of typical drawbacks arising from conventional serotyping protocols. Nevertheless, the resulting data were used as a cornerstone for comparing serotypes with strains' genomic organization.

3.2 | Identification of the O-antigen biosynthesis gene cluster of the T. maritimum type strain

Polysaccharide antigens, including the O-antigen, are usually synthetized by a specialized group of enzymes which are encoded by genes located in an O-AGC (Mostowy & Holt, 2018). Comparative genomics, including analysis of gene synteny, was performed between

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TABLE 2 Oligonucleotides used in this study

Name	Sequence (5′ → 3′)	Amplicon size in bp	Target gene (locus tag) in representative strains
P1F	TAGCCAGTATCTATTATTTGTTTACTG	196 (Types 1 and 2)	MARIT_2530
P1R	AGTTAGCGTGCCATTTATCA		
P2/3F	TTTAGAATCTAGAGATATAAGGATTCC	357 (Type 2) or 717 (Type 3)	CVI1001048_20215 or TM190628A06A_50037
P2/3R	ATCAATTTTTGAAACATAATCTTCAT		
P4F	GAGGCATTTCACATTCTGTAT	985 (Type 4)	USCSE301_230029
P4R	CCATGAGTCAATAATGTTGAGA		
P5F	AACCTGAATATTGGTATTGGAG	872 (Types 1-1, 3-1 and 3-2)	FS0810_80069
P5R	AATAGACATCAGAATCACTATAAGAA		
P6F	GAGAGAAAATTACGCCTAAAAC	476 (Types 2-1 and 3-2)	NCIMB2158_120032
P6R	CAAAAATCTCCCCAACTTTAAC		

T. maritimum (strain NCIMB 2154^T) and *Flavobacterium psychrophilum* (strain JIP02/86), a phylogenetically related species whose O-AGC has been well characterized (Cisar et al., 2019; Rochat et al., 2017). Comparisons with *Tenacibaculum dicentrarchi* (strain 35/09^T), whose genome is available and O-antigen biosynthetic genes were recently described (Saldarriaga-Córdoba et al., 2021), were also performed. This analysis allowed to identify a gene cluster very likely involved in the O-antigen biosynthesis. This locus contains eight and 10 genes out of 16 that are conserved between *T. maritimum* NCIMB 2154^T O-AGC and *T. dicentrarchi* USC 35/09^T (32%–78% protein identity) and *F. psychrophilum* JIP02/86 (26%–71% protein identity) O-AGCs, respectively (Figure S1). Most of these orthologous genes are predicted to be involved in the nucleotide sugar precursors biosynthesis. *Tenacibaculum maritimum* NCIMB 2154^T O-AGC is located between the core genome genes *folC* and *MARIT_2521* (encoding a conserved exported protein), the latter being immediately adjacent to *gyrB*. This locus is about 20 kb long and displays a 30.80% GC, slightly under the median guanine-cytosine (GC) content (32.01%) of the complete genome. All genes of the cluster are transcribed in the same direction, some likely polycistronic.

3.3 | Gene content of the O-antigen biosynthesis gene cluster of the *T. maritimum* type strain's genome

