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Bacterial Fish Pathogens: Outcome of Molecular Studies for Taxonomy, Epidemiology and Identification

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ABSTRACT

Jean-François Bernardet (1996) Bacterial fish pathogens: outcome of molecular studies for taxonomy, epidemiology and identification. *Zoological Studies* **35**(2): 71-77. During the past 3 decades, molecular methods have been progressively introduced for studying bacterial pathogens. These methods have allowed comparisons of bacterial strains and species at the genomic level, and their use has yielded many interesting data. Each of these methods has a certain taxonomic validity range; thus, the molecular method(s) performed in the course of a given study must be adapted to the level of taxonomic data required. For instance, as the 16S rRNA fraction is an excellent "molecular clock", the methods used for investigating the sequence of this molecule (first by oligonucleotide cataloging, then by DNA/rRNA hybridization and actual sequencing) were mostly effective for determining basic relationships among bacteria. Thus, they have yielded interesting information on bacterial phylogeny in the form of dendrograms. Another widely useful molecular method is DNA/DNA hybridization. Utilizing several different techniques, it has proved to be the best way to compare bacterial strains for the purpose of determining whether or not they belong to the same bacterial species. Thus, new bacterial species can be described based on firm genomic grounds. Data obtained by both 16S rRNA investigations and DNA/DNA hybridizations have produced many taxonomic and nomenclatural consequences. Gene detection and production of DNA fingerprints (by restriction endonuclease digestion, hybridization with specific probes, polymerase chain reaction, or any combination of these techniques) potentially allow the detection and identification of bacterial species as well as comparisons of strains within a bacterial species. Thus, these methods became increasingly applicable in diagnosis of infectious diseases and in epidemiological studies. There has been a delay between the application of genomic methods for characterizing human bacterial pathogens and their use for investigating fish-pathogenic bacteria. It is only recently that these techniques have been used for study of such important fish pathogens as *Aeromonas salmonicida*, *Vibrio anguillarum*, *Renibacterium salmoninarum*, and the *Flavobacterium-Cytophaga* group; but the first results are highly encouraging and the future of DNA-based methods in fish bacteriology seems promising.

Key words: DNA/DNA hybridization, 16S rRNA, DNA fingerprints, Molecular probes.

INTRODUCTION

Since the origins of the microbiological sciences, descriptions of bacterial strains and species, as well as their comparison for identification or taxonomic purposes, have relied exclusively on phenotypic characteristics. Early identification and classification schemes were based entirely on

morphological information. Later, investigations on bacterial metabolism allowed the definition of biochemical and physiological characteristics which were integrated with the previous schemes. More precise data were then obtained through chemical analysis of the main components of bacterial cells, i.e., polyamines, respiratory quinones, carbohydrates, proteins, and fatty acids. Some of these phenotypic characteristics proved to be useful chemotaxonomic markers, and very interesting results were obtained by numerical analysis of data from numerous bacterial strains, greatly improving the classification of many bacterial groups. In spite of the considerable progress recently introduced in bacteriological studies which are the topic of this communication, phenotypic characteristics remain instrumental in defining and comparing bacterial strains and species, and they must not be abandoned.

However, it has become increasingly clear that purely phenotypic identification and classification schemes are not entirely reliable, and they frequently "failed to achieve the necessary requirements of predictivity, stability and objectivity" (Priest and Austin 1993). The basic reason for these deficiencies is the purely subjective choice of the "important" phenotypic features to be taken into account, leading to frequent disagreement among bacteriologists. Some very good examples of these problems can be found in fish-pathogenic bacterial species. For instance, the presence of gliding motility was long believed to be of utmost importance for characterizing *Cytophaga* and *Flexibacter* species; it was later demonstrated that this feature is not exhibited by all species assigned to these genera, that several very distant bacterial groups are also able to glide, and that this type of motility is extremely dependent on growth conditions. The presence of a yellow pigment for defining the genus *Flavobacterium* is another example of such a feature whose irrelevance for generic delineation is now well established.

Because the limits of use of phenotypic characteristics were recognized many years ago, molecular methods were progressively introduced, mainly during the last 3 decades. These methods allow a comparison of bacterial strains and species at the genomic level because such macromolecules as DNA and RNA contain a large amount of information. As the bacterial chromosome accumulates mutations in the course of evolution, analysis of its sequence may give information on evolutionary pathways, and comparison of sequences from different bacteria may allow their divergence from a common ancestor to be traced. The composition of many bacterial components such as cellular fatty acids and proteins is more or less affected by growth conditions, but that is not the case with nucleic acids; these molecules are thus the only ones which can be used for objective comparison and classification of bacteria.

