Taxonomy of bacterial fish pathogens

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Running title: Characterization of bacterial pathogens

TABLE OF CONTENTS

Abstract

Bacterial taxonomy has progressed from reliance on highly artificial culture-dependent techniques involving the study of phenotype [including morphological, biochemical and physiological data] to the modern applications of molecular biology, most recently 16S rRNA gene sequencing, which gives an insight into evolutionary pathways (= phylogenetics). The latter is applicable to culture-independent approaches, and has led directly to the recognition of new uncultured bacterial groups, i.e. *'Candidatus'*, which have been associated as the cause of some fish diseases, including rainbow trout summer enteritic syndrome. One immediate benefit is that 16S rRNA gene sequencing has led to increased confidence in the accuracy of names allocated to bacterial pathogens. This is in marked contrast to the previous dominance of phenotyping, and identifications, which have been subsequently challenged in the light of 16S rRNA gene sequencing. To date, there has been some fluidity over the names of bacterial fish pathogens, with some, for example *Vibrio anguillarum,* being divided into two separate entities *(V. anguillarum* and *V. ordalii).* Others have been combined, for example *V. carchariae, V. harveyi* and *V. trachuri* as *V. harveyi.* Confusion may result with some organisms recognized by more than one name; *V. anguillarum* was reclassified as *Beneckea* and *Listonella,* with *Vibrio* and *Listonella* persisting in the scientific literature. Notwithstanding, modern methods have permitted real progress in the understanding of the taxonomic relationships of many bacterial fish pathogens.

Key words: **characterization – phylogenetics – classification – taxonomy – DNA sequencing**

1. Introduction

"What's in a name?" (William Shakespeare; Romeo and Juliet)

The Swedish botanist Carl Linnaeus (1707-1778), who was also known as Carolus Linnaeus and Carl von Linné, is undoubtedly the Father of Taxonomy, and was responsible for developing a system for naming and ranking living organisms. His lasting contribution was the development of a simplified naming system in Latin with consistency across all living organisms, i.e. the binomial system, in which each organism has a unique two-word name – incorporating genus and species. A simplistic view is that Linnaeus made order out of chaos. Yet, for Linnaeus and his contemporaries, the process was comparatively easy, and involved only large organisms, which were clearly visible to the naked eye (= macro-organisms) and easily seen morphological characteristics (= a category of phenotypic characters). Thus, these early classifications (= the process of arranging organisms into groups) were based on limited but easily visible data, and the outcomes were largely obvious, for example a dog is notably different from a horse and would therefore belong in separate species.

The founding father of microbiology, the Dutch textile merchant and lens maker, Antonie van Leeuwenhoek (1632-1723), observed small organisms initially from the proximity of his teeth (= bacteria and protozoa?), and these entities were termed "animalcules", which he wrote about in a letter to the Royal Society in 1676. His careful illustrations suggested morphological variation between the cells. Yet,

another two centuries were to pass before serious attempts at naming and ordering bacteria started. Thus, bacterial taxonomy has progressed from the simplistic approach involving a small number of readily observable characteristics, such as morphology as deduced from observation using light microscopes, to the modern applications of molecular biology. With improvements in knowledge, there have been refinements in taxonomic processes and an increase in reliability. It should be remembered that taxonomy (= the theory of classification, nomenclature and identification) is a man-made process, i.e. the organisms included in any classification have not chosen to be placed in the groups that have been created by human beings. Nevertheless if done properly, taxonomy has value in:

- Understanding biodiversity, namely the range of organisms in a given habitat
- Communication between scientists, thus enabling exchange of information about similar organisms
- Cataloguing information – the name is the key to a catalogue of information about the organism
- Enabling identification, such that new isolates may be readily and reliably identified
- Providing an insight into evolutionary pathways (= phylogenetics).

To be effective, taxonomy should be

- based on a high information content
- reproducible, and
- stable,

otherwise confusion will surely result.

Since the start of bacterial taxonomic processes in the nineteenth century, there has been a progression in the type of information used in the procedure. It may be argued that early bacteriologists had considerable taxonomic insight judging from the conclusions reached from the comparatively simple data that were available. However, taxonomy is a dynamic science, with new developments/methods being incorporated into processes including the descriptions of bacterial species. Since the 1950s, bacterial taxonomy has evolved rationally, encompassing numerical methods [132, 136], chemotaxonomy [e.g. 8, 26], and molecular techniques [63]. Taxonomy has progressed from a highly artificial process involving limited amounts of phenotypic data to the recognition of more natural relationships between organisms, based on comparatively large amounts of varied and reliable data covering multiple aspects of the biology of an organism, and including phenotypic, chemotaxonomic, genotypic and phylogenetic date, i.e. a polyphasic approach. However, the current dominance of 16S rRNA gene sequencing although revolutionising some aspects of bacterial classification needs to be treated cautiously as overreliance on the approach may lead to erroneous conclusions [63]. Nevertheless, it is apparent that sequencing methods are instrumental with the explosion of new species names, which have greeted the arrival of the twenty-first century. Whereas, the information content of many of the new species descriptions is generally high, an unwelcome trend is that many new taxa (= taxonomic groups) are described solely after the study of only single strains. Therefore, the diversity/variability within the new taxon cannot be adequately assessed. Also, it is impossible to determine whether a single strain is effectively an outlier or a median representative of the group [in future years, will it be regarded as typical or atypical of the group?]. However, taxonomy is often ignored by many microbiologists in other specialisms, and there may well be concern that

basic principles could be forgotten, e.g. is the purity and authenticity of cultures always verified before use? Where culturing is not possible, there is the possibility of analyzing the nucleic acids, determining species composition, and even proposing new taxa, i.e. by the use of culture-independent approaches.