Starting immediately downstream *folC* and two adjacent Val-tRNA genes (and orientated 5′ to 3′), *MARIT_2537*, *MARIT_2536* and *MARIT_2535* display significant sequence similarities (35%–60% protein identity; >95% coverage) with genes *wbpA*, *wbpB* and *udg* of *Pseudomonas aeruginosa*, respectively (Figure S1 and additional details in Table S1). Their products are predicted to be involved in the biosynthesis of a nucleotide sugar precursor, probably an uridine diphosphate (UDP)-glucose derivative. *MARIT_2534* encodes a predicted periplasmic protein with poor and partial sequence similarities (below 25% identity) with proteins involved in exopolysaccharide export such as KpsD of *Escherichia coli*, Wza of *Salmonella typhimurium* and AmsH of *Erwinia amylovora*. *MARIT_2533* encodes a predicted transmembrane protein with poor similarity (18.9% protein identity) with WzzE, the enterobacterial common antigen polysaccharide chain length modulation protein of *E. coli* (Rai & Mitchell, 2020). *MARIT_2532* encodes a protein similar (56.5% identity, 98% coverage) to PseB of *Campylobacter jejuni* that possess both C6 dehydratase and C5 epimerase activities on UDP-*N*-acetyl-α-D-glucosamine (Schoenhofen et al., 2006). *MARIT_2531*, *MARIT_2530* and *MARIT_2529* encode multi-pass transmembrane proteins with very poor similarities with proteins having any experimental evidence. *MARIT_2528* and *MARIT_2527* encode proteins with poor similarities with predicted epimerases/dehydratases and acetyltransferases, respectively, but possibly involved in nucleotide sugar modifications. The *MARIT_2526* gene product is highly similar (59.5% protein identity; 98% coverage) to PglC of *C. jejuni* and therefore likely encodes the undecaprenyl phosphate transferase that mediates the first step in the biosynthesis of the undecaprenyl-linked nucleotide sugar, thus creating the first membrane-associated intermediate for the O-antigen unit synthesis (Glover et al., 2006). The *MARIT_2525* gene product displays significant similarities (>50% identity) with different pyridoxal phosphate (PLP)-dependent aminotransferases including EpsN of *Bacillus subtilis* and PglE of *C. jejuni* that modify amino sugars using L-glutamate as an amino donor (Schoenhofen et al., 2006). The lysine, located at the amino acid position 200 in *MARIT_2525* and required for covalent bond formation between the ε-amino group of lysine and the aldehyde group of PLP in PLP-containing proteins (Kaundinya et al., 2018), is well conserved. Finally, the *MARIT_2524*, *MARIT_2523* and *MARIT_2522* genes encode proteins highly similar to RmlA, RmlB and RmlC that are predicted to be involved in the biosynthesis of dTDP-L-rhamnose, a nucleotide sugar precursor of the O-antigen. One might conclude that most if not all genes encompassed in this locus encode for proteins whose function are related to nucleotide sugar biosynthesis, transfer, chain synthesis and export and thus are very likely involved in O-antigen biosynthesis of *T. maritimum*.

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3.4 | Comparison of the genomic organization of the O-antigen biosynthesis gene cluster between *T. maritimum* strains

Extensive comparative genomic analysis of the O-AGCs between *T. maritimum* strains revealed a globally well-conserved structural backbone, located in the same chromosomal region between the core genome genes *folC* and *gyrB*. The loci encompass some genes (i.e. *wbpA*, *wbpB*, *udg*, *epsN*, *rmlA*, *rmlB* and *rmlC*) that encode proteins involved in nucleotide sugar metabolism and globally well-conserved between strains. However, O-AGCs display significant and striking differences among *T. maritimum* strains (Figure 1). By grouping the strains harbouring identical or nearly identical O-AGC gene organization, different O-AGCs, hereafter designated 'Types', were identified.

Genomic organization of O-AGCs Type 1-0 and Type 1-1 displays obvious similarities with little variation between them. They differ from the other Types not only because of their intrinsic gene content composition but by harbouring a group of eight genes located elsewhere in their genome (locus 2 in Figure 1) and likely also involved in polysaccharide biosynthesis. This second locus contains genes encoding nucleotide sugar epimerase/dehydratase, aminotransferase, oxidoreductase and acetyltransferase, among others. Most of these genes (five out of eight) are encompassed in the bona fide, unique O-AGC identified in Types 2-0 to 3-2. Types 2-0 and Type 2-1 display an overall conserved genomic structure with little variation among them, which is also the case for Types 3-0, 3-1 and 3-2. Finally, a unique Type 4-0 distantly related to the previously mentioned Types was identified. Therefore, the O-AGCs of *T. maritimum* strains (not taking into account the locus 2 of O1 strains) vary from 19.8 to 27.5 kb in length and contain from 16 genes for strains belonging to Type 1-0 up to 25 genes for strains belonging to Type 3-1.

