Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Physical characterisation of *Tenacibaculum maritimum* for vaccine development

R. van Gelderen[1], J. Carson[2], N. Gudkovs[3] and B. Nowak[1]

1 National Centre for Marine Conservation & Resource Sustainability, University of Tasmania, Tasmania, Australia
2 Fish Health Unit, Tasmanian Aquaculture and Fisheries Institute, Department of Primary Industries and Water, Tasmania, Australia
3 Australian Animal Health Laboratory, CSIRO Livestock Industries, Geelong Victoria, Australia

Keywords
extracellular products, hydrophobicity, Lipopolysaccharide profiles, *Tenacibaculum maritimum*, vaccine development, whole cell protein profiles.

Correspondence
Rebecca van Gelderen, National Centre for Marine Conservation & Resource Sustainability, University of Tasmania, Tasmania, Australia.
E-mail: rvangeld@utas.edu.au

2010/0598: received 12 April 2010, revised 31 May 2010 and accepted 3 June 2010

doi:10.1111/j.1365-2672.2010.04795.x

Abstract

Aims: *Tenacibaculum maritimum* is a well known fish pathogen worldwide, affecting many fish species including Atlantic salmon in Tasmania, Australia. The aim of this study was to characterise and understand the similarities and differences between the isolates in order to select isolates for later pathogenicity and vaccination trials.

Methods and Results: Several physical characterisation tests were carried out: whole cell protein profiles, lipopolysaccharide profiles (LPS), extracellular product profiles (ECP), indirect immunofluorescent antibody test (IFAT) and hydrophobicity. The Tasmanian strains of *T. maritimum* appear relatively homogeneous physically, but antigenically different. All isolates were hydrophobic and produce a variety of ECP profiles. There were two isolates that stand out in all assays (89/4747 and 01/0356-7) and showed great variation from the other isolates. All isolates have been confirmed as *T. maritimum.*

Conclusions: Based on the tests carried out three isolates were chosen for *in vivo* trials: 89/4747, 89/4762, 00/3280.

Significance and Impact of the Study: This is the first study to characterise *T. maritimum* isolates from Tasmanian waters. The opportunity to develop vaccines for the Tasmanian salmonid aquaculture industry is enhanced by a greater understanding of the physical characteristics of pathogens.

Introduction

Tenacibaculum maritimum (formerly *Flexibacter maritimus*) (Suzuki *et al.* 2001) has been identified as the causative agent of salmonid cutaneous erosion disease (SCED)/marine flexibacteriosis in Tasmania (Schmidtke *et al.* 1991). The main clinical sign is the presence of erosive lesions that form on external surfaces. These lesions are characterised by destruction of the epithelial cells and connective tissue, which leaves the musculature exposed. Bacteria isolated from the leading edges of the lesions are slender, Gram-negative rods. The genus *Tenacibaculum* belongs to the family Flavobacteriaceae, phylum Bacteriodetes and *T. maritimum* is an exclusively marine species (Bernardet *et al.*1996; Suzuki *et al.* 2001; Ludwig *et al.* 2008).

Wakabayashi *et al.* (1986) first described *T. maritimum* as a fish pathogen after isolating it from red sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schegeli*) in Japan. Other susceptible fish species include: flounder (*Paralichthys olivaceous*) (Baxa *et al.* 1986) and yellowtail (*Seriola quinqueradiata*) (Baxa *et al.* 1988a) in Japan; Dover sole (*Solea solea*) (Bernardet *et al.* 1990) in Scotland; sea bass (*Dicentrarchus labrax*) (Bernardet *et al.* 1994) in France and turbot (*Scophthalmus maximus*) in Spain (Alsina and Blanch 1993).

In Tasmania, Australia, *T. maritimum* has been isolated from Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss*) and striped trumpeter (*Latris lineata*) (Handlinger *et al.* 1997). The only severe outbreak of the disease occurred during the summer of 1988/89. Farms up to 30 km apart recorded simultaneous

outbreaks of marine flexibacteriosis within marine cages. Higher than average water temperatures (21°C), sunny cloudless days and poor feeding management were identified as contributing factors to the outbreak (Handlinger *et al.* 1997). Control of the disease has been managed by changing feeding practices, fish handling and the use of antibiotics (Carson *et al.* 1992).

However, there is limited information on the biological factors that could initiate further outbreaks of the disease. Patterns of infection are variable and effective management strategies would be difficult to implement (Carson *et al.* 1994). Antibiotics such as trimethoprim and amoxicillin have been utilised in the treatment of marine flexibacteriosis on Tasmanian salmonid farms (Cameron 1991, 1993a,b), but are undesirable for prolonged use with issues of bacterial resistance and tissue residence times. Therefore, development of a safe and effective vaccine against *T. maritimum* in salmonids for the Tasmanian salmonid aquaculture industry requires as a first step a comparison of local isolates of *T. maritimum* to identify candidates for pathogenicity assessment and subsequent vaccination experiments.