2. Bacterial fish pathogens

There has been a steady increase in the numbers of bacterial species associated with fish diseases, with new pathogens regularly recognised in the scientific literature [17]. However, the names of many bacterial fish pathogens have been subjected to taxonomic change over time, with some species split, for example *Vibrio anguillarum* biotype 2 becoming re-classified as a separate species *V. ordalii* [127, 128]. In other cases, different nomenspecies have been combined, for example *V. carchariae, V. harveyi* and *V. trachuri* into *V. harveyi,* which had precedence because it was the first name to be proposed, albeit as the luminous *Achromobacter harveyi* [49, 110, 144]. The oldest known fish **pathogen**, *V. anguillarum,* has undergone name changes to *Beneckea* [19] and *Listonella* [83]; neither of which was widely accepted. However, *Listonella* remains a valid name and is mentioned in the current edition of Bergey's Manual of Systematic Bacteriology, and *Beneckea* has been consigned to the history books. A positive aspect of sequencing methods is that there has been a progression towards the Orwellian notion of "Order out of Chaos" even if scientists do not always appreciate the significance of the data.

3. Isolation of fish pathogens: the culture-dependent approach

With the rapid development in molecular biology, it is not always necessary to culture

152 an organism in order to enable its study, including the allocation of a species name.
153 Thus, the concept of culture-independent techniques was developed and refined.
154 Sensitivity and specificity increased, but without culturing there was an inability to
155 carry out associated studies, such as the determination of pathogenicity factors. The
156 attraction of culture-dependent approaches is that a pure culture may be obtained and
157 deposited in culture collections as reference material for use by others. This raises a
158 concern about the usefulness of cultures. An assumption is made that pathological
159 material may be used for the recovery of a pure culture of the aetiological agent. This
160 will depend on using appropriate media and incubation conditions, and assumes that
161 the organism is in a form that may be cultured and that the microbiologist picks the
162 "correct" colony. If mixed growth occurs or if the pathogen is largely overgrown by
163 opportunists/secondary invaders/saprophytes, then there is concern that the actual
164 pathogen will be missed. In addition, infections resulting from two or more
165 organisms working synergistically will undoubtedly be mis-diagnosed if the
166 diagnostician chooses only one culture for study. However, there are only a
167 comparatively few indications of disease resulting from multiple species, such as
168 *Aeromonas hydrophila* with *A. salmonicida* [17]. It is speculative how many
169 diagnoses (if any) are made of contaminants rather than the actual pathogen.
170 Moreover, it is surprising that only two species of anaerobic bacteria, namely
171 *Clostridium botulinum* and *Eubacterium tarantellae*, have been implicated as fish
172 pathogens [17]. Of course, this could reflect the general lack of use of appropriate
173 anaerobic procedures by microbiologists rather than the absence of anaerobic
174 pathogens.
175
176 **4. Approaches to characterization**

177 **4.1 Phenotype**

178 Traditionally, bacteria were characterized phenotypically, and undoubtedly for some
179 groups, e.g. the Enterobacteriaceae, a wealth of knowledge emerged particularly from
180 the 1950's onwards. Currently, emphasis on phenotype has declined with a
181 concomitant move towards molecular-based approaches. Nevertheless, phenotypic
182 data have a role in polyphasic studies whereby many facets of the biology of an
183 organism are studied [153]. Phenotyping leads the way with diagnoses worldwide;
184 emphasis often being placed on commercial kits and the use of manufacturer's
185 probabilistic databases to achieve an acceptable identification. Although the approach
186 has standardized diagnoses, the weakness is that most identification systems have
187 been developed for medically important bacteria that grow within 24-48 h at 35-37^{o}C.
188 Consequently, the reliability of these kits for use with fish pathogens which need
189 lower incubation temperatures for longer periods needs to be questioned [17, 148]. In
190 particular, the API 20E system includes the use of sugar fermentation reactions, which
191 may be influenced by the presence of plasmids [17]. Moreover, there may be
192 confusion over the interpretation of the profiles. For example, some of the profiles of
193 *A. hydrophila* are similar to those of *A. allosaccharophila* and *A. sobria; Yersinia*
194 *ruckeri* may be confused with *Hafnia alvei;* moreover *Tenacibaculum maritimum* and
195 *Pseudomonas anguilliseptica* are indistinguishable by API 20E [17]. Problems may
196 result when data from rapid commercial kits are used in conjunction with
197 conventional diagnostic schemes, which have been developed for traditional and often
198 laborious phenotypic characters. Also, some of the traditional tests, e.g. the Voges
199 Proskauer reaction, are not noted for their reproducibility and may introduce errors
200 into the taxonomic process and lead to mis-identification [133].
201

Undoubtedly, selective media have proved useful for the recovery of some fish pathogens, with an example including selective kidney disease medium (SKDM) for *Renibacterium salmoninarum* [11]. However, selective media are only available for a minority of all fish pathogens, therefore recovery is dependent on more general culturing methods. Specially developed diagnostic procedures have aided identification of some group, e.g. the glucose motility **deep cultures** have benefitted the recovery and identification of *V. anguillarum* [155].