3.5 | Comparative genomics of O-AGC loci to identify key molecular determinant of *T. maritimum* serotypes

A very strong correlation was observed between the conventional serotypes and the structure of the locus. Indeed, with the exception of a single strain (CVI1001048), all O1 strains belong to Type 1 (Type 1-0 or 1-1), all O2 strains belong to Type 2 (Type 2-0 or 2-1), all O3 strains belong to Type 3 (Type 3-0, 3-1 or 3-2) and all O4 strains belong to Type 4-0 (Table 1). Even though strain CVI1001048 was serotyped O1 using conventional serotyping, the structure of its O-AGC (named Type 2-0) is closely related to the structure of Type 2-1 O-AGCs. In addition, and contrary to Type 1 strains, strain CVI1001048 and all other Type 2 strains do not possess the second locus elsewhere in the genome (Figure 1). The type strain NCIMB 2154^T, which was typed O1 or O2 and even O1/O2 by conventional methods, typically belongs to the Type 1 group and accordingly contains the second locus.

3.6 | Development of an mPCR serotyping scheme

Based on these observations and on the grouping of strains according to the Type they belong to, we noticed that the presence of distinct genes coding transmembrane proteins was the best marker able to discriminate the Types and displaying obvious correlations with the results of conventional serotyping. Indeed, all strains belonging to Type 1 and Type 2 have a gene (*MARIT_2530* in strain NCIMB 2154^T and orthologs) that is absent in strains belonging to Type 3 and Type 4. This gene encodes a protein with predicted 11 transmembrane segments but with very low BLAST-P similarities with proteins having any known function. However, using HHpred to detect remote homologies, eight out of the 10 best hits (probability ranging from 94.84% to 99.94% that the database match is a true positive) belong to O-antigen ligases and O-antigen polymerases family proteins. One might conclude that *MARIT_2530* and its orthologs likely encode a main actor of the O-antigen biosynthesis with oligosaccharyltransferase activity (Whitfield et al., 2020). This gene was chosen as the first target (using the oligonucleotide pair P1F–P1R) for the development of an mPCR able to discriminate Type 1 and Type 2 from the other Types. In order to distinguish between Type 1 and Type 2 strains, we identified a gene (*NCIMB2158_120038* in strain NCIMB 2158 and its orthologs) present in Type 2 strains and absent in Type 1 strains. This gene encodes an eight-segment transmembrane protein with no significant homologies (even using HHpred) and was chosen as the second target. We also took advantage of a gene (*TFA4_10218* in strain TFA4 and its orthologs) only present in Type 3 strains; this gene displays significant similarities to *NCIMB2158_120038* though containing a 360-bp in-frame insertion. Therefore, this second oligonucleotide pair (P2/3F - P2/3R), encompassing this insertion, not only distinguishes Type 1 from Type 2 strains but also discriminates between Type 2 and Type 3 strains, by amplifying a 357- or 717-bp fragment, respectively. The divergent Type 4 O-AGC also encompass a gene (*USCSE301_v1_230029* in strain USC SE 301.1) encoding a predicted 12-segment transmembrane protein without any BLAST-P similarities with proteins having any known function but with significant structural homologies (>99% probability HHpred) with O-antigen ligases and O-antigen polymerases. A third oligonucleotide pair (P4F–P4R) targeting *USCSE301_v1_230029* and its orthologs was designed to distinguish Type 4 strains (see Figure 1 for genes targeted for mPCR).

Because the different O-AGCs also contain genes likely involved in nucleotide-sugar metabolism and unevenly distributed among the strains, we selected two genes (*FS0810_80069* in strain FS08(1) and its orthologs encoding an acyl-CoA acyltransferase family protein and *NCIMB2158_120032* in strain NCIMB 2158 and its orthologs encoding an aminotransferase family protein) to be included as targets (using the oligonucleotide pairs P5F–P5R and P6F–P6R, respectively) to increase the sensitivity of the proposed mPCR serotyping scheme. Therefore, the final scheme uses six primer pairs (Table 2) and is able to distinguish up to eight different O-AGCs: the four Types (Types 1–4) that correspond to the classical serotypes delineated using conventional serum-based methods) and additional variations (i.e. Types 1-0 and

