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Genetic Approaches for the Identification of Genes Involved in the Pathogenesis of Bacterial Infections in Fish

Esther Gómez • Jessica Méndez • Roberto Navais • Pilar Reimundo • David Pérez-Pascual • José A. Guijarro*

Área de Microbiologia. Departamento de Biología Funcional. Facultad de Medicina. IUBA. Universidad de Oviedo. 33006 Oviedo. Asturias. Spain

Corresponding author: * **jaga@fq.uniovi.es, jaga@uniovi.es**

ABSTRACT

Over the last 15 years new approaches have been developed for the identification and study of the functions of genes and proteins involved in the virulence mechanisms of bacteria. Some of these approaches are based on the identification of genes required by the bacteria to survive and grow inside the host. Most of these techniques have recently been applied to bacterial fish pathogens successfully. Some of these techniques involve the "*In vivo* expression technology (IVET)", which identifies specific promoters expressed in bacteria grown inside the fish body but not in conventional culture media; "Signature-tagged mutagenesis (STM)", a useful tool for the screening of null mutants which show a decreased virulence capability in the host; and "Suppression Subtractive Hybridisation (SSH)", a genome-wide technology, which enables the identification of genes present only in virulent strains of bacteria or the identification of genes expressed only inside the fish host. This paper reviews the application of these techniques to important pathogenic bacteria for the aquaculture field.

Keywords: IVET, SSH, STM, virulence

Abbreviations: **DFI**, differential fluorescence induction; **FACS**, fluorescence-activated cell sorting; **GFP**, green fluorescent protein; **HMDA**, hybridisation-monitored genome differential analysis; **IVET**, *in vivo* expression technology; ***Ivi***, *in vivo* induced; **LD_{50}**, 50% lethal dose; **ORF**, open reading frame; **PCR**, polymerase chain reaction; **RDA**, representational difference analysis; **RIVET**, resolvase-mediated *in vivo* expression technology; **SH**, subtractive hybridisation; **SSH**, suppression subtractive hybridisation; **STM**, signature tagged mutagenesis; **TTSS**, type III secretion system

CONTENTS

INTRODUCTION

Until recently, the mechanisms of bacterial pathogenesis were studied by culturing the bacteria in different types of media, environments very different from the body of the fish. This approach contributed greatly to our understanding of bacterial pathogenesis. However, the media cannot replicate the complex environment found inside the host and for this reason it was not possible to gather information about important aspects of the virulence mechanisms of bacteria. Studies performed in *in-vivo* models, by growing the bacteria inside the fish, were difficult to conduct. However, during the decade of the 1990s new techniques were developed that allow the identification of genes

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involved in the processes of attachment, invasion and bacterial multiplication within animals. At the present, these *in vivo* systems permit the examination of many genes involved in the infection process simultaneously. There are two main techniques known as "*in vivo* expression technology (IVET)" and "signature tagged mutagenesis (STM)". Additional methods that permit the identification of virulent genes using genome-wide technology, such as "suppression subtractive hybridization (SSH)", have been developed. The present review describes the use of these technologies applied to bacteria fish pathogens. It is not the purpose of this review to go into technical details or into the different variations of each system.

IN VIVO EXPRESSION TECHNOLOGY

Many research groups used transcriptional fusion systems to analyze the process of gene regulation of bacterial pathogens inside the animal host. The study of changes in bacterial genes transcribed during infection using the combination of a reporter system in conjunction with a promoter trap has been dubbed IVET. IVET is a promoter-trap strategy designed to identify genes whose expression is induced in a specific environment. Bacterial genes identified by IVET are expressed during the invasion and colonization of the animal body but not during laboratory growth conditions, which imply the growth of bacteria in artificial media. These genes are defined as *ivi* (*in vivo* induced) genes. Theory says genes that satisfy these criteria are probably virulence-related genes, but definitely not housekeeping genes. However, IVET does not reveal if a gene is related to virulence or not. For this, a mutant in a specific gene must be constructed and its phenotype determined.

To date, there are four variations of IVET, and each one relies on the generation of transcriptional fusions of genomic sequences to a reporter gene encoding an enzyme. The variation in the four methods lies in the particular reporter gene utilized. Thus, DNA from the bacterium of interest is cloned into a vector that carries a promoterless cassette of two genes. One will be used for the selection of promoter activity *in vivo* (i.e. *cat* gene conferring resistance to chloramphenicol). The other gene (i.e. conferring β-galactosidase activity) will allow screening, amongst the selected bacteria recovered from the host, for those that did not have