4.2 Immunological methods

The development and availability of standardized **immunological (antibodies and kits)** reagents have improved diagnoses considerably [3, 17, 51], and enhanced the reliability of methods for the detection of pathogens, including *Mycobacterium* spp., ***Photobacterium damselae* subsp. *piscicida* [61], *Piscirickettsia salmonis* [141],** *R. salmoninarum* [2] **and *Streptococcus iniae* [70]**. Tentative diagnoses, including of asymptomatic infections, may result from use of monospecific polyclonal or monoclonal antibodies in a range of **antibody-based** procedures, including the **indirect** fluorescent antibody test (**i**FAT), whole-cell (slide) agglutination, precipitin reactions, complement fixation, immunodiffusion, antibody-coated latex particles, co-agglutination using antibody-coated staphylococcal cells, passive haemagglutination, immuno-India ink technique (Geck) or enzyme linked immunosorbent assay [ELISA; **reviewed by 1**], **the latter of which may also be used for serology, i.e. detecting antibodies in the host to specific pathogens [120]. Antibody-based methods are used effectively for detecting exposure to fish viruses, such as Koi herpes virus [4], but bacterial pathogens pose a more complicated picture with cross reactivities likely unless specific known molecules are used to coat the ELISA**

plates rather than whole pathogens [3]. Techniques are often sensitive, specific, rapid and reliable, and in some cases may be used in the field [17]. This is in marked contrast to molecular biology, which may be much slower and relies on specialist, well equipped laboratories.

4.3 Chemotaxonomy

Chemotaxonomy involves the investigation of chemical constituents of bacteria, and is particularly useful for the study of Gram-positive bacteria. The molecules studied include fatty acids, polar lipids, lipopolysaccharide [nature of the chain length of the fatty acid and the sugar in the Lipid A moiety; 89], menaquinones, naphthoquinones, ubiquinones, mycolic acids, peptidoglycan, polyamines, teichoic and teichuronic acids and isoprenoid quinones [125]. Mycolic acids, which are useful taxonomic markers, are present in Gram-positive bacteria with high G+C ratios of their DNA [26], and have been reported for a range of fish pathogens, including *Mycobacterium chelonei* subsp. *piscarium* [8] and *M. shottsii* [117]. The length of the mycolate side chain has been correlated to 16S rRNA gene sequence homology [147]. Specific examples for which reliable chemotaxonomic data exist for Gram-positive bacterial fish pathogens are detailed below:

4.3.1 *Lactococccus piscium*

The long chain cellular fatty acids of *Lactococcus piscium* were reported to be straight chain saturated, mono-unsaturated and cyclopropane-ring types. The major acids corresponded to hexadecanoic acid, Δ 11-octadecanoic acid and Δ 11-methylenoctadecanoic acid [160].

252 **4.3.2** ***Mycobacterium neoarum***

253 The cell wall chemotype has been given as IVA, with glycolated muramic acids,
254 mycolic acids and MK-9, as the predominant isoprenoid quinone, being present [18].

255

256 **4.3.3** ***Nocardia***

257 *Nocardia salmonicida* contains LL-diaminopimelic acid (DAP) and glycine but not
258 meso-DAP, arabinose or galactose in the cell wall (i.e. Type I). The major cellular
259 fatty acids are hexdecanoic, octadecanoic, octadecanoic and 10-methyloctadecanoic
260 acid [60].

261

262 *N. seriolae* contains *meso*-diaminopimelic acid, arabinose and galactose,
263 indicative of chemotype IVA. Mycolic acids containing 44-58 carbon atoms are
264 present. The cellular fatty acids are dominated by *n*-$C_{16:0}$, *n*-$C_{16:1}$ and *n*-$C_{18:1}$; 10-
265 methyl-$C_{19:0}$ is also present as a major component in some isolates. The predominant
266 isoprenoid quinone is tetrahydrogenated menaquinone with 8 isoprene units [60].

267

268 **4.3.4** ***Renibacterium salmoninarum***

269 Chemotaxonomic traits of *R. salmoninarum* have been highlighted in part because of
270 the comparative difficulty with obtaining conventional phenotypic test results. Thus,
271 the cell wall peptidoglycan was deduced to contain D-alanine, D-glutamic acid,
272 glycine and lysine as the diamino acids [44]. The principal cell wall sugar was
273 glucose, although arabinose, mannose and rhamnose were also present; mycolic acids
274 were absent [123]. Methyl-branched fatty acids form >92% of the total fatty acid
275 component of the cells, with 12-methyltetradecanoic (anteiso-C_{15}), 13-

276 methyldecanoic (iso-C_{15}) and 14-methylhexadecanoic (anteiso-C_{17}) as the major
277 components. Straight chain fatty acids generally account for 1% of the total fatty
278 acids, and unsaturated fatty acids are not detected at all. Over 81% of the total fatty
279 acids are composed of the lower melting point anteiso acids, which may contribute to
280 membrane fluidity at low temperatures. Unsaturated menaquinones with nine
281 isoprene units are present. All strains contain diphosphatydylglycerol, two major and
282 six or seven minor glycolipids and two unidentified minor phospholipids [40].

283

284 **4.4 Molecular/genetic methods**

285 Molecular/genetic methods involving 16S rRNA gene sequencing [47], reverse
286 transcriptase-sequencing [75, 124] and polymerase chain reaction (PCR)-based gene
287 sequencing [121] have been useful additions to the armoury of techniques applicable
288 to bacterial taxonomy [125, 147]. DNA hybridization, which is regarded as the "gold
289 standard" for demonstrating the presence of absence of new species, was introduced
290 into bacterial taxonomy during the 1960s [e.g. 34]. Genotypic classification involving
291 sequencing of the 16S and 23S RNA genes [the latter is less popular] is regarded as
292 the definitive standard for determining phylogenetic relationships of bacteria [47,
293 125]. In particular, the genes are regarded as having all the attributes of useful,
294 relevant and stable biological markers being present and homologous in all bacteria.
295 Also, they are not prone to the effects of gene transfer [125]. Yet, the exact homology
296 values have a profound effect on interpretation of the outputs. Thus, homology values
297 of ≤98.7% [97% according to 147] indicates membership of different species, and this
298 correlates well with DNA hybridization results. Yet, occasionally higher homology
299 values may be attributed to distinct species groupings [46]. By themselves, 16S rRNA