Materials and methods

Bacterial strains

Eighteen *T. maritimum* isolates were cultured from skin lesions on Atlantic salmon and rainbow trout in Tasmania from 1989 to 2001 (Table 1). Isolates were grown on marine Shieh's agar, (MSA) (g l^{-1}: peptone L37 (Oxoid, Adelaide, Australia), 5 g; yeast extract (Oxoid), 0·5 g; sodium pyruvate (Oxoid), 0·1 g; sodium acetate (Oxoid), 0·01 g; citric acid (Oxoid), 0·01 g; distilled water, 100 ml; aged seawater, 900 ml; pH, 7·5–7·8), an adaptation of the medium of Shieh (Song *et al.* 1988). The isolates were identified by phenotype (Schmidtke *et al.* 1991) and confirmed as *T. maritimum* by PCR using primers for the 16S rRNA gene (Carson and Wilson 2003). Two additional strains were used for comparison: the type strain of *T. maritimum* (NCIMB 2154^T) and a reference strain Baxa lyl-1 (Baxa *et al.* 1988a). Stock cultures were stored frozen in peptone water with 8% glycerol (Ward and Watt 1971) at −80°C in single use cryovials. Bacteria were maintained in culture on MSA at 25°C or in marine Shieh's broth (MSB) at room temperature (20°C).

Indirect immunofluorescent antibody test (IFAT)

All isolates were tested by IFAT as this technique would be used later in other trials as a confirmatory test for *T. maritimum*. The procedure described by Carson *et al.* (1992) was used. Smears were produced using bacteria from plate culture that were then air-dried and heat fixed. The smears were overlaid with 40 μl of polyclonal rabbit antiserum to strain 89/0329-5 of *T. maritimum* diluted 1 : 100 in PBS (pH 7·2, 0·1 mol l^{-1}) and incubated in a moist chamber for 30 min at 37°C before rinsing in PBS for 15 min. After the removal of excess buffer by blotting, 20 μl of anti-rabbit FITC (Silenus, Melbourne) diluted 1 : 60 in PBS was added to each slide. Smears were incubated at 37°C for 30 min and rinsed for 30 min in PBS, which was changed every 10 min. Slides were coverslip mounted using alkaline glycerol buffer (Johnson and Munday 1993) and examined at ×40 magnification with epifluorescent microscopy using UV illumination. A marine *Flavobacterium* sp. (99/1972-4b), isolated from Atlantic salmon, the primary antibody only and the anti-rabbit FITC only were used as negative controls. All isolates were tested in triplicate.

Whole cell protein and lipopolysaccharide profiles (LPS)

Whole cell and LPS samples were prepared using a 48 h broth culture. After harvesting, whole cell suspensions were washed twice by centrifugation at 2900 ***g*** for 30 min and re-suspended in PBS (pH 7·2). The suspensions were only washed twice because auto-agglutination occurred in most isolates in subsequent washes or re-suspension. Each suspension was standardised to an absorbance of 1·0 at 550 nm in a spectrophotometer. Cells were collected from aliquots of 1·5 ml of suspension by centrifugation at 6500 ***g*** for 3 min and the supernatant removed. Whole cell lysates were produced by resuspending the cell pellet in 500 μl of SDS solubilisation buffer (Laemmli 1970) and heating for 10 min at 100°C. Cell debris from extracts were centrifuged at 6500 ***g*** for 3 min; the supernatant was removed and stored at −20°C.

Lipopolysaccharide analysis was based on the method of Hitchcock and Brown (1983) with minor modifications. Cells were harvested at 16 500 ***g*** for 5 mins and samples were washed once in PBSA (0·14 mol l^{-1} NaCl, 0·01 mol l^{-1} Na_2HPO_4, pH 7·4, 1 mg of bovine serum albumin per ml) and adjusted to 1×10^9 cell ml^{-1}. The cell pellet was re-suspended in 100 μl of 2% SDS and mixed thoroughly. Glass beads (700–1200 μmol l^{-1} diameter, Sigma-Aldrich, Sydney) were added to break up the bacterial pellet as *T. maritimum* tended to form well bound cell aggregates. To assist solubilisation of the pellet, in addition to the glass beads, samples were heated to 60°C.

Proteinase K (Boehringer-Mannheim, Sydney) at a concentration of 20 mg ml^{-1} was added to each sample to give a final concentration of 5 mg ml^{-1}. Samples were then incubated in a Thermomixer (Eppendorf, Sydney) for an hour at 60°C. The temperature was increased to 99°C for 30 min, after which the samples were stored at

Table 1 *Tenacibaculum maritimum* isolates used in this study

Accession no.	Source	Host	Collection location	Year	Location
00/0400-3	DPIW	Atlantic salmon	Tasmania	2000	Pillings Bay
00/0422-3	DPIW	Atlantic salmon	Tasmania	2000	Brabazon Point
00/0813	DPIW	Atlantic salmon	Tasmania	2000	Nubeena
00/1793-1	DPIW	Atlantic salmon	Tasmania	2000	Port Arthur
00/3280	DPIW	Rainbow trout	Tasmania	2000	Rowella
01/0356-7	DPIW	Atlantic salmon	Tasmania	2001	Tinderbox
89/0329-1	DPIW	Atlantic salmon	Tasmania	1989	Dover
89/0329-5	DPIW	Atlantic salmon	Tasmania	1989	Dover
89/1579-2G	DPIW	Atlantic salmon	Tasmania	1989	Recherche Bay
89/4747	DPIW	Atlantic salmon	Tasmania	1989	Nubeena
89/4762	DPIW	Atlantic salmon	Tasmania	1989	Penguin
89/4913-6	DPIW	Atlantic salmon	Tasmania	1989	Tinderbox
90/1123	DPIW	Atlantic salmon	Tasmania	1990	Port Arthur
90/1445	DPIW	Atlantic salmon	Tasmania	1990	Port Arthur
91/0126	DPIW	Atlantic salmon	Tasmania	1991	Nubeena
91/0247	DPIW	Atlantic salmon	Tasmania	1991	Nubeena
96/0457-4	DPIW	Atlantic salmon	Tasmania	1996	Tinderbox
98/3186	DPIW	Atlantic salmon	Tasmania	1998	Satellite Island
Baxa lyl-1	DPIW	Japanese flounder	Japan	1988	
NCIMB 2154^T	DPIW	Sea bream	Japan	1988	

DPIW = Department of Primary Industries and Water.