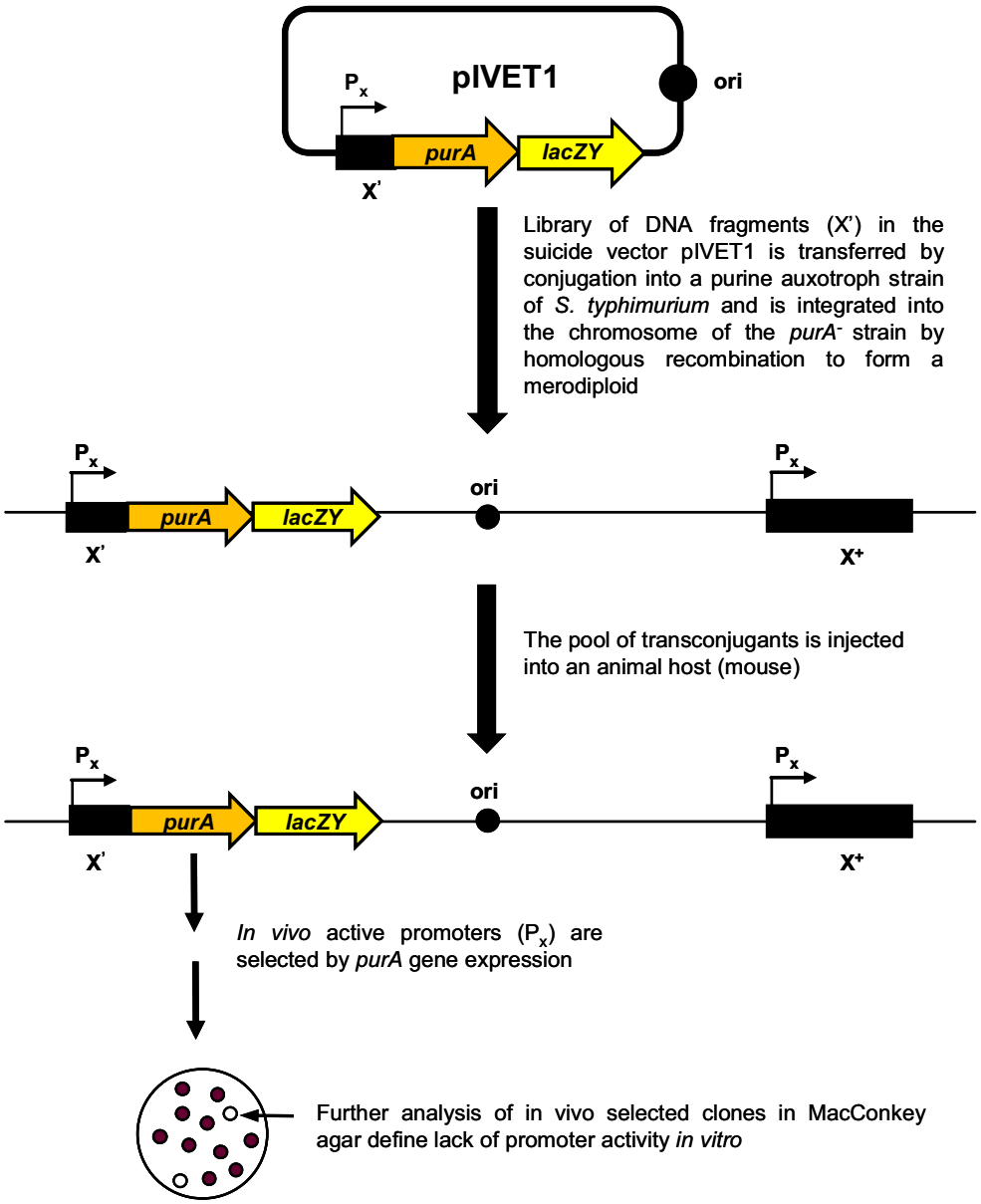

Fig. 1 Auxotrophy complementation-based IVET method described by Mahan *et al.* (1993). Restricted total DNA (X′) is inserted into the 5′ end of a promoterless *purA*/β-galactosidase operon in a suicide plasmid. The constructions are introduced into a *purA*⁻ strain (originally *S. enterica* serovar Typhimurium) and integrated into the chromosome by homologous recombination between the DNA fragment harbored by the plasmid (X′) and the corresponding DNA from the bacterial genome, creating a merodiploid. The resulting pools are introduced into the animal and strains containing infection-induced gene fusions to *purA* are selected in the host and are subsequently screened for lack of *in vitro* expression on *LacZY* indicator plates.

an active promoter *in vitro* (i.e. lack β-galactosidase activity). Therefore, bacterial cells that are resistant to the antibiotic (i.e. chloramphenicol) during host infection and devoid of the enzymatic activity (i.e. β-galactosidase in MacConkey lactose agar) in plates will probably be carriers of a promoter that is specifically active *in vivo*. The original IVET system was the "Auxotrophy complementation-based selection" developed by Mahan *et al.* (1993) in *S. enterica* serovar Typhimurium. The promoter trap consisted of a genomic DNA library of *S. enterica* serovar Typhimurium placed upstream of a transcriptional fusion of the purine biosynthesis gene *purA* and the β-galactosidase reporter genes *lacZY* on a suicide vector (**Fig. 1**). This library was transferred by conjugation into a purine auxotroph strain of *S. enterica* serovar Typhimurium and was integrated by homologous recombination to form partially diploid cells (merodiploids). The *Salmonella* library was injected intraperitoneally in mice. In theory, only those members of the library with functional promoter-*purA* fusions active *in vivo* would be able to complement the auxotrophy of the parent strain and multiply in the host. Recovered bacteria from the host tissue were then screened on selective agar medium containing lactose for a negative *lac* phenotype. In this way, colonies containing promoters active in the host, selected by *purA* auxotrophy complementation and with a lack of expression under environmental laboratory conditions showed by the *lac* negative phenotype were obtained. To date, this system has not been applied to study any fish pathogen.

ANTIBIOTIC-BASED IVET SELECTION FOR *IVI* GENES

In the original utilization of IVET, advantage was taken of the fact that purine auxotrophs of *Salmonella enterica* serovar Typhimurium were rapidly eliminated from the mouse unless they were complemented (Mahan *et al.* 1993). The metabolic requirements of other bacteria under different environmental pressures may not be known. Thus, it was necessary to develop a second variation of IVET that involved the use of antibiotic resistance genes as selectable reporters (Mahan *et al.* 1995). By this strategy, treatment of the infected host with the appropriate antibiotic selects for bacterial strains harboring active gene fusions (**Fig. 2**). This form of IVET can be performed without prior knowledge of the metabolic requirements of the microorganism to be examined, other than their sensitivities to a set of antibiotics. Thus, the primary advantage of this IVET strategy lies in the fact that complementation auxotrophy in the strain under study is not needed. However, this strategy still demands that the pathogen should be transformable and present either homologous recombination for generating chromosomal fusions to IVET reporter genes or plasmid maintenance for generating promoter fusions to IVET reporters on an episomal element. A disadvantage of this approach is that the antibiotic must be administered to the host animal and must penetrate to the site of infection. On the other hand, this requirement provides some flexibility to the IVET selection, since by administering the antibiotic at lower doses or at specific times of infection the breadth of *ivi* genes identified can be increased. Moreover, it allows the identification of genes necessary for the adherence, invasion, survival and multiplication in different organs and also genes encoding tissue-specific virulence factors (Angelichio and Camilli 2002).

