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'*Candidatus* Branchiomonas cysticola' is a common agent of epitheliocysts in seawater-farmed Atlantic salmon *Salmo salar* in Norway and Ireland

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ABSTRACT: The prevalence and geographical distribution of the recently described endosymbiont '*Candidatus* Branchiomonas cysticola' in Atlantic salmon *Salmo salar* gill epithelial cell cysts was investigated in seawater-farmed fish suffering proliferative gill inflammation (PGI). To this end, we developed a specific and sensitive real-time PCR assay for detection of the bacterium. '*Ca*. B. cysticola' was found to be highly prevalent in Atlantic salmon gills sampled over 7 yr and from 17 geographically distant seawater locations in Norway and Ireland. '*Ca*. B. cysticola' was found in significantly greater quantities in fish with large numbers of epitheliocysts, and fluorescence *in situ* hybridization confirmed its localisation within cysts. '*Ca*. Piscichlamydia salmonis', a bacterium previously linked to epitheliocysts, was identified at relatively low levels of infection, apparently independent of epitheliocyst prevalence. These results suggest that '*Ca*. B. cysticola' is the main cyst-forming bacterium in seawater-farmed Atlantic salmon in the studied countries. Our results also suggest a relationship between load of '*Ca.* B. cysticola' and extent of pathological changes. Taken together with a previously described association between epitheliocyst load and severity of PGI in Norwegian salmon, the results could indicate a role for '*Ca*. B. cysticola' in gill diseases such as PGI.

KEY WORDS: '*Candidatus* Branchiomonas cysticola' · '*Candidatus* Piscichlamydia salmonis' · Epitheliocystis · PGI · Proliferative gill disease · Gill pathology · Atlantic salmon

INTRODUCTION

The term 'epitheliocystis' has been widely used to describe a condition characterised by membrane-bound inclusions, epitheliocysts, containing Gram-negative bacteria in gill- and less commonly skin-epithelial cells of fish. Ultrastructural (Molnar & Boros 1981, Paperna et al. 1981, Rourke et al. 1984, Groff et al. 1996, Nylund et al. 1998, Szakolczai et al. 1999) and molecular studies (Draghi et al. 2004, 2007, Meijer et al. 2006, Karlsen et al. 2008, Polkinghorne et al. 2010) have been interpreted as evidence for the involvement of chlamydia-like organisms (CLOs) as causative agents of epitheliocysts. A number of phylogenetically distinct CLOs have been identified associated with intra-epithelial inclusions in diverse fish species. Meijer et al. (2006) identified 3 different CLOs from the leafy seadragon *Phycodurus eques*, silver perch *Bidyanus bidyanus* and barramundi *Lates calcarifer*; Draghi et al. (2007) identified a *Neochlamydia*-like species in Arctic char *Salvelinus alpinus*, and more recently, molecular evidence for a

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novel CLO in a non-teleost species, the leopard shark *Triakis semifasciata*, has emerged (Polkinghorne et al. 2010).

So far, only CLOs had been associated with this type of infection in Atlantic salmon, i.e. '*Candidatus* (*Ca.*) Clavochlamydia salmonicola' in freshwater (Karlsen et al. 2008) and '*Ca.* Piscichlamydia salmonis' in (mainly) seawater in Ireland and Norway (Draghi et al. 2004). However, recently, Toenshoff et al. (2012) identified '*Ca.* Branchiomonas cysticola' as an agent of epitheliocysts in Norwegian Atlantic salmon *Salmo salar*. Also, rRNA gene sequences, consistent with '*Ca.* B. cysticola' had previously been retrieved from both apparently healthy and proliferative gill inflammation (PGI)-affected Norwegian salmon (Steinum et al. 2009).

Disease outbreaks with a similar clinical picture to PGI involving epitheliocystis have also been reported in Scotland and Ireland (Rodger 2007). The main objectives of the present report were therefore to investigate the geographical distribution and prevalence of '*Ca.* Branchiomonas cysticola' in a number of distinct populations of Atlantic salmon through real-time PCR, 16S rRNA gene sequencing analysis and fluorescence *in situ* hybridization (FISH). Furthermore, the importance of this bacterium both in terms of gill pathology and the occurrence of epitheliocystis in fish was explored.