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FIGURE 1 Genomic organization of the *T. maritimum* O-AGCs. The loci were analysed using the Microscope platform. Eight distinct 'Types' were defined by grouping strains harbouring identical or nearly identical O-AGC gene organization. Gene conservation was predicted using both homology and synteny criteria (see Section 2 for details). A second locus (locus 2) is present only in Type 1-0 and Type 1-1 strains. Genes conserved between the second locus and those encompassed in the bona fide O-AGC are shown in dotted line. Oligonucleotides used in the mPCR are indicated by black arrows. The locus_tags for each representative strain are indicated for genes at both extremities of the O-AGCs and for those targeted by the mPCR.

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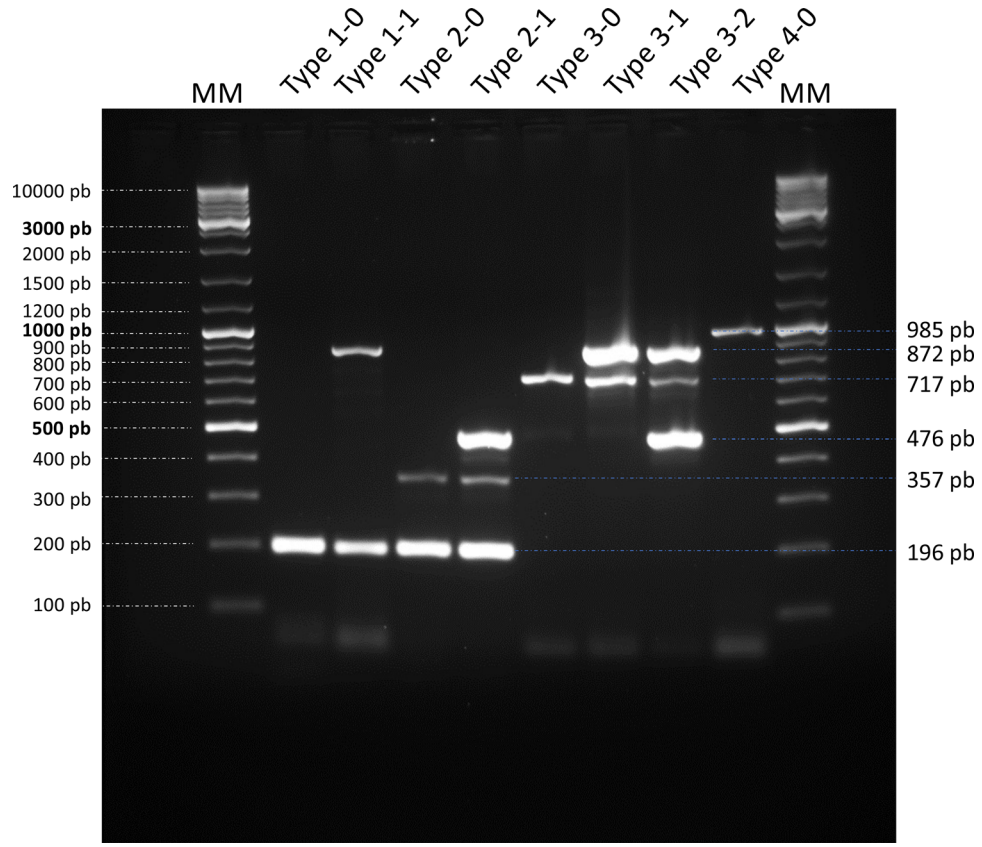


FIGURE 2 Multiplex PCR. Migration of the PCR products after amplification of a representative strain of each O-AGC Type. MM, molecular marker; Type 1-0, representative strain NCIMB 2154^T; Type 1-1, representative strain FS080(1); Type 2-0, representative strain CVI100104801; Type 2-1, representative strain NCIMB 2158; Type 3-0, representative strain 190628A06a; Type 3-1, representative strain TFA4; Type 3-2, representative strain DPIF 89/0239-1; and Type 4-0, representative strain USC SE 30.1.