300 gene sequences are insufficient to describe a new species, but may be used
301 indicatively and in conjunction with DNA:DNA hybridization [147]. However,
302 sequencing has permitted the recognition of new variants. For example, sequencing
303 revealed a new variant among Israeli isolates of *Streptococcus iniae* [74]. Moreover,
304 16S rRNA cataloguing has been useful in providing information about the position of
305 species in existing classifications. Thus, small-subunit rRNA sequencing and
306 DNA:DNA hybridization revealed that *Pasteurella piscicida* was related to
307 *Photobacterium damsela* leading to the proposal that the pathogen be re-classified as
308 *Ph. damsela(e)* subsp. *piscicida* [50]. Furthermore, *R. salmoninarum* was deduced to
309 be a member of the actinomycete subdivision, being related to *Arthrobacter,*
310 *Brevibacterium, Cellulomonas, Jonesia, Micrococcus, Promicromonospora,*
311 *Stomatococcus* and *Terrabacter* [56, 139]. The evolutionary relationship of *R.*
312 *salmoninarum* to *Arthrobacter* was reinforced as the result of genome sequencing,
313 which suggested that the genome of the former had been reduced significantly since
314 its divergence from a common ancestor [156].

315

316 Nucleic acid fingerprinting methods, including amplified fragment-length
317 polymorphism PCR (AFLP), pulsed field gel electrophoresis (PFGE), random
318 amplified polymorphic DNA (RAPD), rep-PCR (repetitive element primed PCR),
319 REP-PCR (repetitive extragenic palindromic-PCR), ERIC-PCR (enterobacterial
320 repetitive intergenic consensus sequences-PCR), BOX-PCR (derived from the boxA
321 element) and ribotyping, provide information at or below the subspecies level [147].
322 Of these, AFLP and ribotyping are extremely useful and standardized.

323

It is unfortunate that with the increasing use of molecular methods, the description of bacterial groups has been often met with the use of minimal phenotypic data, which causes problems for diagnostics especially in laboratories, which are not equipped for molecular biology [17]. In these situations where distinguishing phenotypic feature have not been or could not be provided then the species should be referred to as a geno[mo]species. Nevertheless, molecular methods have revolutionized taxonomy, and led to the description of an increasing number of new taxa. The methodologies may be culture-independent, allowing for the study of uncultured organisms but there are issues with genomic fluidity [125]. "*Candidatus"* describes uncultured prokaryotes for which phylogenetic relationships have been determined, and authenticity confirmed by methods such as *in situ* probing [125].

Sequencing of the 16S rDNA is becoming an accepted procedure for the identification of fish pathogens, for example *V. harveyi* [116] and confirming its synonymy with *V. carchariae* [49, 110], and has been instrumental in the recognition of new pathogens, including *Streptococcus dysgalactiae* [99], *S. parauberis* [previously recognised as *S. uberis* genotype II; 37] and *Vagococcus salmoninarum* [128, 154], and confirmed the presence of *Lactococcus garvieae* in Taiwan [29].

DNA:DNA and RNA:DNA hybridization, 16S RNA cataloguing, and 5S and 16S rRNA sequencing techniques have been used with increasing regularity and success. A review of PCR with emphasis on validation of the techniques and problems with diagnosis has been published [57]. PCR has been used successfully to identify hard-to-isolate fish pathogens, such as *Mycobacterium* spp. in sea bass

348 (*Dicentrarchus labrax)* [72] and *M. chelonei* in a cichlid oscar *(Astronotus ocellatus)*
349 [91]. Moreover, PCR has been useful with distinguishing different species from
350 within the same genus, such as *Lactococcus garvieae* from *L. lactis* [162], from
351 related genera, i.e. *L. garvieae, S. difficilis, S. iniae* and *S. parauberis* [88], and with
352 an admirable level of specificity [7].

353 The sensitivity of PCR is clearly a positive attribute particularly with slow
354 growing and/or nutritionally fastidious pathogens that are otherwise difficult to study
355 in the laboratory. Of relevance, a PCR was developed [77, 78], which detected only
356 22 cells of *R. salmoninarum;* a sensitivity of 10 cells was reported by others [85].
357 Similarly, PCR detected only 10^2 colony forming units (CFUs) of *N. seriolae* in
358 yellowtail [95].
359
360 A recent development is multi locus sequence analysis (MLSA), which
361 permits the genotypic examination of micro-organisms by comparison of the
362 sequences of multiple, i.e. 12 or more, house-keeping genes. The benefit of using
363 multiple genes is that the outputs are more informative and less likely to generate
364 results that are distorted by recombination of single loci [125]. The resulting
365 phylogenetic trees are capable of recognizing deeply branching clusters and permit
366 the delineation of groups within a species or genus [138].

367

368 **5. New species of fish pathogens recognized by 16S rRNA sequencing**

369 16S rRNA sequencing has helped the description of fish pathogens where phenotypic
370 characterization alone does not permit their incorporation in classifications. For
371 example, a new disease of Atlantic salmon (*Salmo salar*) was linked to the

372 *Streptobacillus moniliformis* and the fusobacteria group on the basis of sequence
373 homology; biochemical traits did not permit identification [86]. The newly described
374 cause of a mycobacteriosis in Chesapeake Bay (USA) striped bass *(Morone saxatilis)*
375 was equated to a new species, *M. shottsii,* with confirmation by 16S rRNA sequence
376 homology in which the pathogen was linked most closely to *M. marinum* and *M.*
377 *ulcerans (*similarity = 99.2%) [117]. In one study, *M. gordonae* was identified by 16S
378 rRNA sequencing [122]. **Furthermore, phylogenetic analysis based on 16S rRNA**
379 **gene sequencing together with partial sequences from the 65 kDa heat-shock**
380 **protein (hsp65) and the beta-subunit of the bacterial RNA polymerase (*rpoB*)**
381 **genes and the 16S- 23 S internal transcribed spacer 1 (ITS 1) region named other**
382 **novel mycobacteria as *M. stomatepiae* and *M. barombii* [115].**

383

384 During an examination of 16S rRNA sequences, two isolates of motile
385 aeromonads from diseased elvers in Spain were described as a new species,
386 *Aeromonas allosaccharophila* [87], albeit phenotypically heterogeneous [59]. This
387 heterogenicity has caused problems for reliable phenotypic-based diagnoses.