MATERIALS AND METHODS

Tissue sampling and storage

Archived materials from 14 Norwegian and 3 Irish marine Atlantic salmon farms were studied (Table 1). The Norwegian samples comprised material collected in farms in southern, western and central Norway during longitudinal studies performed in 2004 (described by Steinum et al. 2010) and from 3 additional farms sampled on single occasions in 2005 and 2007. The Irish samples were obtained in 2009/2010 from 3 farms in the west (1 farm, longitudinal study) and north-west (2 farms, diagnostic investigation of PGI-like disease) of the country. All fish were sampled during autumn. In each case, a section of the second left gill arch was fixed in 10% buffered formalin for later processing for histology and a piece of the third left gill arch was collected in RNA*later*™ (Ambion) for isolation of DNA/RNA and subsequent PCR screening. Previously examined samples from 4 Norwegian (Steinum et al. 2010) and 2 Irish freshwater hatcheries were also screened for '*Ca.* Branchiomonas cysticola'.

Histological examination

Paraffin-embedded and formalin-fixed gills were sectioned (5 µm), stained with haematoxylin and eosin (H&E) according to standard histological techniques (Bancroft & Stevens 1996), and one section

Table 1. *Salmo salar*. Origins of samples. The gill samples studied comprised a subset of samples selected from different longitudinal studies. The table shows location, year of sampling, presence or absence of gill disease, number of fish sampled from each site and farm status. Criteria for classification of an outbreak of gill disease at the farm level were gill pathology, respiratory distress and increased mortality levels. PGI: proliferative gill inflammation

Farm ID	Farm status for gill disease	Year sampled	Fish examined (N)	Fish with PGI-like pathology (N)
Seawater sites				
Irl-A	Outbreak	2010	20	12
Irl-B	Outbreak	2009	25	20
Irl-C	Outbreak	2009	10	6
Nor-A[a]	Outbreak	2004	19	14
Nor-B[a]	Outbreak	2004	17	14
Nor-C[a]	Outbreak	2004	7	1
Nor-D[a]	Outbreak	2004	5	2
Nor-F[a]	Outbreak	2004	10	8
Nor-G[a]	No outbreak	2004	7	0
Nor-H[a,b]	No outbreak	2004	11	10
Nor-J[a]	No outbreak	2004	8	0
Nor-K[a]	No outbreak	2004	8	0
Nor-L[a]	No outbreak	2004	8	1
Nor-M[a]	No outbreak	2004	7	1
Nor-N[b]	No outbreak	2005	10	5
Nor-O	Outbreak	2007	15	14
Nor-P	Outbreak	2007	15	15
Freshwater sites				
Irl-1	No disease	2009	10	0
Irl-2	No disease	2009	10	0
Nor-1[a]	No disease	2004	10	0
Nor-2[a]	No disease	2004	10	0
Nor-3[a]	No disease	2004	10	0
Nor-4[a]	No disease	2004	10	0

[a]Farms previously examined for the presence of possible aetiological agents other than '*Ca.* Branchiomonas cysticola' by Steinum et al. (2010)
[b]Most fish examined were diagnosed with PGI, although no outbreak was recognized

per fish was examined by light microscopy. Gills were evaluated for pathological changes according to criteria relating to hyperplasia and cell death. In addition, inflammation and circulatory disturbances, such as lamellar haemorrhage and thrombosis, were evaluated in Norwegian fish (according to Kvellestad et al. 2005) and lamellar fusion in Irish fish. Pathological changes were graded based on the fraction of the filament length affected and were applied as follows: none (Grade 0); slight (Grade 1), where <10% (approximately) of the filament was affected; moderate (Grade 2) with 10 to 50% affected; and finally, extensive (Grade 3), with >50% affected. Epitheliocyst numbers were categorised into not detected, few, some or many, as previously described (Steinum et al. 2010). Since slightly different qualitative criteria were utilised for characterisation of pathological changes in Norwegian and Irish samples, results for the 2 countries were analysed separately.

DNA extraction and efficiency control

After homogenization of approximately 18 mg of gill soft tissues (scraped from the gill arch with a scalpel) with the Roche MagNA lyser, DNA was extracted from half the homogenate volume using the Roche High Pure PCR Template Preparation kit according to the manufacturer's instructions. Consistency of DNA extraction was checked in 29 random samples from both the Irish and Norwegian fish with the Atlantic salmon elongation factor alpha 1 gene assay (Bruno et al. 2007). DNA extraction efficiency was found to be essentially identical (mean ± SD cycle threshold, C_t-value: 20.6 ± 0.8), which allowed meaningful comparisons of C_t-values between samples.