1-1, Types 2-0 and 2-1 and Types 3-0, 3-1 and 3-2). Migration of the PCR products after mPCR amplification can virtually provide six bands ranging from 196 to 985 bp. Their presence/absence and combinations allow the unambiguous assignation of any isolate to its Type (Figure 2). Using the strains whose genomes have been sequenced and available in our laboratory, a 100% correlation was observed between the in silico-predicted Type and the banding pattern obtained by mPCR.

3.7 | Screening a collection of *T. maritimum* isolates using mPCR

In addition to the 40 strains mentioned in Table 1, we used the proposed mPCR serotyping scheme to screen a collection of 124 *T. maritimum* isolates retrieved from a wide variety of geographic areas and host fish species during the last 50 years. Most of these isolates have been previously typed by various methods including MLST (Habib et al., 2014) and MALDI–TOF MS (Bridel et al., 2020). All amplifications were successful and the results are show in Table S2. Strikingly, and taking into account the whole dataset (i.e. the 40 WGS-types strains and the 124 mPCR-types isolates), the isolates are unevenly distributed among the Types: isolates belonging to Types 1-0, 2-1 and 3-1 are widely predominant, whereas isolates belonging to Types 1-1, 2-0,

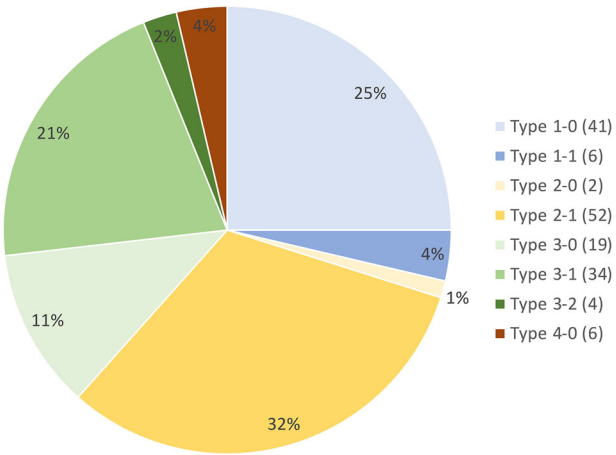


FIGURE 3 Distribution of the isolates according to their Types. The number of isolates is given in parenthesis.

3-2 and 4-0 are scarce (Figure 3). In addition, our results revealed no clear association between mPCR Type, host fish species or geographic origin of the isolates. When many representatives retrieved from a single fish species were available, some tendencies can be noticed (Figure S2). For example, among the 57 isolates retrieved from sea

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bass, 25 and 23 belong to Types 2-1 and 3-1, respectively. Among the 45 isolates retrieved from batfish, about half belong to Type 1-0. On the other hand, isolates originating from Tasmania, most of which isolated from Atlantic salmon, belong to Types 2-1, 3-0, 3-1, 3-2 or 4-0, while isolates retrieved from batfish in French Polynesia belong to Types 1-0, 2-1, 3-0 or 3-1. It is also interesting to note that a major serological diversity is present in the isolates from sea bass (six different types were detected), while sea bream isolates only belong to Types 1-0 and 1-1. On the other hand, no isolates retrieved from Atlantic salmon belong to Types 1-0 and 1-1. Although a limited number of isolates from turbot is available, representatives of four diverse serotypes (Types 1-0, 2-1, 3-0 and 4-0) were detected. Of importance, no clear correlation was observed between the mPCR Types and the MALDI groups, but tendencies could be observed between the mPCR Types and the MLST types, as most isolates belonging to a specific ST display the same mPCR Type. However, the distribution of Types does not follow the phylogeny based on core genome proteins (Figure 4). For instance, strains Tasmania_0759 and Tasmania_0809, both retrieved from Atlantic salmon and tightly clustered in the phylogenetic tree, belong to Type 3-2 and 2-1, respectively. The same lack of correlation between strain position in the phylogenetic tree and the Type they belong to can be observed in many branches of the tree.