388

389 *Francisella* became recognized as the cause of a new disease of Atlantic cod
390 (*Gadus morhua*) in Norway in which the affected fish displayed white granuloma in
391 the viscera and skin. Isolates were recovered, and determined to possess the key
392 phenotypic characters of *Francisella,* viz non-motile, strictly aerobic Gram-negative
393 intracellular coccobacilli which produced H_2S from cysteine-containing media [94].
394 16S rRNA sequencing revealed a 99.17% homology to *Francisella philomiragia*
395 [100], although a slightly higher value of 99.3% was published [94] with the proposal

396 for a new subspecies, i.e. *Francisella philomiragia* subsp. *noatunensis*, to
397 accommodate the organisms. There was 92.2-99.0% homology with *Francisella*
398 *philomiragia* housekeeping genes, *groEL, shdA, rpoB, rpoA, pgm* and *atpA*. A
399 DNA:DNA hybridization of 68% was recorded between the fish pathogen and
400 *Francisella philomiragia* [94].
401
402 *Pasteurella skyensis* was recovered from diseased Atlantic salmon in Scotland,
403 linked to the family Pasteurellaceae by phenotypic analysis, and elevated to a new
404 species largely as a result of 16S rRNA sequencing that identified the closest
405 neighbour as *Pasteurella phocoenarum* [homology = 97.1%; 24].
406
407 *Piscirickettsia salmonis* was named to accommodate isolates from diseased
408 salmon in Chile, of which LF-89 was studied in detail [48] with 16S rRNA
409 conforming to the gamma subdivision of the Proteobacteria with similarities to the
410 family Rickettsiales, and in particular *Wolbachia persica* (similarity = 86.3%) and
411 *Coxiella burnetii* (similarity = 87.5%) more than to representatives of *Ehrlichia,*
412 *Rickettsia* or *Rochalimaea* leading to the description of a new genus and species [48].
413 Other rickettsias not conforming exactly with *Piscirickettia salmonis* have been
414 described. For example, an organism recovered from white sea bass (*Atractascion*
415 *nobilis*) was reported to have a 96-3-98.7% 16S rDNA homology with *Piscirickettsia*
416 *salmonis* [9], which was considered by the authors to be too low for a confirmed
417 identity. A Tasmanian isolate from Atlantic salmon was distinct from *Piscirickettsia*
418 in terms of sequence alignment of the 16S rRNA, and for the present regarded as a
419 rickettsial-like organism [RLO; 31].
420

421 *Pseudomonas plecoglossicida,* the causal agent of bacterial ascites of ayu
422 *(Plecoglossus altivelis),* was described as a new species as a result of 16S rRNA gene
423 sequence analysis confirming distinctiveness from *P. putida* biovar A. DNA:DNA
424 hybridization confirmed the isolates to be a new centre of variation insofar as <50%
425 homology was recorded with other pseudomonads, including *P. putida* [98].
426
427 *Streptococcus phocae* was recognized as a cause of systemic disease in
428 Atlantic salmon farmed in Chile. Phenotypic testing linked the pathogen with the
429 streptococci, notably *Gemella,* but analysis of 16S rRNA genes provided a link to *S.*
430 *phocae* [118].
431
432 *Tenacibaculum soleae* was recovered from diseased sole *(Solea senegalansis)*
433 in Spain, and confirmed as a new species largely on account of 16S rRNA homology
434 values of 94.8-96.7% with other members of the genus [111].
435
436 Two groups of bacteria were recovered from Atlantic salmon with winter ulcer
437 disease/syndrome [81], of which one cluster was found to be closest to *Moritella*
438 *marina* (43% re-association by DNA:DNA hybridization), and was named as *V.*
439 *viscosus*. By 16S rDNA sequencing, the closest match was with *Moritella* [81] and
440 *M. marina* (99.1% sequence homology) so that the organism was re-classified to
441 *Moritella,* but as a new species, as *M. viscosa* [22], despite the high sequence
442 homology [125]. Separately, 19 Icelandic and one Norwegian isolate from shallow
443 skin lesions on Atlantic salmon, and the type strain of *V. marinus* NCIMB 1144 were
444 identified as *V. marinus* after an examination of phenotypic data and analyses by

445 numerical taxonomy [21]. On the basis of 16S rRNA sequencing, the species was
446 transferred to *Moritella as M. marina* [152].

447

448 **5.1 New and uncultured fish pathogens: *'Candidatus"***

449 Molecular techniques have permitted the recognition of uncultured pathogens
450 belonging to new groupings for which the name of '*Candidatus*' has been used.
451 '*Candidatus* Arthromitus' has been recovered from rainbow trout (*Oncorhynchus*
452 *mykiss*) with summer enteritic syndrome, which is a gastro-enteritis [35, 93]. The
453 organism was observed in histological preparations to which nested polymerase chain
454 reaction was used, with confirmation by sequencing [36]. *'Candidatus* Piscichlamydia
455 Salmonis*'* was detected by RT-DGGE in intracellular inclusions, i.e. epitheliocysts, in
456 Atlantic salmon with proliferative gill inflammation [142]. *'Candidatus*
457 Clavochlamydia Salmonicola' is an intracellular organism, causing epitheliocystis in
458 Atlantic salmon, which was recognized as novel as a result of 16S rRNA sequencing
459 [65].