Real-time PCR

The '*Ca.* Branchiomonas cysticola' 16S rRNA gene assay was performed using the Taqman universal real-time PCR master mix (Applied Biosystems) with primers BPf2 and BPr2 (final concentration 0.5 µM, Table 2), which defines a 95 bp amplicon, and probe BPp2 (final concentration 0.25 µM, Table 2). The amplification was run on the Mx3005P™ real-time PCR system using the default quantitative 2-step program with annealing and synthesis at 60°C for 1 min. Negative controls using DEPC-treated water (Invitrogen) and a positive sample, confirmed by sequencing, were included in each run. The '*Ca.* Piscichlamydia salmonis' 16S rRNA gene assay was performed as previously described (Steinum et al. 2010) with primers 16S RTfor, 16S RTrev and 2 fluorescence resonance energy transfer probes (Tib-molbiol, Table 2). The observed C_t-values were divided into 3 equally large intervals (related to C_t-values identified for '*Ca.* B. cysticola' which displayed the largest range), re-

Table 2. PCR primers and probes used in the present study. Lightcycler and Taqman probe labelling and modification are shown: 5'-fluorescein amidite (FAM) and 3'-black berry quencher (BBQ), 3'-fluorescein (FL), 5'-LC640 dye (640) and 3'-phosphate (PH). For the other fluorescence *in situ* hybridization (FISH) probes, see Toenshoff et al. (2012)

Oligonucleotide	Sequence (5'→3')	Source
Real-time assays		
BPf2	AAT ACA TCG GAA CGT GTC TAG TG	This study
BPr2	GCC ATC AGC CGC TCA TGT G	This study
BPp2 (probe)	FAM-CTC GGT CCC AGG CTT TCC TCT CCC A-BBQ	This study
16SRTfor	CCG CAA GGA CAA CTA CAC	Steinum et al. (2010)
16S Rtrev	ATC GAC TTA GGC AGT CTC G	Steinum et al. (2010)
16S RTFL (probe)	AAC CCA ACA CCT CAC GGC ACG A-FL	Steinum et al. (2010)
16S RTLC (probe)	640-CTG ACG ACA GCC ATG CAG CAC-PH	Steinum et al. (2010)
FISH probe		
Psc-197	CTT TTC AGA GTC CCC CGC	This study
Sequencing primers		
BraCy_14F	TGC TTG CAC TAT GTT GGC	This study
BraCy_1420R	CTG CTT CTG GTC AAC CCC	This study
Reverse primer 3	ACT GCT GCC TCC CGT	Dewhirst et al. (1992)
Reverse primer 5	CTA CCA GGG TAT CTA ATC	Dewhirst et al. (1992)
538C	GTA GTC CAC GCC GTA AAC G	Pettersson et al. (1996)
V4 (modified)	GAT TTG TCA CCG GCA GTA TC	This study
V5	GTC CAC ACT CCT ACG GGA GGC	Lunder et al. (2000)
V6	GGG GAY GAC GTC AAG TC	Lunder et al. (2000)

sulting in the following grading scale: 0 = not detected (no C_t-value); 1 = low load ($C_t > 29.9$); 2 = moderate load ($23.6 \leq C_t \leq 29.9$); and 3 = high load ($C_t < 23.6$). A practical cut-off for the assay based on our experiences would be $C_t = 38.0$.

Standard curve construction and assay evaluation

An *Escherichia coli* Topo Cloning vector was used to clone the '*Ca.* Branchiomonas cysticola' real-time PCR amplicon. A 10-fold dilution series with the linearized vector-insert construct (by *Hin*dIII digestion) and a starting concentration of 99.85 ng DNA μl^{-1} (equal to ~1.7×10^{10} copies μl^{-1}) was analysed to make a standard curve. The '*Ca.* B. cysticola' assay was thoroughly tested for specificity both *in silico* and practically against a taxonomically broad panel of 34 bacterial species including both relevant fish pathogenic and environmental isolates. The template quality of this panel was confirmed using universal bacterial 16S rRNA primers A-18 and S-20 (Suau et al. 1999).

16S rRNA gene sequencing and sequence analysis

An almost full-length 16S rRNA gene sequence (GenBank accession no. JQ723599) was obtained from Irish farm Irl-A (fish no. 5) using primers BraCy_14F and BraCy_1420R and sequenced with 6 additional primers (Table 2). All sequencing was performed according to standard protocols using DYEnamic™ ET terminator reagents and the MegaBace 1000 sequencing system (GE Healthcare Bio-Sciences). Subsequent sequence analysis was performed with the Vector NTI Suite 11 software (InforMax) and NCBI blastn searches (Altschul et al. 1990). Following purification using the Nucleospin^R purification kit (Macherey-Nagel), '*Ca.* Branchiomonas cysticola' real-time PCR amplicons were cloned into the TA TOPO dual promoter vector (Invitrogen). Plasmid DNA was extracted with a Nucleospin^R Plasmid kit (Macherey-Nagel) and the amplicon sequenced using M13 primers.