−20°C. Prior to loading, samples were thawed and mixed with 134 μl of 2 × SDS solubilisation buffer. Each sample was heated in a water bath at 100°C for 10 mins and then centrifuged at 16 000 ***g*** for 5 mins. Controls for LPS analysis were *Escherichia coli* ATCC 25922 (positive) and *Lactococcus garvieae* TCFB 0713 (negative).

ECPs

Extracellular products (ECPs) were produced by the cellophane overlay method (Liu 1957). For each isolate, three plates of MSA were incubated for 72 h at 25°C as this yielded the greatest quantity of ECP. This also allowed minimal growth-phase effects as different proteases are produced at different times of the growth curve (Gudmundsdóttir 1996). After incubation, the culture was washed off the cellophane with 1 ml of PBS (pH 7·2) and centrifuged at 4000 ***g*** for 20 min. The supernatant fluid (ECP) was filter sterilised (0·22 μmol l^{-1} pore size) and stored in aliquots at −80°C. Protein content was determined using the bicinchoninic acid (BCA) method (Pierce Protein Research Products, Thermo Scientific, Melbourne, Victoria, Australia). Duplicate cultures of each strain of bacteria to produce ECP were used to confirm reproducibility of the results.

Analysis of proteins and LPS

ECPs and whole cell proteins were separated by SDS-PAGE (Laemmli 1970) with a 5% stacking gel and 12% resolving gel. Samples were heated to 100°C for 10 mins prior to loading (10 μl). Electrophoresis was carried out for 1 h at 40 mA and 200 V. Gels were stained with 0·125% Coomassie Blue R-250.

LPS was run on a NuPAGE™ (Invitrogen, Melbourne, Australia) 12% Bis-Tris Gel for 1 h at 200 V. Gels were silver stained according to the method of Tsai and Frasch (1982) but as this did not reveal banding, therefore Western blotting was used to detect LPS bands (Fig. 1).

Western blots were run using the method of Towbin *et al.* (1979) with a single modification. As NuPAGE™ Bis-Tris gels were used, 10% methanol was added in the transfer buffer instead of 20% methanol. LPS was transferred onto nitrocellulose membranes using an Invitrogen Novex (Melbourne) XCell II Blot Module (semi-dry transfer) for an hour at 30 V. After transfer, the nitrocellulose membranes were rinsed in PBSA and then blocked for 30 mins with 3% skim milk powder in Tris-saline (Ab SM). Membranes were washed with Tris-saline and incubated for 30 mins with antisera against *T. maritimum* 89/0329-5 in rabbit (dilution, 1 : 100) in Ab SM. After being washed twice in Tris-saline–0·05% Tween 20, and once in Tris-saline, the membranes were incubated for 1 h with anti-rabbit horseradish peroxidase (HRPO) (Bio-Rad, Sydney, Australia) diluted 1 : 1000 in Ab SM. Membranes were washed as previously described above. Bands were visualized by incubating the membranes in 30% w/v H_2O_2 in Tris-saline and a 4-chloro-1-napthol tablet according to the manufacturer's directions (Sigma-Aldrich, Sydney, Australia) solubilised in 100% methanol combined

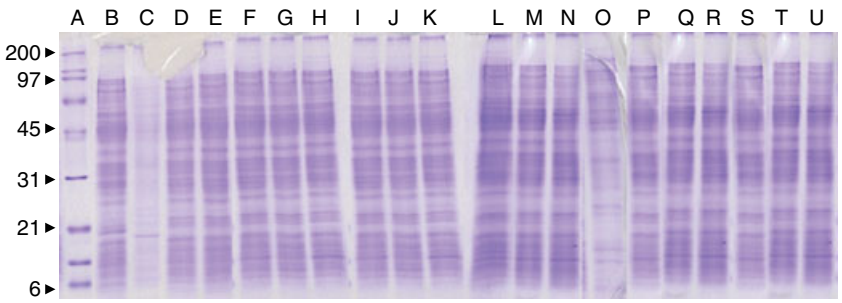

Figure 1 SDS-PAGE whole cell protein profile of *Tenacibaculum maritimum* isolates stained with Coomassie Blue-R. Lanes: A, Broad-range molecular weight markers (Bio-Rad) (kDa); B, 89/4762; C, 89/4747; D, 00/0400-3; E, 00/0422-3; F, 00/0813; G, 00/1793-1; H, 89/0329-1; I, 89/0329-5; J, 89/1579-2G; K, 89/4913-6; L, 90/1123; M, 90/1445; N, 91/0126; O, 91/0247; P, 01/0356-7; Q, 96/0457-4; R, 98/3186; S, Baxa lyl-1; T, 00/3280; U, NCIMB 2154^T. Numbers on the left indicate molecular sizes (in kDa).

together immediately prior to use. The reaction was stopped by the addition of water.