APPLICATIONS OF ANTIBIOTIC-BASED IVET SELECTION TO FISH BACTERIAL PATHOGENS

Yersinia ruckeri

Y. ruckeri is the aethiological agent of the enteric redmouth disease (ERM) or yersiniosis. This pathology mainly affects salmonids and causes important economic losses in rainbow trout and salmon aquaculture. Fernández *et al.* (2004) applied this variation of IVET to study the fish pathogen *Y. ruckeri* (**Fig. 2**). In this case the pIVET8 vector was used (Mahan *et al.* 1995). This vector contains promoterless chloramphenicol acetyltransferase (*cat*) and *lacZY* genes (**Fig. 2**). Before these genes there is a unique restriction site where DNA could be inserted to generate transcriptional fusion with these genes. This plasmid behaves as a suicide vector in *Y. ruckeri* (Fernández *et al*. 2004). *In vivo* active promoters are selected by chloramphenicol and MacConkey or EMB agar media is used to identify *lac* negative phenotypes. The use of this vector led to the isolation of 14 different *ivi* clones (Fernández *et al.* 2004). Some of them contained putative open reading frames (ORFs) encoding proteins similar to known virulence factors from other bacteria, such as a type IV secretion system, a haemaglutinin, a haemolysin, a group of proteins important for the adherence of the bacteria to the host, and genes required for iron acquisition by a catecholate siderophore (Fernández *et al*. 2004, 2007a).

The catecholate siderophore (ruckerbactin) was analyzed further (Fernández *et al.* 2004). The genetic organization of the ruckerbactin biosynthetic and uptake loci was similar to that of the *Escherichia coli* enterobactin gene cluster. Regulation of expression by iron and temperature for genes *rucC* and *rupG*, putative counterparts of *E. coli entC* and *fepG*, respectively, involved in the biosynthesis and transport of the iron siderophore complex, respectively, were confirmed. Moreover, LD_{50} values 100-fold higher than those of the wild-type strain were obtained with the *rucC* isogenic mutant, showing the importance of ruckerbactin in the pathogenesis caused by this microorganism. This result validates the correct application of IVET to fish.

Another gene identified by IVET was a putative protein involved in the secretion/activation of a haemolysin (*yhlB*) (Fernández *et al.* 2007b). The *yhlB* gene precedes another ORF (*yhlA*) encoding a *Serratia*-type haemolysin. Other toxins belonging to this group have been identified in genomic analyses of human-pathogenic *yersiniae*, although their role and importance in pathogenicity have not been defined yet. It was shown that higher levels of expression were obtained at 18°C, the temperature of occurrence of disease outbreaks, than at 28°C, the optimal growth temperature. The expression of the haemolysin also increased under iron-starvation conditions. This finding confirmed the decisive role of iron and temperature as environmental clues that regulate and coordinate the expression of genes encoding extracellular factors involved in the virulence of *Y. ruckeri*. LD_{50} and cell culture experiments, using *yhlB* and *yhlA* insertional mutant strains, demonstrated the participation of the haemolysin in the virulence of *Y. ruckeri* and also its cytolytic properties against the BF-2 fish cell line.

The *tra* operon or *tra* cluster, also identified by IVET, encoding a type IV secretion system and composed of eight genes (*traHIJKCLMN*), was shown to be similar both in sequence and organisation to the *tra* operon cluster found in the virulence-related plasmid pADAP from *Serratia entomophila* (Méndez *et al*. 2009). Interestingly, the *tra* cluster of *Y. ruckeri* is chromosomally encoded, and no similar *tra* cluster has been identified yet in the genomic analysis of human pathogenic *yersiniae*. A *traI* insertional mutant was obtained by homologous recombination. Coinfection experiments with the mutant and the parental strain, as well as LD_{50} determinations, indicate that this operon is involved in the virulence of this bacterium. Additional IVET selected genes are now under study, for instance: a *tad* cluster of genes involved in the pili synthesis, a tricarboxylic transport system and a haemaglutinin (Fernández *et al.* 2004).

IVET VARIANTS

Differential fluorescence induction (DFI) is a promoter-trapping technique that utilizes the green fluorescent protein (GFP) as a selectable marker to monitor promoter activity. It was originally developed to isolate acid-inducible genes in *S. enterica* serovar *typhimurium* (Valdivia and Falkow 1996, 1997). In DFI, DNA fragments of the pathogen chro-