460

461 **6. Taxonomic developments associated with specific bacterial fish pathogens**

462 From the early literature, a question-mark has hung over the reliability of some
463 bacterial names insofar as there was often negligible evidence to support the use of
464 those names. Concern may also be expressed about the value of studies based on only
465 single isolates where concern about the reasons for choice of the culture may be aired.
466 Some of the controversy surrounding specific diseases/pathogens follows:

467

468 **6.1 Motile aeromonas septicaemia**

469 *Aeromonas hydrophila* (= *A. formicans* and *A. liquefaciens)* would appear to have
470 worldwide distribution and to be a pathogen, causing motile aeromonas septicaemia,
471 of many species of freshwater fish. Indeed, there are reports of a spread into marine
472 fish, notably ulcer disease of cod [76]. Since its initial recognition in the literature, a
473 wealth of knowledge has been accumulated about many facets of its biology [see 17].
474 A new variant *A. hydrophila* subsp. *dhakenis,* which was originally covered from
475 children with diarrhoeae in Bangladesh, was determined to be pathogenic to rainbow
476 trout [103]. However overall, there has been some doubt about the role of *A.*
477 *hydrophila* as a pathogen, and in some cases it may well be present in fish tissue only
478 as a secondary invader [17]. Moreover with developments in the taxonomy of motile
479 aeromonads [28], the accuracy of some of the early published identifications may be
480 justifiably questioned. Could other motile aeromonads be associated with fish disease
481 and may have been confused previously with *A. hydrophila* [103]?

482

483 It is clear that there is phenotypic, serological and genotypic heterogeneity
484 within the descriptions of fish pathogenic *A. hydrophila* [e.g. 6, 84], and other motile
485 aeromonads have been implicated as the aetiological agents of (fish) diseases. Thus, 8
486 isolates reported as pathogenic to eel in Spain were identified by numerical taxonomy
487 with *A. jandaei* [41, 42]. Certainly, the current approach of allocating species names
488 as a result of the examination of 16S rRNA gene sequences has encompassed fish
489 pathogenic motile aeromonads. For example, isolates from diseased fish which were
490 recovered in *Aeromonas* DNA Hybridization Group 2 (= *A, hydrophila)* were equated
491 with a new group, *A. bestiarum* [5]. Subsequently, *A. sobria* (*A. sobria* biovar sobria
492 and *A. veronii* biovar sobria were reported as pathogenic to rainbow trout [103].
493 Indeed, *A. sobria* has been previously found to have a role as a fish pathogen, with

494 isolates recovered from wild spawning gizzard shad (*Dorosoma cepedianum)* in
495 Maryland, USA during 1987 [148, 149]. Also, *A. veronii* has been recovered from
496 Siberian sturgeon (*Acipenser baerii*) with identification of the pathogen resulting from
497 phenotyping and 16S rRNA gene sequencing [82].

498

499 **6.2 *Aeromonas salmonicida***

500 *Aeromonas salmonicida* is one of the oldest described fish pathogens, being isolated
501 initially from diseased hatchery-maintained brown trout (*Salmo trutta*) in Germany,
502 and named as 'Bacillus der Forellenseuche' or bacillus of trout contagious disease. The
503 history of the organism reveals a plethora of synonyms including *Bacillus devorans*,
504 *Bacterium salmonica*, *Bacterium salmonicida*, *Bacillus truttae* and *Bacillus*
505 *salmonicida* [17]. The 7th edition of *Bergey's Manual of Determinative Bacteriology*
506 (1957) placed the pathogen in the genus *Aeromonas* within the family
507 Pseudomonadaceae [134]. Later, there was a transfer to the family Vibrionaceae and
508 subsequently to its own family, i.e. the Aeromonadaceae [30]. Re-classification was
509 based primarily on phenotyping [54]. Thus in 1953, the first detailed description of
510 the pathogen was published, and from an examination of 10 isolates, it was concluded
511 that *Bacterium salmonicida* was homogeneous in cultural and biochemical
512 characteristics [54]. Numerous studies have addressed the homogeneity of the species
513 [e.g. 12]. The basic description is of an organism, which comprises non-motile
514 [motility and *flaA* and *flaB* flagellar genes have been reported; 92, 154], fermentative,
515 Gram-negative rods, which produce a brown water-soluble pigment on tryptone-
516 containing agar, do not grow at 37ºC, and produce catalase and oxidase [17]. Cultures
517 have the ability to dissociate into rough, smooth and G-phase (= intermediate)

518 colonies [38]. The pathogen has spread from its dominance in salmonids to cyprinids
519 and marine flatfish [17]. An ongoing issue surrounds the intraspecies structure, i.e.
520 the validity of subspecies *achromogenes, masoucida, pectinolytica, salmonicida* and
521 *smithia,* and the status of so-called atypical isolates.

522

523 *A. salmonicida* subsp. *salmonicida,* isolates of which have been obtained
524 almost exclusively from outbreaks of furunculosis in salmonids, is regarded as
525 homogeneous, and is referred to as "typical" [104]; all other isolates are considered as
526 heterogeneous and "atypical" [17]. So called atypical strains may demonstrate weak,
527 slow or non-pigment production [73, 97], catalase [62] or oxidase-negativity [e.g. 62,
528 157], nutritional fastidiousness for blood products [10], slow growth, i.e. ≥5 days
529 compared with 1-2 days for typical isolates [10, 62] and be pathogenic for fish other
530 than salmonids, e.g. cyprinids [e.g. 10, 53, 62] and marine flatfish, namely dab
531 (*Limanda limanda*), plaice (*Pleuronectes platessa*), flounder *(Platichthys flesus)* and
532 turbot *(Scophthalmus maximus)* [107, 158, 159], and cause ulceration rather than
533 furunculosis [17, 52]. The deviation in characteristics from the typical to atypical
534 isolates has made typing difficult [15, 58, 80]. Even 16S rDNA sequencing has not
535 helped with the clustering of atypical forms [e.g. 161].