Before giving more details concerning the main genomic methods increasingly used in bacteriology, some information about the DNA base composition (guanine-plus-cytosine content of DNA) should be discussed. Among other techniques, the relative amount of guanine-plus-cytosine (%G + C) in double-stranded bacterial DNA can be determined through its thermal denaturation mid-point (Marmur and Doty 1962). The range in base composition within the bacterial world is extremely wide (from about 25% to 75%) but it is constant for a single bacterial species. As the base composition of a bacterial DNA is not related to the sequence of its bases, 2 very different organisms may share identical %G + C values. Conversely, if 2 bacterial species exhibit very different %G + C values, they are likely to be only distantly related. Thus, the DNA base composition alone can readily demonstrate that 2 bacteria are very different, but it cannot be used as the only criterion for grouping 2 bacteria together. However, the G + C content is still considered an useful characteristic of a bacterial genus, providing that many other phenotypic and genomic features are also taken into account. When considering the wide range in %G + C values among certain bacterial genera, such as *Flavobacterium, Flexibacter* and *Cytophaga* (all of which include some fish-pathogenic species), it is very clear that they are, in fact, very heterogeneous and that they certainly should be split into several genera (Holmes et al. 1984, Reichenbach 1989).

Several procedures are now based on the study of bacterial DNAs and RNAs, but it is very important to realize that each of them has a certain taxonomic validity range; thus, the molecular method(s) performed during the study of a given bacterial group must be adapted to the level of taxonomic information required (De Ley 1992). For instance, the value of %G + C can be used as one of the characteristics defining a bacterial genus, but it gives no information at either the level of the species or of higher taxa, such as families.

It is not the purpose of this short review to give an exhaustive list of the genomic methods presently used in bacteriology. In the following paragraphs, only the main techniques as well as

some examples of the results obtained with bacterial fish pathogens will be discussed; these examples are taken from the literature as well as from our own recent studies.

ANALYSIS OF RNA

Among the 3 kinds of RNA present in all bacteria, mRNA, tRNA, and rRNA, the latter is the only one for which analysis has produced useful taxonomic information to date. Due to its small size, 5S rRNA apparently contains little information. 16S and 23S rRNAs are much larger molecules and may yield more valuable data (Priest and Austin 1993). Until now, 16S rRNA has been, by far, the most widely used. This molecule may reveal relatedness between distantly related organisms because, due to its essential function in the cell, its structure has virtually remained unmodified throughout the course of evolution. But it may also be used to compare rather similar bacteria because some regions in its sequence have evolved more rapidly than others. Thus, analysis of 16S rRNA sequence may be efficient in a very wide taxonomic range, from the level of the species to very deep branching between different phyla (Woese 1987). This is why, during the last decade, rRNA studies have yielded so much interesting, and often surprising, data concerning bacterial phylogeny, most conveniently presented in the form of dendrograms. Some of these data have had spectacular taxonomic and nomenclatural consequences.

In order to present the 3 different procedures used for investigating 16S rRNA, we shall use as an example the large bacterial group which includes several fish-pathogenic species, i.e., the *Bacteroides-Flavobacterium-Cytophaga* group.

"Oligonucleotide cataloging" was the 1st method used; the 16S rRNA molecule was fragmented into oligonucleotides, which were then sequenced separately. This method showed that the *Bacteroides-Flavobacterium-Cytophaga* group is comprised of 2 major branches, one including the members of the genus *Bacteroides*, the other grouping the genera *Flavobacterium, Flexibacter* and *Cytophaga* (Paster et al. 1985).

This bacterial group was then extensively investigated when DNA/rRNA hybridization techniques became available. A probe consisting of the labelled rRNA of a reference strain is hybridized with a collection of DNAs from other bacteria fixed on filters; the thermal resistances of the hybrids are then tested and compared in order to draw a dendrogram. This technique, in which many strains and species can be included, yields a much more detailed view of the branching levels among the taxa belonging to the *Bacteroides-Flavobacterium-Cytophaga* group (Vandamme et al. 1994). These data demonstrated that the fish-pathogenic *Flexibacter* species (e.g., *Flexibacter columnaris*, *F. psychrophilus* and *F. maritimus*) are in fact quite distant from the *Flexibacter* and *Cytophaga* type species. Because the 2 freshwater species belong to a very coherent rRNA cluster comprising the type species of the genus *Flavobacterium*, *F. aquatile*, it was proposed to include both species within this genus, under the names *Flavobacterium columnare* and *Flavobacterium psychrophilum*. This study also resulted in an emended description of the genus *Flavobacterium* and of the family *Flavobacteriaceae* (Bernardet et al. 1996).