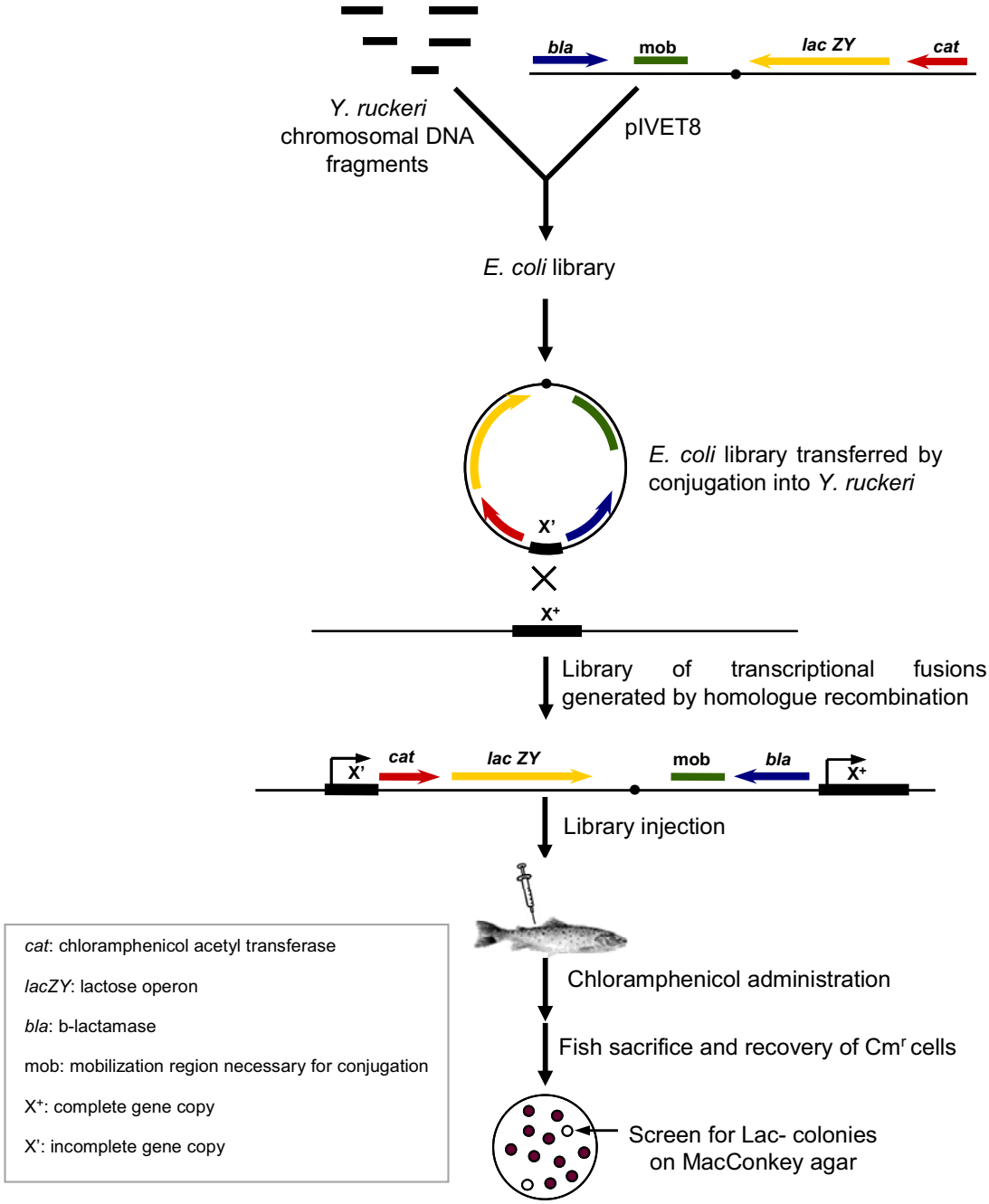

Fig. 2 Graphic depiction of antibiotic-based IVET in *Y. ruckeri*. The first step is the construction of a fusion library in *E. coli* by ligating random genomic fragments of *Y. ruckeri* DNA into the promoterless pIVET8 vector. The library is moved into *Y. ruckeri* by conjugation with *E. coli*. The pIVET8 vector cannot replicate in *Y. ruckeri*, therefore transconjugants must contain transcriptional fusion generated by homologue recombination between the *Y. ruckeri* DNA fragment contained in the vector and the corresponding sequence in the *Y. ruckeri* genome creating a merodiploid. These transconjugants are injected into the fish and the antibiotic chloramphenicol is used for strain selection containing infection-induced gene fusions to *cat* gene. After promoter selection in the host, bacterial cells were recovered from the fish and subsequently screened for lack of *in vitro* expression on *LacZY* indicator plates. Reprinted from **Fernández L, Márquez I, Guijarro JA** (2004) Identification of specific *in vivo*-induced (*ivi*) genes in *Yersinia ruckeri* and analysis of ruckerbactin, a catecholate siderophore iron acquisition system. *Applied Environmental Microbiology* **70**, 5199-5207, with kind permission from the American Society for Microbiology, ©2004.

mosome are cloned upstream from the promotorless green fluorescent protein gene (*gfp*). The resulting plasmids are introduced into the pathogen and the pool of transformants is inoculated into a host. Fluorescent bacteria are recovered directly from host tissues using fluorescence-activated cell sorting (FACS). Then, bacteria are grown *in vitro* in the absence of host cells and those with low fluorescence are isolated. These clones contain *ivi-gfp* fusions. DFI allows for the identification of bacterial promoters induced in distinct anatomical sites and at different stages of infection. Furthermore, unlike other methodologies, the use of the GFP reporter allows for single cell, temporal and spatial monitoring of pathogen gene expression in infected animals (Bumann and Valdivia 2007). No references related to the application of this variant to fish-pathogenic bacteria have been found.

The resolvase–mediated *in vivo* expression technology (RIVET) was initially developed to identify *Vibrio cholerae* genes induced during infection of mice (Camilli and Mekalanos 1995; Lee *et al.* 2001). In RIVET, an antibiotic resistance marker flanked by two resolvase-recognition sites is integrated into the chromosome of the bacterium. Subsequently, a promoterless copy of a resolvase-encoding gene is introduced into a plasmid and used to trap promoters by monitoring changes in the antibiotic resistance phenotype. Importantly, this approach does not rely on selective pressure during the animal experiments, since promoter activations are irreversibly trapped by the excision of the anti-