4 | DISCUSSION

Serological techniques are important for diagnosis and epidemiological studies as well as for antigenic characterization to select strains for vaccine development and efficiency follow-up. *Tenacibaculum maritimum* was initially described as a very homogeneous group of bacteria (Wakabayashi et al., 1984), but different serological studies have revealed the existence of several serotypes, thus revealing some antigenic diversity (Avendaño-Herrera et al., 2004; Avendaño-Herrera et al., 2005; Fernández-Álvarez et al., 2018; Piñeiro-Vidal et al., 2007). In addition, the antigenic differences are mainly due to differences in the 'O' chains of the LPS (Avendaño-Herrera et al., 2004). Moreover, it has been suggested that a vaccine produced against one *T. maritimum* serotype may not be effective against another (Romalde et al., 2005). Indeed, the unique vaccine commercially available so far exclusively targets the O2 serotype affecting turbot[1]. However, conventional protocols for the serotyping of *T. maritimum* isolates suffer several drawbacks. For instance, different methods have been proposed: microagglutination, slide agglutination, immunodiffusion, indirect immunofluorescence, enzyme-linked immunosorbent assay and dot blot assay (see Fernández-Álvarez & Santos [2018] for a recent and exhaustive review). The choice of reference strains and the procedure used to extract antigens (from whole cells to purified O-antigen) in order to raise antisera greatly varied among studies. Furthermore, the use of unabsorbed or cross-absorbed sera (and the choice of the strain(s) used to perform reciprocal absorption) has also important consequences. For example, the type strain NCIMB 2154^T was typed O1 or O2 and even O1/O2 by conventional methods. Therefore, these discrepancies probably originated either from limits of the conventional serotyping method or, less plausibly, from subtle variations within the coding sequences or altered regulation of some key genes. In addition, conventional serotyping protocols require to raise antisera from animals (sometimes with variable immune responses) and some isolates display strong, weak or lack of reactivity, or even cross-reactivity with multiple antisera. Hence, because traditional serotyping is costly, labour-intensive and requires significant technical expertise, molecular serotyping relying of the presence/absence of relevant genes is a promising alternative. In full expansion for the molecular serotyping of human pathogens (Howell et al., 2015; Ludwig et al., 2020; Nakaue et al., 2021), such schemes were recently developed for animal and especially fish pathogenic bacteria (Rochat et al., 2017; Torres-Corral & Santos, 2020). The strategy is based on the genomic identification of molecular markers that determine the serotype. In this study, we first identified a locus in the *T. maritimum* type strain genome with similarities with the O-AGC of *F. psychrophilum* and *T. dicentrarchi* (Figure S1). We noticed that the metabolic pathway of dTDP-L-rhamnose biosynthesis also requires the product of *rmlD*, which is not part of the O-AGC and is located elsewhere in the *T. maritimum* genome (*MARIT_0966*). This organization was also observed in the *F. psychrophilum* genome in which *rmlC* and *rmlD* genes are not located in the O-AGC. As the *rmlABC* genes are located immediately downstream to *MARIT_2525* and *MARIT_2526*, it is tempting to speculate that an amino-modified dTDP-L-rhamnose is the first nucleotide sugar to be loaded on the undecaprenyl carrier (C55-P) on the cytoplasmic face of the inner membrane. It was also noteworthy that most of the genes encompassed in the *T. maritimum* O-AGC encode for proteins that display similarities with proteins from phylogenetically distantly related bacteria (e.g. *P. aeruginosa*, *C. jejuni* and *B. subtilis*) (Table S1). Therefore, and as previously reported in diverse bacterial species (Holt et al., 2020; Wang et al., 2010), horizontal gene transfer and homologous recombination likely play instrumental roles in the evolution of *T. maritimum* O-AGCs.