The 3rd and most recent procedure for comparing 16S rRNAs is the actual sequencing of the complete molecules or of the corresponding genes. Because this technique is rather difficult to handle, it is not possible to include many strains in this kind of study, and only the type strains are usually sequenced. This method is more discriminatory and powerful than DNA/rRNA hybridization for revealing deep phylogenetic relationships (Woese 1987). rRNAs of many species belonging to the *Bacteroides-Flavobacterium-Cytophaga* group have now been sequenced, and the dendrograms are very similar to those drawn from DNA/rRNA hybridization (Gherna and Woese 1992, Nakagawa and Yamasato 1993). Sequence analysis of rRNAs also has revealed that the fish-pathogenic gliding bacteria are phylogenetically very distant from the true myxobacteria, with which they have long been confused (Woese 1987).

The rRNAs of several other fish-pathogenic bacteria have been studied, including *Aeromonas* (Martinez-Murcia et al. 1992), *Renibacterium* (Gutenberger et al. 1991), and *Vibrio* (Ruimy et al. 1994), resulting in important taxonomic and phylogenic data. As with the other molecular methods, the use of RNA analysis must be restricted to the taxonomic range in which it is really applicable. Indeed, several new bacterial species have been published on the basis of their rRNA sequences alone. This practice, and the resulting problems, have recently been discussed by several authors (Fox et al. 1992, Martinez-Murcia et al. 1992, Stackebrandt and Goebel 1994), who all concluded that identity of 16S rRNA sequences is not always correlated with high DNA homology.

DNA/DNA HYBRIDIZATION

In contrast to the previous procedure, DNA/DNA hybridization is mostly effective in determining the relationships between bacteria at the species level. The similarities in the base sequences of 2 bacterial species is revealed by DNA reassociation experiments: the 2 DNAs are mixed and denatured, and the amount of hybrid DNA molecules formed after renaturation is assessed. The greater the level of hybridization, the greater the similarity between the 2 DNAs. Several techniques exist, but one of the most widespread uses thermal denaturation, reassociation in free solution, radiolabelling of one of the DNAs, and use of S1 nuclease for separating the hybridized molecules from the remaining single strands. The determination of the thermal stability of the hybrids (ΔT_m) may be necessary when the DNA relatedness is not very high (Popoff et al. 1981).

DNA/DNA hybridization has been extensively used for more than 2 decades for determining the DNA relatedness (or DNA homology) between bacterial strains. It is thus possible to know whether several strains belong to the same species, and to compare strains to valid species in order to determine if they belong to one of them, or if they represent a new species. The problem is to decide the level of DNA relatedness at which 2 bacterial strains are accepted as belonging to the same species. A group of taxonomists proposed the following rule: "The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA/DNA relatedness and with 5 °C or less ΔT_m. Both values must be considered" (Wayne et al. 1987). This report also strongly suggests that such genospecies should also be clearly defined by several phenotypic characteristics.

The usefulness of DNA homology studies is thus mainly in taxonomy. By using DNA/DNA hybridization, we were able to delineate the fish pathogenic species *Flexibacter columnaris*, *F. maritimus* and *F. psychrophilus*. These species formed 3 distinct genomic groups, with very high levels of DNA homology within each group and very little or no DNA homology between the groups or with any other species in the genus *Flexibacter* and related genera. Emended descriptions of these species were proposed and type strains were designated (Bernardet and Grimont 1989). A similar study was performed with *Pseudomonas anguilliseptica* strains isolated from several fish species in France; DNA/DNA hybridization with the type strain, and with several other reference strains, confirmed the original identification based on several phenotypic features (Michel et al. 1992).

Many other fish-pathogenic bacteria have been studied by DNA/DNA hybridization. This method was instrumental in creating the new species *Flexibacter ovolyticus*, a pathogen of halibut eggs, as the overall phenotypic characteristics were very similar to those of *Flexibacter maritimus* (Hansen et al. 1992). Other pathogens of marine fish for which DNA homology was investigated include several *Vibrio* species (Staley and Colwell 1973).