Fluorescence *in situ* hybridization

Both fresh gill tissues and deparafinized sections of gill tissues from 1 Irish and 3 Norwegian farms displaying many epitheliocysts were selected for FISH. FISH utilising specific fluorescence labelled oligonucleotide probes for '*Ca.* Branchiomonas cysticola' (BraCy-129) and '*Ca.* Piscichlamydia salmonis' (Psc-523, Psc-197) was performed as described by Toenshoff et al. (2012). A general bacterial probe mix (EUB338I, II, III) labelled with Fluos (Daims et al. 1999) and 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to DNA, were also used for bacterial localisation during hybridization. FISH probe efficiency was tested on cloned target sequences (Schramm et al. 2002). Non-sense probes were used as negative controls.

Statistical analysis

C_t-values were converted to ordinal measures (described above), and median (median absolute deviation [MAD]) C_t-values were calculated. Negative samples were awarded C_t-values of 38.1 to allow inclusion of all samples in the analysis. For all fish, cyst levels and severity of pathological change were categorised as described by Steinum et al. (2010). The strength and direction of any association between the cross-tabulated ordinal data was analysed using Goodman and Kruskal's gamma test. Following calculation of the gamma statistics, the significance of associations were tested using the *Z*-test and *z*-scores. The critical *z*-score value is 2.58 (at the 0.01 level of significance), i.e. all values above this threshold indicate a significant positive association.

RESULTS

Histopathological findings

In total, 123 out of 202 fish examined histologically, 85/147 (~58%) and 38/55 (~69%) from Norway and Ireland, respectively, displayed pathological changes that met our pre-set criteria. The percentage of PGI diagnosed fish in Norway was higher at 83/109 (~76%), and more similar to that seen in Ireland if the farms with virtually no gill problems were omitted (≤1 diagnosed fish per farm).

In the Irish fish, our findings ranged from mild hyperplasia of the lamellar tips and occasional fusion of lamellae, to extensive hyperplastic inflammatory lesions obliterating the normal lamellar architecture and widespread fusion and death of epithelial cells. The Norwegian material from 2004 was described previously (Steinum et al. 2010). In short, pathological changes consistent with PGI criteria (hyperplasia, inflammation, cell death and circulatory disturbances such as lamellar haemorrhage and thrombosis) were

observed in fish from all farms both severely and mildly affected. However, all fish, irrespective of farm status, displayed slight inflammation of the lamellar epithelium especially at the apical margins. Trichodinids were the most commonly observed ectoparasites, particularly in fish from the 2 farms with the highest mortality. Pathological changes consistent with PGI criteria were also found in a majority of fish sampled from 3 Norwegian farms in 2005 and 2007.

Epitheliocysts were observed in 84 out of the 123 (~68%) fish that met our pre-set pathological criteria. In comparison, only 33 out of the remaining 79 (~42%) apparently healthy fish from both countries displayed epitheliocysts. Generally, the highest number of epitheliocysts was observed in fish with moderate to extensive pathology. In these fish, they appeared to be generally scattered throughout the proliferative gill epithelia. Alternatively, in fish with few epitheliocysts, the cysts were usually observed near the apical tips of lamellae.

'Ca. Branchiomonas cysticola' real-time PCR assay evaluation

The results of the standard curve construction for the '*Ca.* Branchiomonas cysticola' specific real-time PCR assay (slope = −3.313, amplification efficiency E = 98.7% and *y*-intercept = 38.25) suggest the assay to be highly sensitive and efficient. *In silico* analysis of the real-time PCR assay primers and probe demonstrated that cross-reaction with any sequence available on the public databases was highly unlikely. Further, no cross hybridization or amplification was identified on *in vitro* testing of the taxonomically broad panel of bacteria (Table 3).