We took advantage of the availability of the whole-genome sequences of 40 *T. maritimum* strains to perform genomic comparisons of the O-AGCs. By grouping the strains harbouring identical or nearly identical O-AGC gene organization, we identified different 'Types', with obvious links with the serotypes obtained using conventional procedures. This genome-based association study allowed us to develop a robust mPCR serotyping scheme able to discriminate up to eight different allelic combinations. This scheme is able to detect subgroups within each serotype and therefore performs better than conventional serotyping. We successfully applied our typing scheme to an additional collection of 124 isolates. In the pioneering works using conventional serotyping methods, some correlations were observed between serotype and host fish species: O1 isolates were retrieved from sole, O2 isolates from sea bream and sea bass and O3 isolates from turbot (Avendaño-Herrera et al., 2003). However, further studies revealed no strict association between serotype and host fish species. For instance, isolates retrieved from sea bass belong to serotype O1, O2 or O3 (Avendaño-Herrera et al.,2005; Yardimci & Timur, 2016), isolates retrieved from turbot belong to serotype O1, O2 or O3 (Avendaño-Herrera et al., 2003; Avendaño-Herrera et al., 2004; Castro

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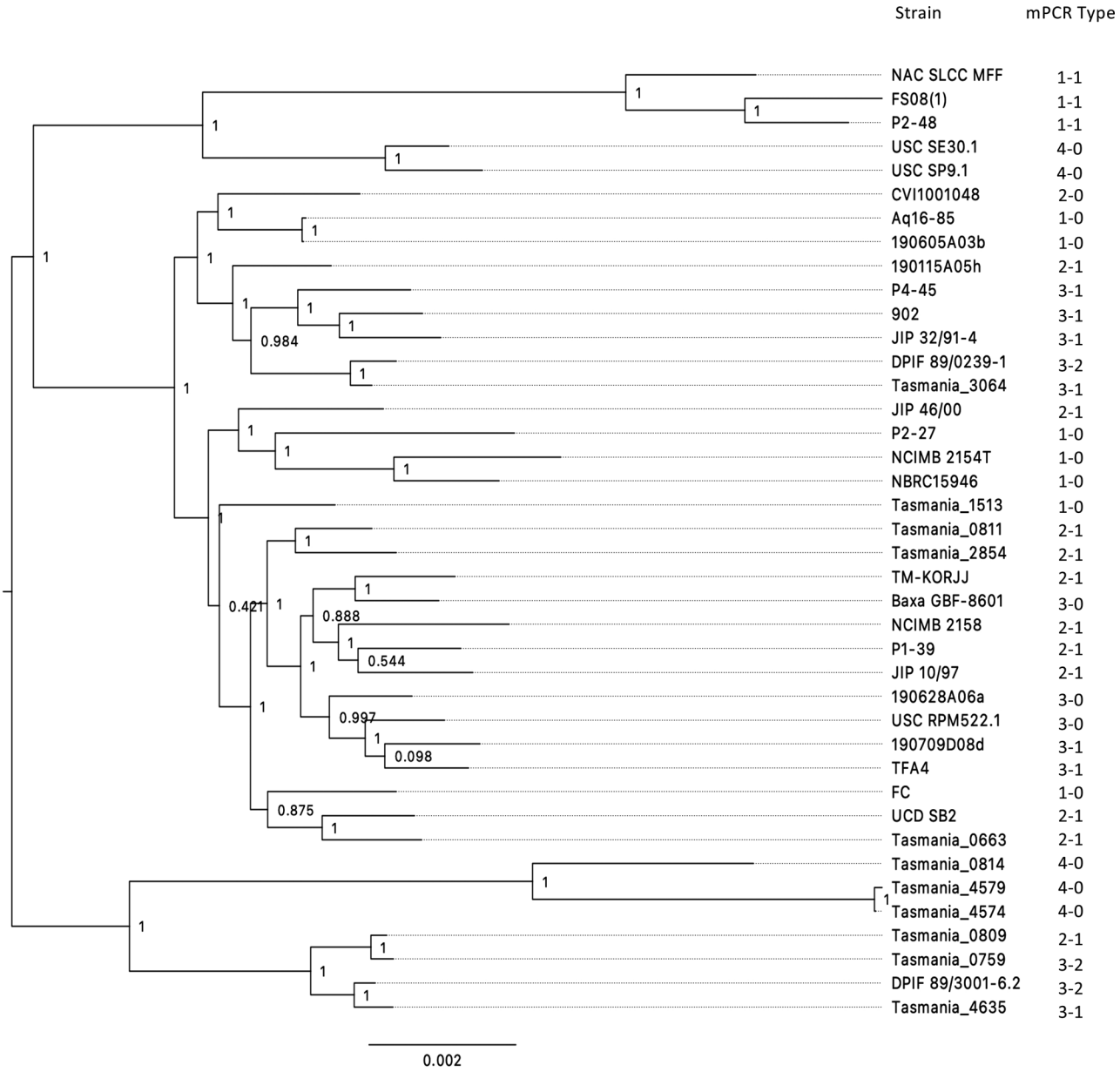