Hydrophobicity

The hydrophobicity assay was modified from the method described by Rosenberg *et al.* (1980). Twenty-four hour MSB cultures of *T. maritimum* were centrifuged for 30 min at 2000 ***g***. Cell pellets were resuspended and washed twice in PBS (pH 7·2) and diluted to an initial OD (OD_I) of 0·6 with a spectrophotometer ($\lambda = 600$ nm). In a glass test tube, 1·2 ml of each bacterial suspension was added along with 0·2 ml of the hydrocarbons *n*-hexadecane (Merck Chemicals, Melbourne, Australia), *n*-octane (Sigma-Aldrich) or *p*-xylene (Merck Chemicals). Each bacterial suspension-hydrocarbon test was prepared and assayed in triplicate. The suspension was agitated vigorously for 2 min, and then allowed to separate for 20 min into the hydrocarbon and aqueous phases.

From each tube, 1 ml of the aqueous phase was transferred to a cuvette and the final OD (OD_F) of each suspension measured ($\lambda = 600$ nm). The hydrophobicity of each bacterial suspension to each hydrocarbon was expressed as the percentage partitioning into the hydrocarbon phase:

$$\text{Partitioning into hydrocarbon}(\%) = [(OD_I - OD_F)/OD_I] \times 100$$

For each hydrocarbon of each isolate the percentage partitioning was ranked. Each rank was added together and then divided by three (i.e. three hydrocarbons). This gave a final rank that could be sorted in order of greatest to lowest overall hydrophobicity.

Results

Out of the eighteen local isolates, 16 produced the same whole cell protein profile as the type strain NCIMB 2154^T and reference strain Baxa lyl-1 (Fig. 2). These isolates showed banding patterns from 118·4 kDa down to 9·2 kDa. Major single bands are located around 12, 15·5, 25, 45·6, and 99·5 kDa with a triplet at 20 kDa. The two local isolates that varied (89/4747 and 01/0356-7) showed similarities to the other isolates, however there was a distinct band at 17·6 kDa for 89/4747 and bands at 135, 22 and 12·7 kDa for 01/0356-7 which indicate they are different from each other (Fig. 2).

Eight different ECP groupings were detected from all 20 isolates (Table 2). Bands that all isolates have in common occurred at 46·5 and 73 kDa. Isolates 01/0356-7 and 89/4747 showed the greatest variation, making up individual groups. Strains used for comparison, NCIMB 2154^T and Baxa lyl-1, showed identical profiles to each other, which was again different from the local isolates

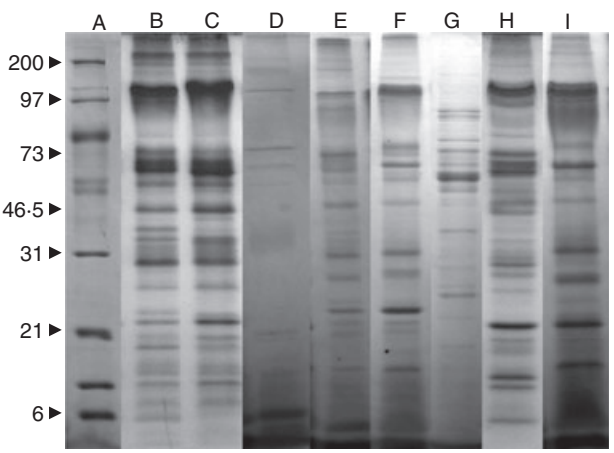

Figure 2 SDS-PAGE of extracellular product profiles of *Tenacibaculum maritimum* isolates stained with Coomassie Blue R. Greyscale analysis of gels was used to establish differences. Lanes: A, Broad-range molecular weight markers (Bio-Rad) (kDa); B, Group 1; C, Group 2; D, Group 3; E, Group 4; F, Group 5; G, Group 6; H, Group 7; I, Group 8. Numbers on the left indicate molecular sizes (in kDa).

Table 2 Extracellular product profiles groupings of *Tenacibaculum maritimum* isolates based on SDS-PAGE analysis

Groups	Isolates accession numbers
1	89/1579-2G, 89/0329-5, 89/4762, 96/0457-4
2	89/4913-6, 90/1445, 90/1123, 91/0126, 91/0247
3	89/4747
4	89/0329-1, 00/0813
5	00/0422-3, 00/3280
6	01/0356-7
7	00/0400-3, 00/1793-1
8	Baxa lyl-1, NCIMB 2154^T

(Fig. 3). There was no correlation of isolates between year groups or geographical locations within Tasmania.

LPS profiles indicated that there are at least two serotypes amongst the isolates as 89/4747 and 01/0356-7 did not show any banding using the Western blotting technique (Fig. 4). However, in this study, it was not determined if these isolates (89/4747 and 01/0356-7) had a shared serotype. Laddering appeared uniform across most other isolates; the major exceptions were 89/0329-5, Baxa lyl-1, 89/4762 and 91/0126 which had a distinct band at 55 kDa and isolates 00/1793, 00/0400-3 and 98/3186 showed a different LPS profile. Based on differences in the profiles, isolates were grouped together (Table 3).

All isolates of *T. maritimum* were hydrophobic (Fig. 5). In all cases, isolates showed the lowest hydrophobicity towards *n*-hexadecane and the highest to *p*-xylene, with moderate hydrophobicity to *n*-octane. Two exceptions were isolates 89/4762 and Baxa lyl-1 that possessed strong hydrophobic tendencies with all three hydrocarbons. Overall, all isolates were hydrophobic and as surface area of the alkane molecule decreased, hydrophobicity increased. After final rankings, isolate 89/4762 was found to be the most hydrophobic. Isolates 89/4747 and 01/0356-7 were IFAT negative; all other 18 isolates were IFAT positive, including Baxa lyl-1 and NCIMB 2154^T.