Table 3. Bacteria representing a broad taxonomic range of species used to test the '*Ca.* Branchiomonas cysticola' real-time PCR assay for specificity. NCIMB: National Collections of Industrial, Marine and Food Bacteria; NVI: Nowegian Vet Institute; NCFB: National Collection of Food Bacteria; ATTC: American Type culture Collection; DSM: German Collection of Microorganisms (and cell culture)

Bacterial species	Source
Aeromonas salmonicida subsp. *salmonicida*	NCIMB 1102
Aeromonas salmonicida subsp. *achromogenes*	NCIMB 1110
Aeromonas salmonicida subsp. *smithia*	NCIMB 13210
Arthrobacter globiformis	NCIMB 8907
Bacillus cereus	NVI 3588
Brochothrix thermosphacta	NCFB 1676
Burkholderia mallei	Clinical isolate[a]
B. thailandensis	Clinical isolate[a]
B. pseudomallei	Clinical isolate[a]
Carnobacterium piscicola	ATCC 1985
Escherichia coli	NCIMB 10000
Francisella noatuensis subsp. *noatunensis*	NCIMB 14265
Micrococcus luteus	Clinical isolate[b]
Moritella viscosa	NCIMB 2263
Mycobacterium avium subsp. *avium*	DSM 44156
M. marinum	NVI 5222
Nocardia asterioides	NVI 6532
Pasteurella skyensis	NVI 4464
Photobacterium phosphoreum	NCIMB 1953
Piscirickettsia salmonis	NVI 5692
Proteus mirabilis	NCIMB 10823
Pseudomonas fluorescens	NCIMB 10067
P. aeruginosa	NCIMB 12469
Renibacterium salmoninarum	Clinical isolate[b]
Rhodococcus equi	Clinical isolate[b]
R. erythropolis	NCIMB 11148
Serratia marcescens	NCIMB 10351
Staphylococcus aureus subsp. *aureus*	NCIMB 11787
Streptococcus algalactiae	NCIMB 1334
Tenacibaculum sp.	NCIMB 3846
Vibrio anguillarum	NCIMB 1291
Vibrio salmonicida	NCIMB 2262
Vibrio splendidus	NVI 4286
Yersinia ruckeri	NVI 353

[a]Norwegian Institute of Public Health
[b]NVI Norwegian Veterinary Institute

Real-time PCR screening and *'Ca.* Branchiomonas cysticola' 16S rRNA gene sequencing

In the 3 Irish seawater farms, all with outbreaks of PGI-like disease, *'Ca.* Branchiomonas cysticola' and '*Ca.* Piscichlamydia salmonis' were detected by real-time PCR in 45/55 fish (~82%) and 25/55 fish (~45%), respectively. The C_t-values observed ranged from 17.8 to 36.3 for *'Ca.* B. cysticola' and from 26.9 to 38.0 for '*Ca.* P. salmonis'.

In the 9 Norwegian seawater farms in which either several fish with PGI diagnosis or outbreaks had been recognized, *'Ca.* Branchiomonas cysticola' and '*Ca.* Piscichlamydia salmonis' were detected in 106/109 fish (~97%) and 72/109 fish (~66%), respectively. The corresponding C_t-values ranged from 17.8 to 35.7 and from 25.7 to 37.5, respectively. In the 5 remaining Norwegian seawater farms with virtually no gill problems, *'Ca.* B cysticola' was detected in 27/38 fish (~71%) with a range in C_t-values from 25.0 to 37.9 (median 30.6 ± 2.7 MAD; for all other median values, see Table A2). Notably, '*Ca.* P. salmonis' was not detected at all.

In all samples investigated, '*Ca.* Branchiomonas cysticola' was detected in 112/117 (~96%) and 66/85 (~78%) fish with and without visibly obvious epitheliocysts, respectively (Table A1). The relationship between '*Ca.* B. cysticola' load and number of epitheliocysts observed histologically was investigated. The load of the bacterium appeared positively associ-