FIGURE 4 O-AGC organization (Types) and evolutionary relationships between the *T. maritimum* strains. Types are shown on a tree attempting to capture the phylogenetic relationships between the 40 *T. maritimum* strains used in this study for which complete genomes were available. The tree was constructed with 2034 core genome proteins (see Section 2 for details). Local support values are indicated at nodes. The bar at the bottom provides the scale of branch lengths and the unit corresponds to the number of amino acid change per sites.

et al., 2007; Fernández-Álvarez et al., 2018; Piñeiro-Vidal et al., 2007) and sole isolates belong to serotype O1, O3 or O4 (Avendaño-Herrera et al., 2003; Avendaño-Herrera et al., 2005; Fernández-Álvarez et al., 2018). In addition, Fernández-Álvarez et al. (2018) stated that serotype O1 is the dominant one regardless of the source of the isolates (i.e. host fish species or geographic location). The same conclusions were drawn when isolate genotypes (determined using either MLST, MALDI–TOF or complete genome-based typing approaches) were compared to the background information on the isolation source (host fish, year and geographic origin) that could account for only a limited amount of the total genetic variance. Indeed, only trends were observed between the geographic origin of the isolates and the genotypes (Bridel et al., 2020; Habib et al., 2014). Therefore, the lack of any obvious correlation between the mPCR Type and the host fish species was not surprising and only trends were noticed. Yet, it is difficult to state if these trends are the consequences of sampling bias or if they arise from hidden functional links (e.g. bacterial fitness, virulence, colonization of a specific host). Anyhow, this situation is in sharp contrast with the conclusions obtained with *F. psychrophilum* where obvious links between the serotype and the host fish species were noticed (Li et al., 2021; Rochat et al., 2017). Strikingly, in *T. maritimum* the Types distribution does not follow the core genome-based phylogeny (Figure 4). This

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result is probably the consequence of allelic exchanges mediated by horizontal gene transfer in relation to the high recombination rate observed in this bacterium (Bridel et al., 2020; Habib et al., 2014).

In conclusion, our data revealed a significant diversity of the O-AGCs in *T. maritimum* and provided a valuable mPCR-based serotyping scheme for this important fish pathogen. This scheme should help to improve our knowledge of the serotype distribution at different scales in order to facilitate future epidemiological studies and to help the development of vaccines, including the selection of appropriate strains for autogenous vaccination. If necessary, the proposed mPCR scheme could be enriched with additional targets for a more accurate discrimination of isolates. The O-AGCs genomic structure described in this study may also serve as a milestone for future complete genome-based typing approaches (Bentley & Lo, 2021; Lee et al., 2021; Mostowy & Holt, 2018; Zhang et al., 2019).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Pierre Lopez performed the mPCR development and isolate typing and contributed to data analysis and to the writing of the manuscript. Sébastien Bridel performed genome comparisons and O-AGCs identification. Denis Saulnier contributed to the writing of the manuscript. Beatriz Magariños and Beatriz S. Torres performed the serotyping. Jean François Bernardet managed the *T. maritimum* bacterial strains collection and contributed to the writing of the manuscript. Eric Duchaud wrote the manuscript, contributed to data analysis and coordinated the work. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

New sequences used in this study have been deposited in the PubMLST database and are freely available in the PubMLST web site (https://pubmlst.org/bigsdb?db=pubmlst_tenacibaculum_isolates&page=query).

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