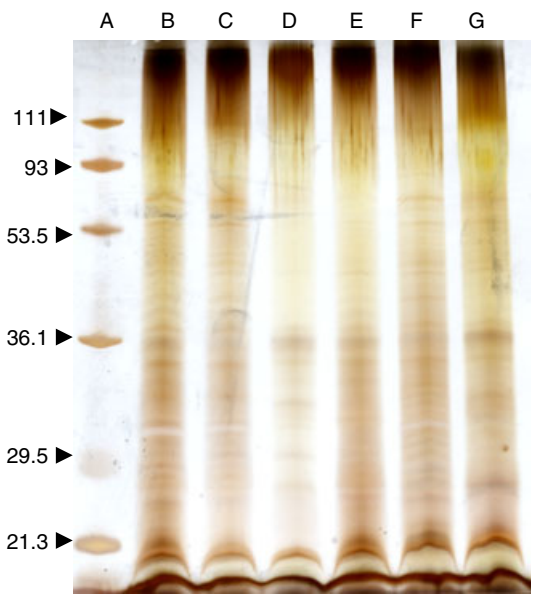

Figure 3 SDS-PAGE of silver stained lipopolysaccharide profiles of *Tenacibaculum maritimum* isolates. Lanes: A, Low-range molecular size markers (Bio-Rad) (kDa); B, 89/0329-1; C, 89/0329-5; D, 89/1579-2G; E, 89/4913-6; F, 98/3186; G, 96/0457-4;. Numbers on the left indicate molecular sizes (in kDa).

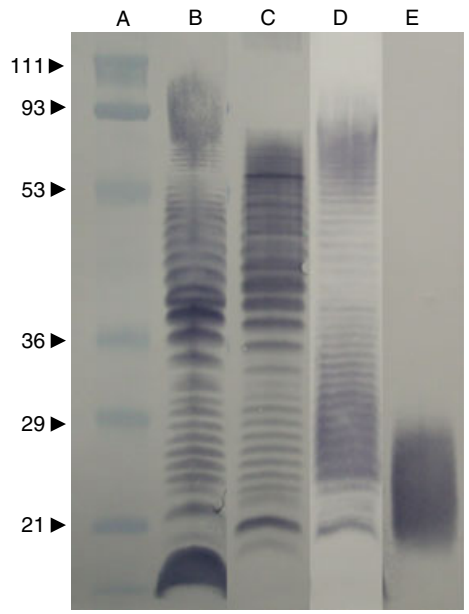

Figure 4 Nu-Page Western blot of lipopolysaccharide profiles of *Tenacibaculum maritimum* isolates. Lanes: A, Low-range molecular weight markers (Bio-Rad) (kDa); B, Group 1; C, Group 2; D, Group 3; E, Group 4. Numbers on the left indicate molecular sizes (in kDa).

Table 3 Lipopolysaccharide profiles groupings of *Tenacibaculum maritimum* isolates based on Western blot analysis

Groups	Isolates accession numbers
1	89/0329-1, 89/1579-2G, 89/4913-6, 96/0457-4, NCIMB 2154^T, 00/3280, 91/0247, 00/0813, 00/0422-3, 90/1445, 90/1123
2	98/3186, 00/1793, 00/0400-3
3	89/0329-5, Baxa lyl-1, 89/4762, 91/0126
4	89/4747, 01/0356-7

Discussion

The aims of this study was to determine if all local strains were homologous in respect of an arbitrary reference

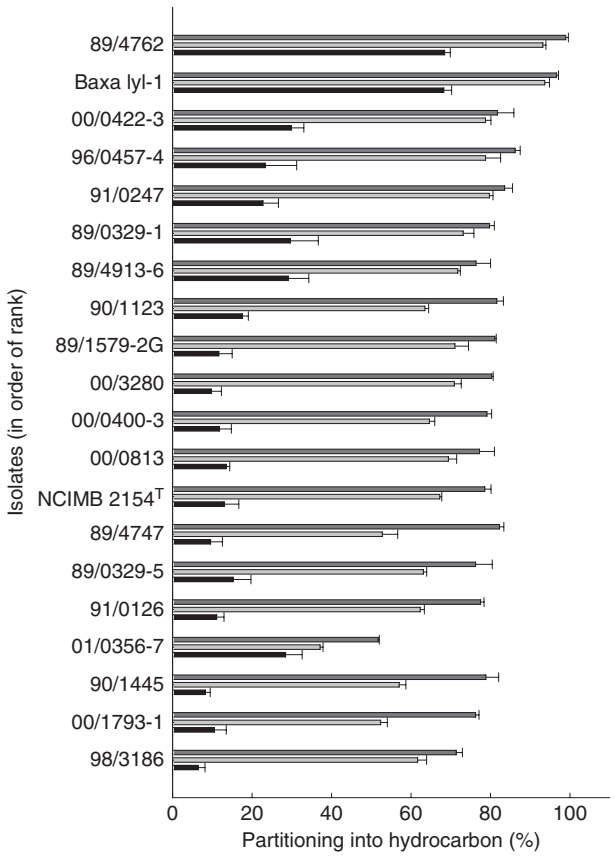

Figure 5 Graph showing the partitioning of each *Tenacibaculum maritimum* isolate into the three hydrocarbons (±SE). (■) *p*-xylene; (□) *n*-octane and (■) *n*-hexadecane.

strain and the type strain of *T. maritimum*, and homologous in respect to shared antigens. Establishing homogeneity is important so that strain variation can be assessed and taken into account for subsequent pathogenicity trials and for nominating a representative strain that can be used for a prototype vaccine in Atlantic salmon.