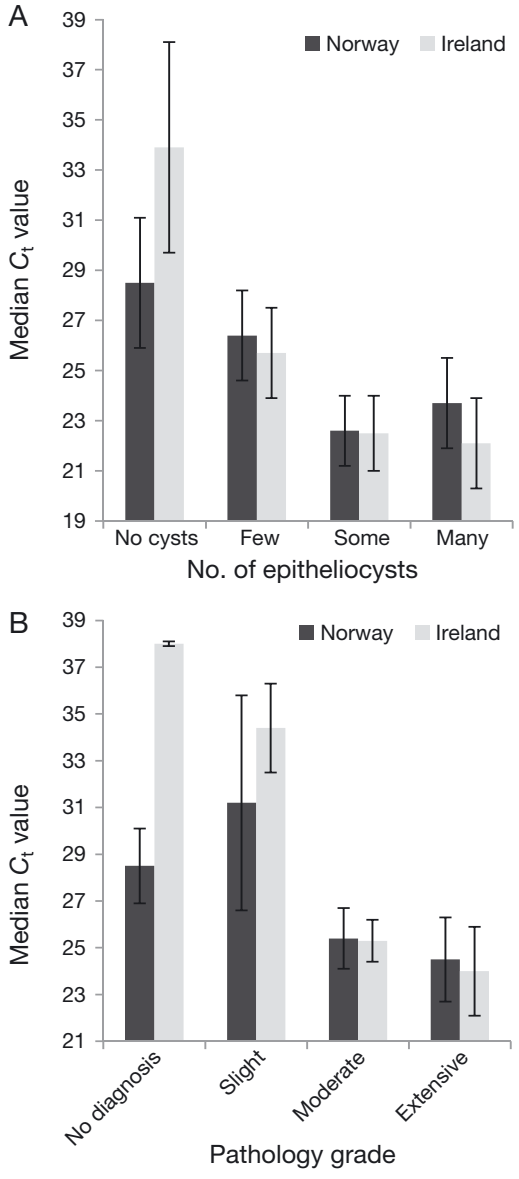

Fig. 1. *Salmo salar*. Correlation of '*Ca.* Branchiomonas cysticola' abundance with epitheliocysts and pathology. (A) Relationship between '*Ca.* B. cysticola' load in soft gill tissues of Irish and Norwegian seawater-reared Atlantic salmon, here denoted by semi-quantitative PCR (median cycle threshold [C_t]-values), and the categorical classification of samples according to epitheliocyst number (no cysts to many cysts). The median absolute deviation (MAD) is represented by error bars. (B) Relationship between '*Ca.* B. cysticola' load in the same material and severity of pathological change (no diagnosis or slight to extensive). The first columns representing median C_t-values in fish from Ireland classified as without cysts or significant pathology is 33.9 and 38.0, respectively, as most '*Ca.* B. cysticola' negative samples from Ireland were in these categories. The median C_t-values of only the positive samples was 26.9 (±3.5) and 26.4 (±2.6), respectively. Including the negative samples had little effect on median C_t-values for all other categories, but the MAD inevitably increased

ated with increasing number of epitheliocysts, but also with increasing severity of pathological changes in both the Norwegian and the Irish dataset (Fig. 1, Table A2). A statistically significant correlation was calculated for the whole material (gamma = 0.62, *z*-score = 5.37) which can be classified as good to strong, thus suggesting a correlation between increasing '*Ca.* B. cysticola' load and number of epitheliocysts in sample material from both countries.

The almost full length 16S rRNA gene sequence obtained from one sample from an Irish farm displayed 99.8% sequence identity with the previously published sequence of *'Ca.* Branchiomonas cysticola' from Norway (GenBank accession no. JN968376, Toenshoff et al. 2012), which indicates a high degree of conservation in geographically disparate isolates.

Observations in freshwater hatcheries

Sixty pre-smolts from 6 freshwater hatcheries in Norway and Ireland were also screened for '*Ca.* Branchiomonas cysticola' using the real-time PCR. The bacterium was not detected. No significant gill pathology was observed in any of these fish. Some of the Irish fish displayed epitheliocysts consistent in appearance with those caused by '*Ca.* Clavochlamydis salmonicola' (Mitchell et al. 2010).

In situ detection of *'Ca.* Branchiomonas cysticola'

Prior to testing gill tissues with FISH, both '*Ca.* Branchiomonas cysticola' and '*Ca.* Piscichlamydia salmonis' probes were tested successfully on cloned PCR products containing the target sequences of interest. FISH performed on samples from both Norway and Ireland produced strong signals within cysts using both EUB-Mix (a probe mix targeting most bacteria) and the specific '*Ca.* B. cysticola' probe (Fig. 2A) in all 5 fish tested (Irish farm Irl-A, Fish 1, 5, 9, 21 and 23). In all cases, homogenous signals were observed, indicating occupation of the cysts by a single bacterial species, i.e. '*Ca.* B. cysticola'. The '*Ca.* P. salmonis' probes did not generate any visible signal within the cysts, but faint signals were apparent diffusely distributed throughout the tissues. Similar results were observed when hybridizations were carried out on fresh, squeezed gill tissues, with clear signals from cysts using the '*Ca.* B. cysticola' probes (Fig. 2B), and only faint signals which might be only