536 Smith [131] recognized heterogeneity in the species description of *A.*
537 *salmonicida*. She examined six isolates of non-pigmented *A. salmonicida*, which
538 were clustered as Group I in her numerical taxonomy study, for which a separate new
539 species name was proposed, i.e. *A. achromogenes,* but the proposal was not adopted
540 widely. A second non-pigmented group was described by Kimura [67], and named as
541 *A. salmonicida* subsp. *masoucida.* Schubert [129] considered these non-pigmented

542 isolates as subspecies, and coined the names of *A. salmonicida* subsp. *achromogenes*
543 and *A. salmonicida* subsp. *masoucida,* respectively. Pigmented strains (= typical)
544 were classified as *A. salmonicida* subsp. *salmonicida* [129]. The precise relationship
545 of the subspecies has been the subject of detailed discussion. In particular, it was
546 contended that subsp. *achromogenes* and *masoucida* were more closely related to *A.*
547 *hydrophila* than to *A. salmonicida* [112]. Later, it was mooted that subsp. *masoucida*
548 bridged typical *A. salmonicida* and *A. hydrophila* [105]. Yet, *A. salmonicida* subsp.
549 *masoucida* is non-motile, sensitive to *A. salmonicida* bacteriophages, possesses an
550 antigenic profile specific to *A. salmonicida,* and shares a DNA homology of 103%
551 with *A. salmonicida* [84]. By PCR, a combination of *achromogenes* with *masoucida*
552 could be justified, but this was not substantiated by ribotyping and RAPD analyses
553 [15]. Phenotypic data suggest a case for combining subsp. *masoucida* with
554 *salmonicida,* and subsp. *achromogenes* with *Haemophilus piscium,* which is the
555 causal agent of ulcer disease of trout [135]. Examination of the small subunit rRNA
556 gene sequences revealed 99.9% homology of an authentic strain of *H. piscium* with *A.*
557 *salmonicida* subsp. *salmonicida* [145]. So far, the comparative uniqueness of subsp.
558 *smithia* has been indicated from several studies [e.g. 15]. The complication is with
559 aberrant strains of *A. salmonicida* from fish species other than salmonids.

560 DNA homology was used to reveal that all isolates of *A. salmonicida*
561 (including *A. salmonicida* subsp. *masoucida)* were highly related, i.e. 96-106%
562 homology, when hybridized against *A. salmonicida* subsp. *salmonicida* [84]. It was
563 opined that *A. salmonicida* subsp. *masoucida* and some atypical isolates did not
564 warrant separate subspecies status, because they were regarded as variants of other
565 well-recognized groups. Also as a result of genotypic analyses, it was reported that
566 typical and atypical isolates of *A. salmonicida* were very closely related, with minimal

567 divergence [90]. Using DNA:DNA re-association, it was concluded that typical *A.*
568 *salmonicida* were recovered in a homogeneous group, whereas the atypical
569 representatives were more diverse [20]. From numerical taxonomy and DNA:DNA
570 hybridization, similar conclusions regarding the homogeneity of typical isolates of *A.*
571 *salmonicida* [12]. However using 16S rRNA sequencing techniques, it was reported
572 that subspecies *achromogenes* and *masoucida* were indistinguishable, and only
573 differed from subspecies *salmonicida* by two bases [87].

574

575 The relation of *A. salmonicida* to other aeromonads has been discussed. Eddy
576 [39] focused on the inability of *A. salmonicida* to produce 2,3-butanediol from
577 glucose, and the absence of motility, which were in contrast to the genus description
578 [71]. A new genus, i.e. *Necromonas,* was proposed with two species, namely *N.*
579 *salmonicida* for the typical isolates and *N. achromogenes* for the non-pigmented
580 strains [131]. This proposal was not formally widely accepted, although Cowan [32]
581 used the suggestion in his landmark identification scheme for medically important
582 bacteria. Subsequent serological and bacteriophage sensitivity data supported the
583 relationship between *A. salmonicida* and the motile aeromonads. Common antigens
584 between *A. hydrophila* and *A. salmonicida* subsp. *masoucida* and other isolates of *A.*
585 *salmonicida* were reported [68, 105]. Furthermore, serological cross-reactions
586 between *A. salmonicida* and motile aeromonads were discussed [79]. Moreover, *A.*
587 *hydrophila* cultures were found to be sensitive to *A. salmonicida* bacteriophages [113,
588 114]. The outcome of all the studies is that DNA homology supports the classification
589 of *A. salmonicida* in the genus *Aeromonas* [e.g. 20, 84, 105].

590

There are certainly outstanding questions about the validity and taxonomic placing of *Haemophilus piscium* [135], but an authentic reference strain was not deposited any in any recognized culture collection at the time of its first isolation. Later, it was concluded that the organism was not a *bona fide Haemophilus* because of the lack of requirement for haemin or NAD [66]. In particular *H. piscium* differed from the type species of the genus, *H. influenzae,* in the inability to reduce nitrate or alkaline phosphatase and to grow at 37ºC, in conjunction with a higher G+C ratio of the DNA. It was commented that there was only a low similarity between *H. piscium* and other *Haemophilus* spp. in a numerical taxonomic study [27]. A link with atypical, achromogenic *A. salmonicida* was made [105]. This link was reinforced by bacteriophage sensitivity, when it was concluded that *H. piscium* is an atypical *A. salmonicida* [150]. Other workers have supported this view [e.g. 15]. However with the absence of an authentic, original type strain, the definitive taxonomic position of *H. piscium* is only speculative.