Based on the cell envelope, including whole cell analysis, *T. maritimum* has been described by other authors as a homogeneous species (Bernardet *et al.* 1994; Avendaño-Herrera *et al.* 2004). Initial observations of the local *T. maritimum* isolates using whole cell protein profiles indicate a relatively homogeneous group. These findings are similar to that of Pazos (1997), who reported *T. maritimum* isolates with a large level of similarity between isolates for proteins with molecular mass in the range of 14·4–97·4 kDa. While minor banding is evident above 100 kDa with the Tasmanian isolates, major bands could be seen from 12 to 99·5 kDa. For the purpose of this study, assessment of the protein profiles was intended to be qualitative only, and although the gel loading volume was constant (10 μl), protein concentration of preparations was not standardised. No weight can be applied to relative differences in band intensity between the different isolates. With the exception of two isolates (89/4747 and 01/0356-7), all other local isolates showed the same profile to that of the type strain NCIMB 2154^T. Similarly, by IFAT, all isolates were positive using the polyclonal antibody to isolate 89/0329-5 except for isolates 89/4747 and 01/0356-7 suggesting these two isolates represent some other distinct serogroup or groups.

LPS analysis revealed further antigenic heterogeneity within the group of isolates, with three distinct patterns evident compared to the reference strain NCIMB 2154^T. Initial attempts at silver staining LPS were successful only with the positive control *E. coli* ATCC 25922. While this method produced clear laddering for *E. coli* isolates, it proved unsatisfactory for *T. maritimum* as the banding appeared to be masked possibly by protein (Fig. 1). Although cell preparations had been digested with proteinase K, it is known that some proteins are resistant to digestion with proteinase K (Sørensen *et al.* 1992) which might account for the failure to disclose the LPS moieties using the method of Hitchcock and Brown (1983). The underlying LPS profiles however could clearly be shown by Western blotting using polyclonal antiserum to 89/0329-5.

Although initially described as antigenically homogeneous (Wakabayashi *et al.* 1986), *T. maritimum* is has been reported to be antigenically heterogeneous (Ostland *et al.* 1999; Avendaño-Herrera *et al.* 2004). Antigenic differences have been demonstrated among *T. maritimum* isolates from Atlantic salmon using immunoblot analysis of LPS (Ostland *et al.* 1999) where differences were detected among isolates and between the isolates and reference strains (NCIMB 2153, 2154^T). These differences have been attributed to the O-antigen side chains, which may be associated with different strains from outbreaks on both temporal and geographical aspects (Ostland *et al.* 1999). The current study reported here supports evidence of antigenic variation, though it appears that most of the strains form a homogeneous group with little LPS variation, despite isolates being collected over a 12 year period from two different hosts: Atlantic salmon and rainbow trout.

While most of the isolates seemed to be homogeneous antigenically, as assessed by IFAT, strains 89/4747 and 01/0356-7 appeared different. The identity of both strains had been confirmed previously by PCR using 16S rRNA gene primers for *T. maritimum* (Carson and Wilson 2003) and had a phenotype consistent with *T. maritimum* (Schmidtke *et al.* 1991), yet there were differences in the whole cell and LPS profiles as well as serotyping by IFAT. We concluded that these two isolates may represent a different serotype or serotypes but requires further analysis.

Previous work by Avendaño-Herrera *et al.* (2004) reported distinct serotypes for *T. maritimum* in turbot (*Scophthalmus maximus*), sole (*S. solea*) and gilthead sea bream (*Sparus aurata*). They also found different serotypes according to the source of the isolation. For example, strains isolated from turbot reacted only with the antiserum derived from turbot isolates. Similar findings were noted for sole and gilthead sea bream strains with the antiserum obtained against the sole isolate (Avendaño-Herrera *et al.* 2004). The relationship between serotype and host evident from this study was not found with the salmonid strains. The antiserum to 89/0329-5 isolated from Atlantic salmon reacted with isolate 00/3280 from rainbow trout, the Baxa lyl-1 strain from Japanese flounder and NCIMB 2154^T from red sea bream.

The primary purpose of evaluating serotype in this study was to establish the extent of serological diversity that might exist among isolates from Atlantic salmon for the purpose of selecting a strain that might serve for developing a vaccine for use in Tasmania. Establishing the serological relationship to strains of *T. maritimum* from other hosts and determining their serotype once such a schema is developed will provide context to serotype observations which have been generated thus far.

ECPs are compounds secreted by bacteria and thought to facilitate invasion of the host (Dalsgaard 1993). Although toxic activities of *T. maritimum* have been investigated previously (Baxa *et al.* 1988b), this is the first account of ECP protein profiles for *T. maritimum*. The ECP findings suggest significant heterogeneity within the species.

Heterogeneity of ECPs is present in other marine pathogenic bacterial species including *Aeromonas salmonicida* (Gudmundsdóttir 1996). Out of 32 strains analysed, six different protease groups were detected. All typical strains belonged to a single group, while atypical strains showed significant differences from the typical strains and between each other. It was suggested that ECP type was more strongly associated with geographic location than the host fish species. Our results indicate that host may not be an important factor since isolate 00/3280 from rainbow trout grouped with 00/0422-3 from Atlantic salmon. Both isolates however are from salmonids which may account for the similarity in ECP profile. The type strain NCIMB 2154^T and Baxa lyl-1 are also from different host species, yet are grouped together having identical protein profiles.