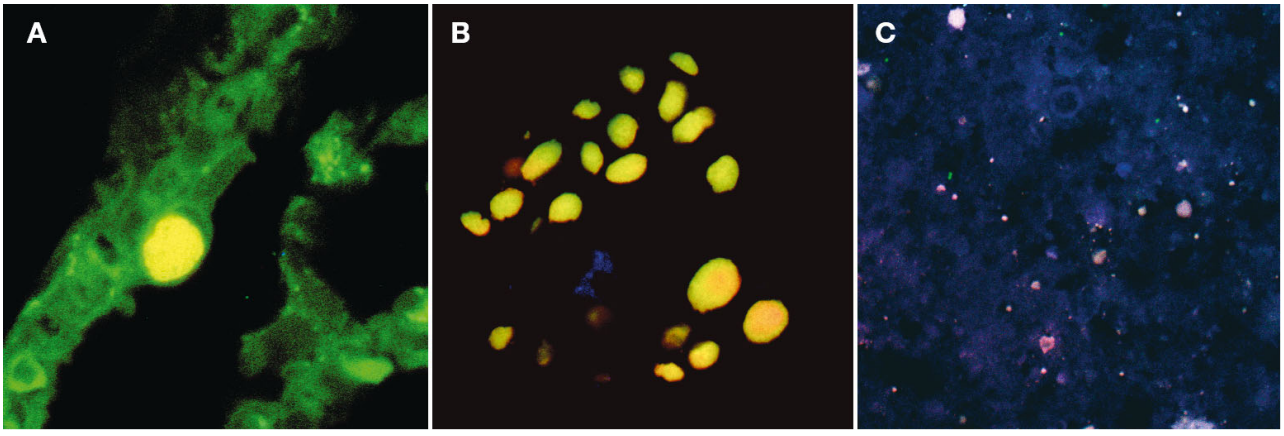

Fig. 2. *Salmo salar*. Detection of '*Ca.* Branchiomonas cysticola' in Atlantic salmon from Ireland by fluorescence *in situ* hybridization (FISH). (A) Formalin-fixed and paraffin-embedded gill tissues; (B) and (C) fresh, squeezed gill tissues. The betaproteobacterial probe Btwo23A labelled with Fluos and the '*Ca.* B. cysticola'-specific probe BraCy-129 labelled with Cy3 were used in (A) and (B). The general bacterial probe EUB-Mix labelled with Cy5 and the '*Ca.* Piscichlamydia salmonis' specific probes Psc-523 and Psc-197 labeled with Cy3 were used in (C)

fish tissue autofluorescence using the '*Ca.* P. salmonis' probes (Fig. 2C). DAPI staining did not identify cysts other than those staining positive for '*Ca.* B. cysticola' within the tissues.

DISCUSSION

We found '*Ca.* Branchiomonas cysticola', a bacterium recently associated with epitheliocysts in sea-farmed Atlantic salmon (Toenshoff et al. 2012), to be highly prevalent in more than 200 Atlantic salmon sampled over a 7 yr period from 17 widely distributed locations in Norway and Ireland. '*Ca.* B. cysticola' therefore appears to be common in both diseased and apparently healthy Atlantic salmon gills.

A significant correlation between '*Ca.* Branchiomonas cysticola' load estimated by real-time PCR and epitheliocyst number enumerated by histology was observed (Fig. 1A, Table A1). Further, strong positive FISH signals utilising '*Ca.* B. cysticola' probes were observed within cysts in both Irish and Norwegian fish (Fig. 2). The combined results from FISH and real-time PCR suggest that '*Ca.* B. cysticola' is the primary, and possibly only, bacterium residing in the epitheliocysts studied. These results support the findings of Steinum et al. (2010), who concluded that at least 1 bacterium other than '*Ca.* Piscichlamydia salmonis' was responsible for cysts observed during that study.

Although '*Ca.* Branchiomonas cysticola' is present in many fish without apparent gill pathology, our results indicate a relationship between increasing numbers of the bacterium and severity of PGI in the gills (Fig. 1B). This suggests that the negative effects of '*Ca.* B. cysticola' may be load dependent. However, the dispersion observed in the levels of '*Ca.* B. cysticola' makes it challenging to identify a statistically significant relationship. Registering pathological changes on a continuous scale as well would most likely have solved the issue, if it was practicable. A relationship has previously been identified between epitheliocyst number and PGI severity in Norwegian salmon (Steinum et al. 2010). This, taken together with the results showing '*Ca.* B. cysticola' to be the main cyst-forming bacterium, indicates that this bacterium may play an aetiologic role in gill diseases such as PGI. Such a 'dose-dependent' pathological effect has also been suggested for another microorganism associated with PGI, i.e. the microsporidean *Desmozoon lepeophtherii* (Steinum et al. 2010, Nylund et al. 2011). However, in the absence of infectious challenge evidence, whether the high tissue loads of '*Ca.* B. cysticola' are the cause or result of proliferative gill disease remains unclear. As is the case with other agents linked to PGI, e.g. '*Ca.* Piscichlamydia salmonis' and *D. lepeophtherii* (Steinum et al. 2010), '*Ca.* B. cysticola' does not completely satisfy molecular guidelines for establishing causation (Fredricks & Relman 1996) as a singly responsible agent. It remains our opinion that PGI is most likely of multifactorial aetiology.