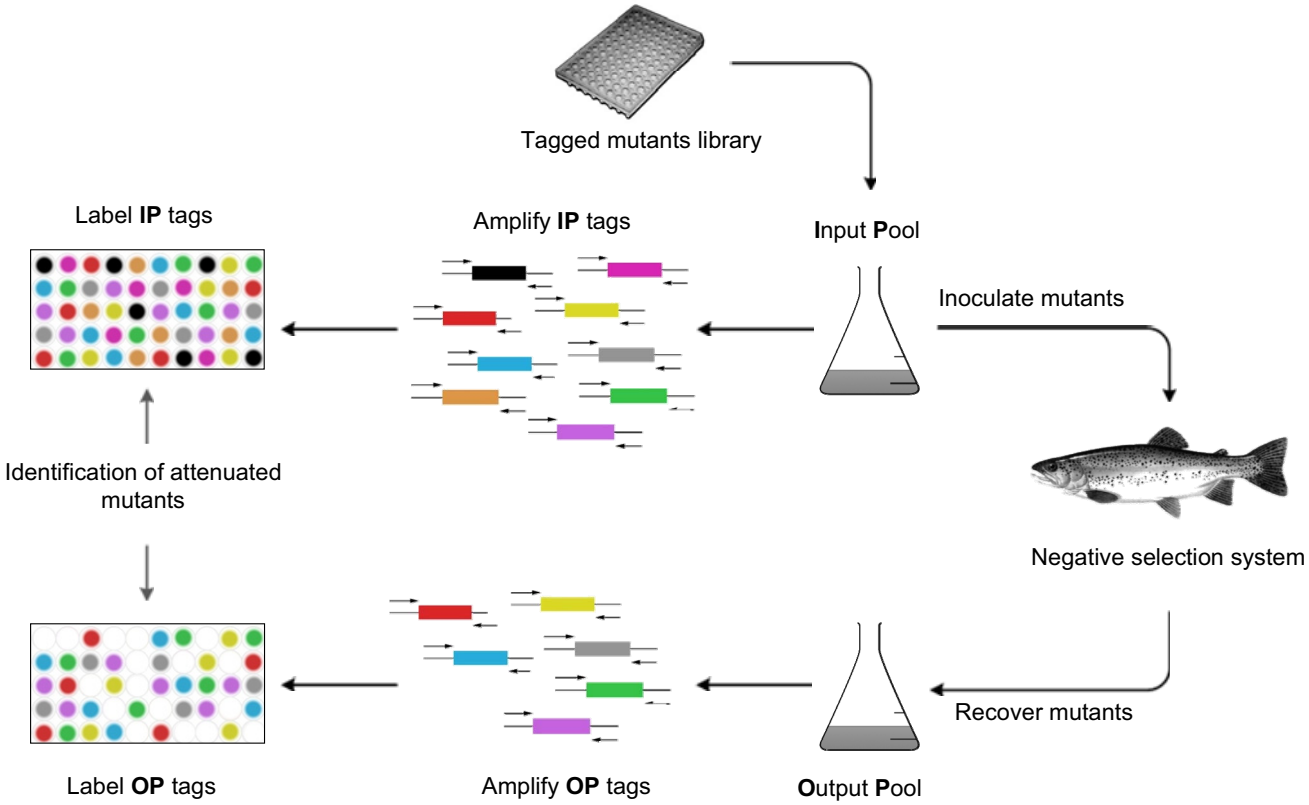

Fig. 3 Graphic description of the STM technique applied to bacterial fish pathogens. A transposition-based mutant library, each of them carrying a unique and exclusive DNA tag, is obtained as a first step. This pool of mutants is inoculated into the fish, which acts as the negative selection system. At the same time, tags are amplified from this input pool and are probed against the different tags, which were previously transferred to a membrane by dot-blot. After infection the recovered mutants (output pool) are subjected to the same procedure. In this way, a weak or absent hybridisation signal from the output pool compared to the input signal permits the identification of the attenuated mutants.

biotic resistance marker and can be identified after recovery of the bacterium under investigation from the host (Mahan *et al.* 2000; Angelichio and Camilli 2002). There is no reference of this technique applied to fish-pathogenic bacteria.

SIGNATURE-TAGGED MUTAGENESIS (STM)

Transposable elements have been extensively used to study microorganisms. STM is an evolution of traditional transposon mutagenesis that allows massive screening of transposon-insertion mutants for the identification of virulence genes in pathogenic bacteria (Autret and Charbit 2005). STM combines the power of insertional mutagenesis and negative selection with a detection system, which permits the identification of individual attenuated mutants from a complex mutant pool (Saenz and Dehio 2005).

The key to STM is to label each mutant with a unique identifying DNA 'signature-tag'. STM uses a library of mutants generated by transposition, each of them harbouring a tag that allows them to be identified specifically. In this way, if a mutant cannot grow in a particular host, its tag, which was present in the pool injected into the animal, will not be detected among the recovered bacteria. Due to its design, STM only identifies certain classes of virulence factors, more specifically essential factors for growth in a host animal but not in *in vitro* conditions (Mecsas 2002). The original STM method was designed to detect new virulence genes of the target organism *S. enterica* serovar Typhimurium (Hensel *et al.* 1995). The essence of the STM technique is conducted in two stages: first of these consists in obtaining a large mutant library by transposition, each of them carrying a unique and exclusive DNA sequence called a "tag"; this mark allows each mutant to be specifically identified. In the second phase, a pool of mutants is inoculated into a host animal and those with limited growth in the host are selected. This selection system is based on the delay or absence of growth of mutants as a result of the interruption of key genes for the development of the infection. After the selection of interesting mutants (considered to be possible attenuated mutants), these can be identified by their specific tag (**Fig. 3**). Therefore, the system is based on negative selection screening (Shea *et al.* 2000). The main tag-detection systems are hybridisation and PCR amplification. The original STM method used DNA colony blots for hybridisation between target DNAs and labelled tags. However, this system is relatively insensitive and can be difficult to perform with some pathogens. As a variation to this strategy, Lehoux *et al.* (1999), developed a PCR-based detection system (called PBSTM from *PCR-based Signature Tagged Mutagenesis* concept), that consists in the employment of defined oligonucleotides for tag construction and PCR for rapid screening of bacterial mutants *in vivo*. This simple STM method can be adapted to any bacterial system and used for genome scanning in various growth conditions (Lehoux *et al.* 1999; Lehoux and Levesque 2000).

Since this first STM approach, various screenings followed a similar protocol with different modifications that increased the versatility of the method and allowed the use of this technique in different microorganisms (Saenz and Dehio 2005). For instance, there are several different transposition systems for generating mutants; besides the original transposon, miniTn*5*, several studies have applied other kinds of transposons, such as *magellan2* or *Himar1*, which are derived from the *mariner* family. Their activity is not dependent on host factors and thus they are applicable to a broad variety of organisms (Saenz and Dehio 2005). Moreover, a Tn*917*-based STM was used for the screening of new genes involved in the survival and virulence of some Gram-positive bacteria (Choi and Kim 2009). One of the most important aspects of STM is the negative selection system. The majority of screenings used animals or *in vivo* models to identify attenuated mutants because of the high selective pressure that the microorganisms are subjected to. However, as in IVET, there are other ways to conduct the

screening in absence of an adequate *in vivo* model, as in *in vitro* cell culture systems or animal and cell culture simultaneous assays to discard mutants that may be attenuated in the animal model due only to growth defects (Burall *et al*. 2004).