Attachment of bacterial pathogens to different substrates is considered an important step in colonisation of host cells as this has the potential to initiate disease (Vatsos *et al.* 2001). A number of techniques are employed to assess non-specific and specific attachment to different substrates e.g., salt aggregation test (SAT), phase partitioning with hydrocarbon solvents, and adherence to nitrocellulose filters (NCF). While more than one assay system is normally used to obtain a robust measurement of hydrophobicity (Santos *et al.* 1990), a single assay was used here because the focus of this study was to assess similarities and differences between a range of isolates. Also, implementing other assays was found to be problematic because of the profound auto-agglutination of *T. maritimum*. Of the methods available, phase partitioning with hydrocarbons was found to be more accommodating than other techniques for assessing surface hydrophobicity of *T. maritimum*. As expected, all isolates were hydrophobic, consistent with other *T. maritimum* hydrophobicity studies (Sorongon *et al.* 1991; Magariños *et al.* 1996; Kawahara and Kusuda 1998).

Using the phenotypic markers described, the isolates were grouped and ranked in order to make selections for later pathogenicity and vaccination trials (van Gelderen *et al.* 2009, in press). The three isolates chosen for the pathogenicity trials were: 89/4747, 89/4762, 00/3280. Isolate 89/4747 was chosen due to its differences with other isolates for the characterisation tests and in particular because it may represent a distinct serotype. Isolates 00/3280 and 89/4762 showed similarities to each other and most other isolates in whole cell protein profile and serotype. However, in the ECP groupings, 89/4762 occurred within a common group, whereas 00/3280 was grouped with only one other isolate with a distinct electropherotype. This contrasts with the LPS groupings, where 00/3280 was similar to most other isolates while conversely 89/4762 was different and placed into a minor group. Isolate 89/4762 was also the most hydrophobic of all the isolates and was included to determine if this characteristic was a marker of virulence.

The data collected from this study enabled the researchers to make educated decisions about the choice of isolate tested based on similarities and differences between them. It provided more information and sound reasoning for choices, rather than random selections. Isolate 89/4762 was used in the final vaccination study (van Gelderen *et al.* 2009).

References

Alsina, M. and Blanch, A.R. (1993) First isolation of *Flexibacter maritimus* from cultivated turbot (*Scophthalmus maximus*). *Bull Eur Ass Fish Pathol* **13**, 157–160.

Avendaño-Herrera, R., Magariños, B., Lopez-Romalde, S., Romalde, J.L. and Toranzo, A.E. (2004) Phenotypic characterization and description of two major O-serotypes in *Tenacibaculum maritimum* strains from marine fishes. *Dis Aquat Organ* **58**, 1–8.

Baxa, D.V., Kawai, K. and Kusuda, R. (1986) Characteristics of gliding bacteria isolated from diseased cultured flounder, *Paralichthys olivaceus*. *Fish Pathol* **21**, 251–258.

Baxa, D.V., Kawai, K. and Kusuda, R. (1988a) Detection of *Flexibacter maritimus* by fluorescent antibody technique in experimentally infected Black Sea bream fry. *Fish Pathol* **23**, 29–32.

Baxa, D.V., Kawai, K. and Kusuda, R. (1988b) *In vitro* and *in vivo* activities of *Flexibacter maritimus* toxins. *Rep USA Mar Biol Inst Kochi Univ* **10**, 1–8.

Bernardet, J.F., Campbell, A.C. and Buswell, J.A. (1990) *Flexibacter maritimus* is the agent of ''black patch necrosis'' in Dover sole in Scotland. *Dis Aquat Organ* **8**, 233–237.

Bernardet, J.F., Kerouault, B. and Michel, C. (1994) Comparative study on *Flexibacter maritimus* strains isolated from farmed sea bass (*Dicentrarchus labrax*) in France. *Fish Pathol* **29**, 105–111.

Bernardet, J.-F., Segers, P., Vancanney, M., Berthe, F., Kersters, K. and Vandamme, P. (1996) Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.

Cameron, D.E. (1991) Tissue levels of trimethoprim in Tasmanian Atlantic salmon in relation to dose and duration of administration. In *Proceedings of the Saltas Research Review Seminar* ed. Valentine, P. pp. 117–126. Hobart, Tasmania: Saltas.

Cameron, D.E. (1993a) The optimisation of trimethoprim dose rate for Atlantic salmon with regard to therapeutic tissue levels and withholding period. In *Seeking and Solving: Papers from the Saltas Research and Development Review Seminar* ed. Valentine, P. pp. 121–127. Hobart, Tasmania: Saltas.

Cameron, D.E. (1993b) Evaluation of amoxicillin for the therapy of salmonid cutaneous erosion disease. In *Seeking and Solving: Papers from the Saltas 1993 Research and Development Review Seminar* ed. Valentine, P. pp. 129–133. Hobart, Tasmania: Saltas.

Carson, J. and Wilson, T. (2003) Development of sensitive, high-throughput one-tube RT-PCR-enzyme hybridisation assay to detect selected bacterial fish pathogens. *Dis Aquat Org* **54**, 127–134.

Carson, J., McCosh, P. and Schmidtke, L. (1992) Pathogenicity of *Flexibacter maritimus* in rainbow trout. In *Barriers and Breakthroughs. Proceedings of the SALTAS Research Review Seminar* ed. Valentine, P. pp. 89–99. Hobart, Tasmania: Saltas.

Carson, J., Schmidtke, L. and Lewis, T. (1994) Development of a vaccine against disease: results of efficacy testing of three types of vaccine. In *Proceedings of the SALTAS Research Review Seminar* ed. Valentine, P. pp. 149–158. Hobart, Tasmania: Saltas.