Very valuable results have been obtained by combining rRNA studies and DNA/DNA hybridization, particularly for allocating new isolates to existing or new taxa. A recent example is the description of the newly characterized fish pathogen *Flavobacterium scophthalmum* (Mudarris et al. 1994). Several bacterial strains were isolated from the marine environment and from diseased turbot in Scotland; an extensive phenotypical study demonstrated that all the isolates were very similar, and that they probably belonged to the *Bacteroides-Flavobacterium-Cytophaga* group (Mudarris and Austin 1989). In a DNA/rRNA study, several probes (consisting of the radiolabelled rRNAs from representative species of the main rRNA branches comprising this group) were hybridized with DNAs from the 2 isolates. The resulting data showed that the turbot pathogen belongs to a branch grouping several *Flavobacterium* species from human or environmental origins. Subsequent DNA/DNA hybridizations with all the species in this branch demonstrated that the turbot isolates indeed belong to a new species, *Flavobacterium scophthalmum*. Further molecular studies resulted in a nomenclatural change, as the whole branch received a new generic name, *Chryseobacterium* (Vandamme et al. 1994). This example shows that rRNA analysis may not be informative at the level of the species but, as it assigns an unknown bacterium to a certain rRNA branch, subsequent DNA/DNA hybridizations are thus only necessary with other species on the same branch.

DNA FINGERPRINTING

An outstanding development of molecular methods in bacteriology has mostly been due to the introduction of several essential techniques that characterize the DNA molecule: agarose gel electrophoresis, fragmentation by restriction endonucleases, cloning and sequencing, Southern

DNA/DNA hybridization, and polymerase chain reaction (PCR). These techniques, used individually or in various combinations, allow the detection of given genes (using specific molecular probes) and the production of DNA fingerprints (Versalovic et al. 1993). Length polymorphism of DNA fragments is widely used; when such fragmentations result from digestion of the DNA by restriction enzymes, they are called Restriction Fragment Length Polymorphisms (RFLP). All these methods may be applied to plasmid, chromosomal, or total bacterial DNA; they yield very important informations on bacterial disease epidemiology, and they are sometimes used to perform diagnostic tests. In both cases, they offer the advantages of reproducibility, sensitivity, and discriminatory ability; in the case of epidemiological studies, their ability to differentiate distinct isolates is an additional advantage (Versalovic et al. 1993). Indeed, they may represent new typing methods which can replace or complement more classical typing techniques such as serology. Again, an exhaustive list of such methods will not be given, but some of the most important ones are detailed and some examples of their recent use in fish bacteriology are provided.

1. A 1st group of molecular methods uses restriction endonuclease digestion, but no DNA amplification. Plasmid profiles can be obtained and plasmid-based RFLP analysis may be performed; a specific probe may then detect a given gene after Southern transfer of the restriction fragments. The most interesting results for characterizing pathogenic bacteria concern such plasmid-borne features as resistance to certain antibiotics and some virulence factors. Until now, few studies have been published concerning the plasmids of bacterial fish-pathogens; one recent example is the study of the plasmid content of *Vibrio anguillarum* (Olsen and Larsen 1993). When chromosomal DNA is digested by restriction enzymes, chromosomal-based RFLP profiles are obtained, but they usually give no useful information because of the large number of bands. More meaningful results are obtained after Southern transfer and hybridization with specific probes revealing the presence of particular genes or DNA sequences.

A good example is the use of probes which specifically hybridize with rRNA operons. These operons are present in several copies in the bacterial chromosome; as the numbers and locations of the rRNA operon copies differ widely among bacterial species and strains, the restriction pattern (ribotype) revealed by a specific probe may yield useful taxonomic information (Grimont and Grimont 1986). Ribotyping has been successfully applied to the fish pathogens *Vibrio anguillarum* (Olsen and Larsen 1993) and *Aeromonas salmonicida* (Nielsen et al. 1994). We are currently performing a similar study on a collection of *Pseudomonas anguilliseptica* strains. We tested the activity of several restriction endonucleases on bacterial DNA and selected those giving the best patterns after Southern transfer and hybridization with a 16 + 23S-rRNA-specific labelled probe. The initial data show that certain enzymes are able to define several ribotypes related to the geographical origin of the strains (Bernardet et al. unpublished data).