In practice, the range of possible mutations detected by this method is restricted. For example, genes encoding toxins and extracellular matrix-binding proteins are extremely difficult to identify (Chiang *et al*. 1999). It detects neither genes that are critical for causing disease but do not significantly affect survival of the bacterium in the host; nor mutations in essential genes, in contrast to IVET (Mahan *et al*. 1993, 1995). The same happens if there is genetic redundancy for a virulence function (Shea and Holden 2000). Basically, this technique should identify a subset of genes that are required for growth *in vivo*.

HIGHLIGHTS FROM STM STUDIES OF BACTERIAL FISH PATHOGENS

Edwarsiella ictaluri

E. ictaluri is the leading cause of mortality in channel catfish (*Ictalurus punctatus*) culture, but little is known about its pathogenesis. The use of Tn*5*-based STM in a waterborne infection model (in this case the detection of attenuated mutants was made by PCR) resulted in the identification of 50 mutants that were unable to infect and/or survive in catfish (Thune *et al*. 2007). Eight insertions occurred in genes encoding proteins associated with virulence in other pathogens, including three in genes involved in LPS biosynthesis, three in genes involved in type III secretion systems (TTSS), and two in genes involved in urease activity (which, for a facultative intracellular pathogen, could be important to passage through the gastrointestinal tract or to survival in the acid phagosomes of macrophages). In addition, a TTSS mutant retained its capacity to invade catfish cell lines and macrophages but was defective in intracellular replication. The mutant was also able to invade catfish tissues in numbers equal to those of invasive wild-type *E. ictaluri* bacteria but it showed poor replication and was slowly eliminated from the tissues, while the wild type increased in number. So its attenuation illustrates the importance of the secretion system in the pathogenesis of *E. ictaluri* in catfish and in intracellular survival. This study has also identified a number of housekeeping genes whose mutants have growth- or replication-related phenotypes (Thune *et al*. 2007).

Lactococcus garvieae

L. garvieae is the aetiological agent of lactococcosis, which affects cultured freshwater and marine fish. There is little information about the precise pathogenic mechanisms by which this bacterium is able to defeat the host defences and cause disease. That is why the STM study carried out by Menéndez *et al*. (2007) represents the first report of a genome-wide scan for virulence factors in this bacterium. Concerning some technical aspects of this study, it should be mentioned that hybridisation was the detection method and rainbow trout individuals (*Oncorhynchus mykiss*) were infected by intraperitoneal injection. Briefly, it consisted in a library of 1250 *L. garvieae* UNIUD074-tagged Tn*917* mutants that were screened for the ability to grow in fish. Among them, 29 mutants were identified which could not be recovered from rainbow trout following infection. Twenty mutants had Tn*917*-flanking DNA encoding proteins with identities to known proteins. Sequence analysis of the tagged Tn*917*-interrupted genes in these mutants indicated the important role for bacterial growth within the fish of transcriptional regulatory proteins homologous to GidA and MerR; the metabolic enzymes asparagine synthetase A and α-acetolactate synthase; the ABC transport system of glutamine and a calcium-transporting ATPase; the *dltA* locus involved in alanylation of teichoic acids; and hypothetical proteins containing EAL and Eis domains, among others, suggesting the importance of their respective functions for infection.

To verify that selected mutants carried a single chromosomal insertion of Tn*917*, the individual mutant strains were subjected to Southern analysis, which indicated that insertions occurred singly in the chromosome of *L. garvieae* mutants. In addition, *in vivo* competition assays were performed to validate the results of the STM screening and to quantify the degree of virulence attenuation of individual mutants. The mutants displayed a range from subtle to severe attenuation. In this sense, the results obtained in the analysis of the *dltA* mutant were particularly relevant. They showed that this locus was necessary for full growth and pathogenesis of *L. garvieae* in rainbow trout (Reimundo *et al.* 2010).

Mycobacterium marinum

M. marinum is a fish, amphibian and human pathogen. Besides its importance in aquaculture, *M. marinum* has an additional interest because it is genetically closely related to the human tuberculosis etiologic agent, *M. tuberculosis* and the knowledge obtained from the study of the virulence determinants of *M. marinun* could be useful for a better understanding of those from *M. tuberculosis*. With this in mind, Ruley *et al.* (2004) used the STM method to identify attenuated mutants of *M. marinum* in goldfish (*Carassius auratus*) as a model of mycobacterial pathogenesis. After negative selection of mutants and their corresponding DNA sequence analyses, 35 mutants with unique transposon insertion were identified from an initial pool of 1,008. In addition, 28 of the 35 *M. marinum* mutants (85%) had transposon insertion into putative virulence genes encoding proteins homologous to proteins of *M. tuberculosis*. Among the identified loci, there were genes belonging to the PPE/PE family, which encodes a signature Pro-Glu (PE) amino acid sequence whose function is unknown but which might be involved in macrophage replication, persistence in granulomas and antigenic diversity (Ramakrishnan *et al.* 2000; Bouley *et al.* 2001); genes involved in cell-wall related function such as the methoxymycolic acid synthase gene (*mma*A) or a gene homologous to an enoyl-coahydratase, which is related to elongation of the fatty acid chains in mycobacteria (Kikuchi and Kusaka 1984); metabolic genes such as an L-carnitine dehydratase homologue; genes related to signal transduction pathways; polyketide synthase (*pks* genes); and genes involved in amino acid or nucleic acid synthesis such as *cys*D and *cys*Q, necessary for cysteine biosynthesis. To establish the attenuation of these STM mutants, competition assays were used, and they showed that 90% of selected mutants were really attenuated. It is important to emphasize the fact that some of the genes identified as virulence genes were recognized in the mouse model for *M. tuberculosis*, validating the use of *M. marinum* as a surrogate for *M. tuberculosis* (Ruley *et al*. 2004).