A lack of congruence has been reported between the results of molecular (PCR, RAPD and ribotyping) and phenotypic methods in taxonomy of aeromonads [15]. Moreover, there are problems of inter-laboratory differences and lack of standardisation in test methods [33]. The outcome is that the definitive classification of *A. salmonicida* has not been achieved, to date.

6.3 Enteric redmouth (ERM)

613 There has been discussion about the taxonomic position of the aetiological agent of
614 ERM. Strong agglutination with *Salmonella enterica* subsp *arizonae* O group 26, and
615 a weak reaction with O group 29 was reported [119]. In addition, biochemical
616 similarities with enterics, notably *Enterobacter liquefaciens, Serratia marcescens*
617 subsp. *kiliensis* as well as *Salmonella enterica* subsp. *arizonae* were mentioned [119].
618 Serological cross-reactions were also recorded with *Hafnia alvei* [143]. Nevertheless,
619 a new species, i.e. *Yersinia ruckeri* was described although there was only a 30-31%
620 DNA homology with *Y. enterocolitica* and *Y. pseudotuberculosis* [43]. This compares
621 to DNA homologies of 24-28% and 31% with *Serratia marcescens* and *Serratia*
622 *liquefaciens,* respectively [140]. Indeed, it has been suggested that the causal agent of
623 ERM should belong in a new genus of the Enterobacteriaceae [23]. A complication
624 developed when a new non-motile form of the pathogen was recovered from rainbow
625 trout. By 16S rRNA sequencing and a homology of 100%, the organisms were linked
626 to *Y. ruckeri* but regarded as a new biogroup [16]. Similar non-motile variants were
627 also recovered from previously vaccinated rainbow trout in Spain [45].

628

629 **6.4 Vibriosis**

630 The causal agent of 'red-pest' in eels was first designated as *Bacterium anguillarum*
631 [17]. Subsequently, an outbreak among eels in Sweden led to the use of the name
632 *Vibrio anguillarum*. Numerous studies have pointed to heterogeneity in *V.*
633 *anguillarum* initially with the delineation of two sub-groupings/biotypes [e.g. 19].
634 This increased to three [130] and then four sub-groups/phena within the species
635 definition [64, 106]. Ribotyping has confirmed the heterogeneity [102], although a
636 single taxon, homogeneous by ribotyping but heterogeneous by LPS profiles, plasmid

637 composition, serogrouping, and BIOLOG-GN fingerprints and API 20E profiles was
638 described [13, 14]. Biotype II became recognized as a separate species, i.e. *V. ordalii*
639 [126], which is homogeneous by plasmid profiling, ribotyping and serogrouping,
640 accommodates two LPS groups, but is heterogeneous by BIOLOG-GN fingerprints
641 and API 20E profiles [14].

642

643 Serology has been widely used for diagnosis, but has complicated the
644 understanding of *V. anguillarum* [25], and the establishment of serotypes has to some
645 extent traversed species boundaries. With *V. anguillarum,* serogroup 02 and 05, there
646 are common antigens with *V. ordalii* [96] and *V. harveyi* [13], respectively. Initially,
647 three serotypes were recognised for isolates from salmonids from the northwest USA,
648 Europe, and the Pacific-northwest (USA) [104]. This number increased to six [71],
649 and then 10 [137] and upwards [55, 102]. Serogroup O1 dominates the number of
650 isolates available for study and the relative importance to fish pathology [13, 108,
651 109]. Serogroup O2 has been further subdivided into serogroup O2a and O2b [146].

652

653 *V. anguillarum* was re-classified initially to *Beneckea* [19] and then to a newly
654 proposed genus *Listonella* [83], but the changes were not widely accepted.

655

656 **7. The role of phylogenetics in bacterial fish pathology**

657 The techniques described above are relevant for the taxonomy of bacterial fish
658 pathogens. Yet, molecular methods, namely sequencing of the 16S rRNA gene,
659 permit the study of evolutionary relationships, i.e. phylogenetics, which may be

viewed as phylogenetic trees, which are interpreted by cladistics and used in defining taxa. The approach is essential in the study of the evolutionary tree of life, but is it strictly necessary for fish pathology and the recognition of species? One concern is the comparative fluidity by which genes may be exchanged, such as by horizontal gene transfer, and the impact of this movement on the outcome of the taxonomic/phylogenetic process.

8. Conclusions

There has been a resurgence of interest in bacterial taxonomy partially because of the current focus on biodiversity and the development of reliable molecular methods, notably 16S rRNA sequencing. Undoubtedly, these molecular approaches have led to greater confidence and accuracy in the reporting of bacterial names. Nevertheless, it is conceded that bacterial taxonomy is a specialist subject, which is not of interest to all fish pathologists. However, it cannot be overstated that there is a real value for good taxonomy as a means of communication. In terms of fish pathology, taxonomy enables the recognition of new pathogens, improvements in the understanding of relationships between taxa, an appreciation of variation within existing nomenspecies including the recognition of new subspecies and biogroups, and facilitates accurate commentary about all aspects of biology from epizootiology to pathogenicity.

For the future, a range of new techniques, including *in situ* hybridization, probe hybridization, microarray techniques and restriction enzyme digestion, are entering taxonomic use, and are likely to be used in fish pathology. The impact of these new approaches is difficult to predict, but will undoubtedly be incorporated in some fish bacteriology laboratories.

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