Another kind of molecular probe which can be hybridized with RFLP patterns (Southern blot) consists of labelled oligonucleotides (obtained by cloning or chemical synthesis) which will specifically hybridize with particular genes or DNA sequences. If these genes or sequences are species-specific (usually 16S rDNA sequences), this method may allow a very rapid identification of the bacterial species and thus provide a very sensitive and specific diagnosis. Other methods besides Southern blot for hybridization of the probe include such procedures as colony hybridization, dot-blot, sandwich hybridization, and hybridization in liquid medium. These techniques and their use in aquatic bacteriology and for such fish pathogens as *Yersinia ruckeri*, *Vibrio vulnificus*, and *Edwardsiella ictaluri* have been reviewed recently (Vivares and Guesdon 1992). A DNA fragment from *Aeromonas salmonicida*, specific for all strains tested, was used as a specific DNA probe which proved to be a very useful tool in the study of the ecology of the bacterium as well as the mode of transmission of furonculosis (Hiney et al. 1992). Combined with PCR, this probe allowed the detection of approximately two *A. salmonicida* cells; in certain cases, the bacterium was detected in the viscera before any symptoms of the disease had occurred.

2. A 2nd group of molecular methods is based on DNA amplification by PCR, with or without subsequent enzymatic restriction (Versalovic et al. 1993). The oligonucleotide primers used for PCR may or may not be specific.

When specific primers are used, particular genes or DNA sequences are amplified. For instance, the 16S rRNA genes may be amplified and subsequently sequenced (see above). Another application is the amplification and gel electrophoresis of species-specific genes or sequences, allowing a very sensitive detection and identification of bacterial species; it is even possible to detect bacteria in biological samples, without any

prior cultivation. This is, of course, particularly useful in the case of very fastidious bacteria and it is no surprise that PCR-based detection has been used for such fish pathogens as *Mycobacterium marinum* (Colorni et al. 1993) and *Renibacterium salmoninarum* (Magnusson et al. 1993). A specific detection of *Flavobacterium psychrophilum* based on the same procedure has also been recently published (Toyama et al. 1994).

In a 2nd approach, arbitrary sequence (i.e., non specific) oligonucleotides may be used to generate strain-specific DNA fingerprints; two methods based on this principle are called "arbitrary priming PCR" (AP-PCR) and "randomly amplified polymorphic DNA" (RAPD) (Versalovic et al. 1993). These relatively new techniques have not been applied until now to fish-pathogenic species, but we are currently elaborating a RAPD method for typing *Pseudomonas anguilliseptica* and *Flavobacterium psychrophilum* strains. The initial step was to test many different arbitrary primers, in order to eliminate those which do not amplify, and to select those which produce clear profiles of amplified fragments. The 2nd step will be to use the selected primers on a wide selection of strains, to see if some of these profiles are correlated with geographical origin, ribotypes, virulence, or the fish species from which they were isolated.

It is hoped that this short review has clearly shown that these molecular methods have already produced promising results for certain bacterial fish pathogens, and that these methods should certainly be further developed in the near future for purposes of taxonomy, phylogeny, diagnosis, and epidemiology.

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魚類細菌性病原：分類，流行病學及鑑定上之分子研究

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最近三十年分子生物學的方法逐漸被用來研究細菌性病原。運用這些方法可以在基因層次上比較不同細菌品系及種類且可產生許多有意義的資料，這些方法在分類上都各自有特定的準確範圍，因此進行研究時所採用的分子生物學的方法一定要適合所期望的分類資料的層次。例如：16S rRNA這部分是個很好的分子時鐘，研究這個基因的鹼基序列的方法（如首先用寡核糖核酸分解法，續有DNA/rRNA的雜合反應及DNA鹼基的序列分析）能有效的深入探討細菌之間的關係，因而對以支序圖表示的細菌分類能提供有價值的訊息。另一個被廣泛使用的方法是DNA/DNA之雜合反應，數個不同的技術在比較不同細菌的品系上已被證實是優良的方法，於是能用以決定這些品系是否屬於同一物種，並能在堅實之基因組數據的基礎下定義細菌的新種。利用16S rRNA及DNA/DNA之雜合反應所得的資料有許多分類及命名上的價值。基因之偵測及DNA指印法之產生（由限制酶剪切，特定探針之雜合反應，聚合酶連鎖反應或合併這些技術），可以做爲細菌種類的偵測鑑定以及同種細菌中不同品系之比較，這些方法逐漸可應用到傳染性病菌之檢測及流行病學上之研究。運用研究人類細菌性病原之基因方法總是需要一段時間才能用來探討魚類細菌性病原，一直到最近這些技術才被用來研究一些重要的魚類病原，如 *Aeromonas salmonicida, Vibrio anguillarum, Renibacterium salmoninarum* 及 *Flavobacterium-Cytophaga* 等。最初所得的結果是值得嘉獎，以DNA爲基礎之方法來探討魚類細菌學是可期許的。

關鍵詞：DNA/DNA之雜合反應，16S rRNA，DNA指印法，分子探針。

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