Mehta *et al*. (2006) constructed a Tn*5367*-derived *M. marinum* mutant library to analyse their ability to cause infection and survive in the macrophage. They identified 19 mutants out of 529 as attenuated clones in macrophage infection assays; these mutants were classified in 3 categories: I) defective for growth in association with macrophages, II) defective for infection and III) defective for infection and growth in association with macrophages. Five of these mutants were associated with virulence of mycobacteria in animal models, which confirmed the efficacy of the macrophage selection system as a useful method for identifying new virulence factors in mycobacteria. In the same way, the authors complemented different mutants with the interrupted genes to ensure that each phenotype was a result of the transposon insertion. Among these, the genes *pks12*, *fadD29* and *fadD30* (class II), involved in the synthesis of fatty acid-related molecules, were identified. The gene *pks12* is related to virulence in mice (Sirakova *et al*. 2003) and fish (Ruley *et al*. 2004), and plays an important

role in both bacterial multidrug resistance (Philalay *et al*. 2004) and in the synthesis of a polyketide involved in immune recognition (Matsunaga *et al*. 2004). The *fadD30* gene is situated upstream and very close to *pks6* in *M. marinum* and *M. tuberculosis*; the *pks6* gene is involved in virulence in mice (Hisert *et al*. 2004). In the same category was the *mimH* gene, related to the ESAT-6 secretion system located in the *M. tuberculosis* RD1 region, which plays a role in virulence in fish (Gao *et al*. 2004) and mice (Hsu *et al*. 2003; Stanley *et al*. 2003). Finally, the *nrp* gene (Ruley *et al*. 2004) (class I) is related to virulence in fish and it is involved in glycopeptidolipid synthesis (Mehta *et al*. 2006).

Streptococcus iniae and *S. pyogenes*

S. iniae is a fish pathogen that in rare cases can be transmitted to immunocompromised humans. Miller and Neely (2005) performed a large-scale screening of signature-tagged transposon mutants in the zebrafish (*Danio rerio*) model using *Tn4001* transposon as disrupting element. A total of 41 clones out of 1,128 mutants were selected as potentially attenuated. The analysis of the interrupted genes showed that 28 clones had significant homology with others present in Gram-positive pathogens. Among them, were identified genes involved in transport (i.e. putative sodium/decarboxylate symporter, ABC transporter), genes encoding proteins with basic metabolic functions (i.e. a putative nucleotide sugar dehydrogenase or a putative adenylosuccinate lyase), or genes related to transcription regulation. This work demonstrated the utility of an STM screen in the zebrafish animal model in the study of bacterial pathogenesis and host-pathogen interactions.

Kizy and Neely (2009) were able to successfully conduct the first reported study of STM applied to *S. pyogenes* in the zebrafish model. The fish infected intramuscularly with *S. pyogenes* develops similar symptoms to those of a necrotizing fascitis disease state (Neely *et al*. 2002). Previously reported streptococcal virulence genes such as *mga* (*trans*-acting positive regulator), *hasA* (hyaluronan synthase), *amrA* (transcriptional activator), *smeZ* (mitogenic exotoxin), and two genes in the *sil* locus (putative sensor histidine kinase and transcriptional regulator) were identified. All of this validates the usefulness of the system but, in this case, due to the nature of the host, the results obtained from this study should only be used as putative human-pathogen virulence factors.

SUBTRACTIVE HYBRIDIZATION (SH) METHODS

During the last few decades a wealth of nucleotide sequence data from bacterial genomes has become available. In addition, whole genome sequencing projects constitute a more and more attainable objective nowadays. However, if our target is the study of the genomic differences between two close relatives, genome sequencing would still imply an expensive and wasteful approach. For that reason, several tools for identifying differences between two close prokaryotic genomes have been developed (Winstanley 2002). Here we will mainly focus on the applications of the subtractive hybridisation (SH) method and its derivatives to the identification of differences between the genomes of two closely related bacterial fish pathogens.

SH was first reported in 1990 as a technique for isolating genomic DNA absent in yeast deleted mutants (Straus and Ausubel 1990) but the main idea behind the strategy remains the same today: to subtract the common DNA sequences present in two close species or strains, and selectively clone the sequences that are present only in the selected one (named *tester* strain) but absent in the other (named *driver* strain). Since 1990 the initial SH method has been subjected to several modifications and consequently it has appeared under different names (Straus and Ausubel 1990; Lisitsyn *et al*. 1993; Diatchenko *et al*. 1996; Akopyants *et al*. 1998; Cao *et al*. 2006). For example, "representational difference analysis (RDA)" incorporates a PCR-based enrichment step of both the cleaved tester and driver DNAs before the hybridisation and subtraction steps occur. In this way, the complexity of the genomes to be compared is reduced (Lisitsyn 1995; Ermolaeva and Sverdlov 1996). Another SH variation, named "hybridisation-monitored genome differential analysis (HMDA)", incorporates a monitor system into a PCR-based solid subtraction hybridisation that tracks the entire hybridisation process. Subtraction is therefore assured to be completed (Cao *et al*. 2006). Finally, variations in gene expression between two strains under different conditions can also be detected by a subtraction hybridisation method. In this approach total mRNA is first transformed in cDNA and then a subtractive cDNA library is constructed (Winstanley 2002).

SUPPRESSION SUBTRACTIVE HYBRIDIZATION (SSH)

In recent years, SSH has become probably the most attractive and demanded SH alternative. SSH was initially reported by Diatchenko *et al*. (1996) and was first applied to bacteria in a study of *Helicobacter pylori* by Akopyants *et al*. (1998). Nowadays, there is a commercial kit available (Clontech PCR-Select Bacterial Genome Subtraction Kit, BD Clontech UK, Hampshire) which makes the technique relatively easy and accessible for every laboratory (**Fig. 4**). Briefly, the tester DNA (from the strain in which unique sequences have to be identified) is digested with a restriction endonuclease (such as *Rsa*I or *Sau3A*I) and the fragments are divided into two groups, each of which is marked by ligation to a different specialized oligonucleotide adaptor (**Fig. 4**). When the marked DNA is denatured and allowed to hybridise in the presence of an excess of unmarked driver DNA that has been digested with the same enzyme, most tester sequences will form heterohybrids with the driver DNA (DNA from the strain which lacks the sequences present in the tester strain). Tester-specific sequences, however, will remain as single-stranded DNA. When the two portions are eventually mixed and hybridised together, homologous single-stranded DNAs can associate and form hybrids. These hybrids are double-stranded tester-specific molecules with different ends on each strand, which correspond to the adaptors. These sequences form amplifiable fragments that are enriched by PCR with primers designed to bind to adaptor sequences. The PCR products can then be cloned to form a subtracted library and be sequenced (Winstanley 2002). It should be mentioned that the DNA samples should be appropriately cleaved with a restriction enzyme to achieve an optimal PCR amplification of the subtracted fragments since it is a PCR-based method. For the same reason and since a frequently cutting restriction endonuclease is employed, the expected average size of the PCR products will be approximately 1 kb or lower. The most noteworthy reason for the success of this strategy is the suppression PCR effect. Sequences carrying the same adaptor sequence at both ends form a secondary structure that prevents exponential amplifications. In this way, only those sequences with two different adaptors on their ends will amplify and undesirable amplifications will not occur (Winstanley 2002). Since no preliminary information about the genome content of the strains is needed, the principal advantage of SH methods is that they constitute an excellent starting point to study species poorly known from a genetic perspective. In fact, SSH is currently considered the most effective approach for detecting differences between two bacterial genomes which have not been sequenced.