Further characterisation of '*Ca.* Branchiomonas cysticola' and development of a challenge model would greatly enhance our understanding of the role of this novel bacterium in gill disorders. However, culture of '*Ca.* B. cysticola' is likely to prove challeng-

ing, as it may be an obligate intracellular bacterium, and previous efforts to culture epitheliocystis agents have failed. The environmental source of '*Ca.* B. cysticola' is not known. Although the freshwater sampling was not exhaustive, '*Ca.* B. cysticola' was not identified in salmon sampled during the freshwater stage of culture, and we therefore consider it likely that salmon are first infected after seawater transfer. Interestingly, epitheliocyst-like structures have also been observed in marine invertebrates such as molluscs and crustaceans (Fryer & Lannan 1994). Molecular screening of different marine organisms also including representatives of zoo-/phytoplankton and protist parasites, particularly those affecting the gills of fish, may help identify any other host species for this bacterium.

While specific FISH probes clearly identified cloned '*Ca.* Piscichlamydia salmonis' inserts *in vitro,* this bacterium could not be associated with cysts. The failure of FISH to clearly demonstrate '*Ca.* P. salmonis' in real-time PCR positive gills may, conceivably, be attributed to a number of factors including secondary or tertiary structures blocking probe binding *in situ*, the presence of bacterial rRNA at levels too low for detection, possibly due to bacterial dormancy or low physiologic activity (Daims et al. 2005), or that cysts containing '*Ca.* P. salmonis' are so few or small that they are simply overlooked. The location and significance of '*Ca.* P. salmonis', which was only detectable in low to moderate loads in fish from farms with either several PGI diagnosed fish or recognized outbreaks, therefore remains, ambiguous.

In summary, the combination of FISH, specific real-time PCR and sequence retrieval data provide convincing evidence that '*Ca.* Branchiomonas cysticola' is the main epitheliocyst-forming bacterium in sea-farmed Atlantic salmon examined in the present study. We have demonstrated that '*Ca.* B. cysticola' is widely distributed within farmed Atlantic salmon in both Norway and Ireland, highly conserved at the 16S rRNA gene level, and that the bacterial load appears to be related to the severity of pathological changes observed in the gills of affected fish.

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Appendix 1. Additional information

Table A1. *Salmo salmar*. Categorical results from all Atlantic salmon post-smolts in the present study (Irish and Norwegian farms) analysed for association with the Goodman and Kruskal's gamma test. '*Ca.* Branchiomonas cysticola' load versus epitheliocyst number resulted in gamma = 0.62 and *z*-score = 5.37. This association can be considered good to strong and is statistically significant, as the critical *z*-score value (at the 0.01 level of significance) is ± 2.58

'*Ca.* B. cysticola'	Epitheliocysts			
	None	Few	Some	Many
Not detected	19	5	0	0
Low load	28	17	1	1
Moderate load	32	50	7	9
High load	6	6	11	10

Table A2. *Salmo salmar*. '*Ca.* Branchiomonas cysticola' load in soft gill tissues of Irish and Norwegian seawater-reared Atlantic salmon, as estimated by semi-quantitative PCR (median cycle threshold [C_t]-values), compared to epitheliocyst number (0 = no cysts to 3 = many) and severity of pathology (0 = none to 3 = extensive). The median absolute deviation is shown in parentheses

No. cysts	Median C_t	Pathology	Median C_t
Norway			
0	28.5 (2.6)	0	28.5 (1.6)
1	26.4 (1.8)	1	31.2 (4.6)
2	22.6 (1.4)	2	25.4 (1.3)
3	23.7 (1.8)	3	24.5 (1.8)
Ireland			
0	33.9 (4.2)	0	38 (0.1)
1	25.7 (1.8)	1	34.4 (1.9)
2	22.5 (1.5)	2	25.3 (0.9)
3	22.1 (1.8)	3	24.0 (1.9)

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