Dalsgaard, I. (1993) Virulence mechanisms in *Cytophaga psychrophila* and other *Cytophaga*-like bacteria pathogenic for fish. *Annu Rev Fish Dis* **3**, 127–144.

van Gelderen, R., Carson, J. and Nowak, B. (2009) Experimental vaccination of Atlantic salmon (*Salmo salar* L.) against marine flexibacteriosis. *Aquaculture* **288**, 7–13.

van Gelderen, R., Carson, J. and Nowak, B. (in press) Experimentally induced marine flexibacteriosis in Atlantic salmon smolts (*Salmo salar* L.). I. Pathogenicity. *Dis Aquat Organ*.

Gudmundsdóttir, B.K. (1996) Comparison of extracellular proteases produced by *Aeromonas salmonicida* strains isolated from various fish species. *J Appl Bacteriol* **80**, 105–113.

Handlinger, J., Soltani, M. and Percival, S. (1997) The pathology of *Flexibacter maritimus* in aquaculture species in Tasmania, Australia. *J Fish Dis* **20**, 159–168.

Hitchcock, P.J. and Brown, T.M. (1983) Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* **154**, 269–277.

Johnson, A.M. and Munday, B.L. (1993) Toxoplasmosis: pathology, histopathology and serology. In *Australian Standard Diagnostic Techniques for Animal Diseases* ed. Corner, L.A. and Bagust, T.J. Melbourne: CSIRO.

Kawahara, E. and Kusuda, R. (1998) The cell surface hydrophobicity of *Flexibacter maritimus*. *Rep Res Inst Mar Biores Fukuyama Univ* **9**, 1–6.

Laemmli, U.K. (1970) Clevage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Liu, P.V. (1957) Survey of hemolysin production among species of *Pseudomonas*. *J Bacteriol* **74**, 718–727.

Ludwig, W., Euzéby, J. and Whitman, W.B. (2008) Draft taxonomic outline of the *Bacteroidetes*, *Planctomycetes*, *Chlamydiae*, *Spirochaetes*, *Fibrobacteres*, *Fusobacteria*, *Acidobacteria*,*Verrucomicrobia*, *Dictyoglomi*, and *Gemmatimonadetes*. Bergey's Manual Trust, Bergey's Taxonomic Outlines Vol. 4 [online]. Available from: http://www.bergeys.org/outlines/Bergeys_Vol_4_Outline.pdf

Magariños, B., Bonet, R., Romalde, J.L., Martinez, M.J., Congregado, F. and Toranzo, A.E. (1996) Influence of the capsular layer on the virulence of *Pasteurella piscicida* for fish. *Microb Pathog* **21**, 289–297.

Ostland, V.E., LaTrace, C., Morrison, D. and Ferguson, H.W. (1999) *Flexibacter maritimus* associated with a bacterial stomatitis in Atlantic salmon smolts reared in net-pens in British Columbia. *J Aquat Anim Health* **11**, 35–44.

Pazos, F. (1997) *Flexibacter maritimus*: estudio fenotipico, inmunologico y molecular. Tesis doctoral, Universidad Santiago de Compostela, Santiago.

Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett* **9**, 29–33.

Santos, Y., Bandin, I., Nieto, T.P., Bruno, D.W., Ellis, A.E. and Toranzo, A.E. (1990) Comparison of the cell surface hydrophobicity of bacterial fish pathogens by different procedures. In *Pathology in Marine Science* ed. Perkins,

F.O. and Cheng, T.C. pp. 101–115 San Diego: Academic Press.

Schmidtke, L., Carson, J. and Howard, T. (1991) Marine *Flexibacter* infection in Atlantic salmon - characterization of the putative pathogen. In *Proceedings of the SALTAS Research Review Seminar* ed. Valentine, P. pp. 25–39. Hobart, Tasmania: Saltas.

Song, Y.L., Fryer, J.L. and Rohovec, J.S. (1988) Comparison of six media for the cultivation of *Flexibacter columnaris*. *Fish Pathol* **23**, 91–94.

Sørensen, J., Skouv, J., Jørgensen, A. and Nybroe, O. (1992) Rapid identification of environmental isolates of *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida* by SDS-PAGE analysis of whole-cell protein patterns. *FEMS Microbiol Lett* **101**, 41–50.

Sorongon, M.L., Bloodgood, R.A. and Burchard, R.P. (1991) Hydrophobicity, adhesion, and surface-exposed proteins of gliding bacteria. *Appl Environ Microbiol* **57**, 3193–3199.

Suzuki, M., Nakagawa, Y., Harayama, S. and Yamamoto, S. (2001) Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: proposal for Tenacibaculum gen. nov. with *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amylolyticum* sp. nov. *Int J Syst Evol Microbiol* **51**, 1639–1652.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.

Tsai, C.M. and Frasch, C.E. (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**, 115–119.

Vatsos, I.N., Thompson, K.D. and Adams, A. (2001) Adhesion of the fish pathogen *Flavobacterium psychrophilum* to unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) and n-hexadecane. *Lett Appl Microbiol* **33**, 178–182.

Wakabayashi, H., Hikida, M. and Masurmura, K. (1986) *Flexibacter maritimus* sp. nov. a pathogen of marine fishes. *Int J Syst Bacteriol* **36**, 396–398.

Ward, M.E. and Watt, P.J. (1971) The preservation of gonococci in liquid nitrogen. *J Clin Pathol* **24**, 122–123.