APPLICATIONS OF SSH TO FISH BACTERIAL PATHOGENS

Aeromonas hydrophila

A. hydrophila is the causal agent of hemorrhagic septicaemia in a wide variety of freshwater fish species. In this bacterium SSH was carried out by Zhang *et al.* (2000) to detect

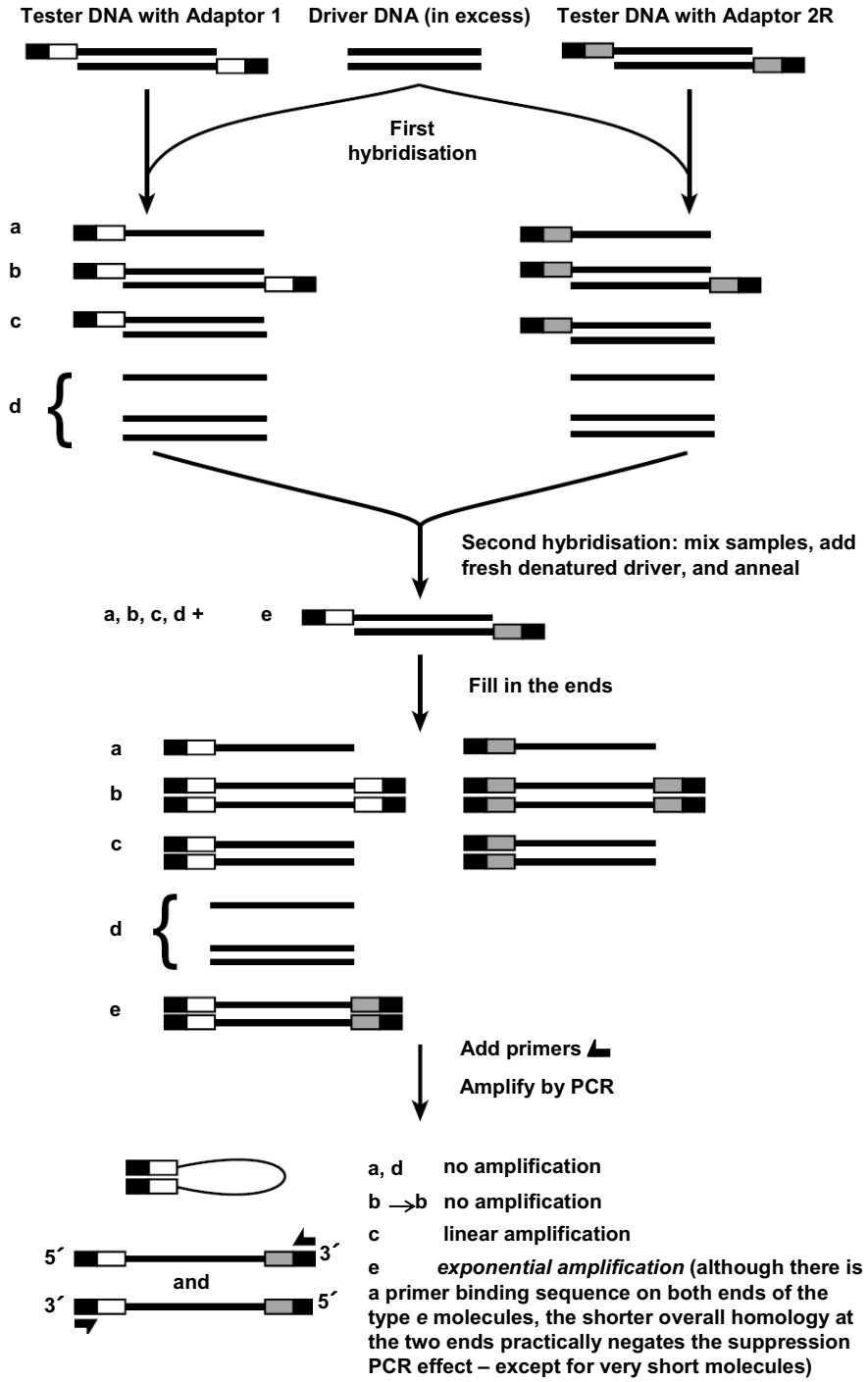

Fig. 4 Scheme of PCR-Select suppression subtractive hybridisation. DNA from tester strain (containing unique sequences) is ligated at the 5′ ends with adaptors 1 and 2R. DNA denaturation, and further hybridisation of tester and driver DNAs give heterohybrids (c) and single-stranded DNA corresponding to tester-specific sequences (a, d). In a second hybridisation between the two amplified mixes, homologous single-stranded DNAs can form hybrids (e). These hybrids correspond with double stranded tester-specific sequences. After filling the ends, double strain-specific sequences containing adaptor 1 and 2R (e) are amplified by PCR using primers that bind to the adaptor molecules. The amplified products are then cloned and sequenced. Figure based on the Clontech PCRSelectTM Bacterial Genome Subtraction kit User Manual (Clontech Laboratories, Inc., Mountain View, CA).

genomic differences between two strains of *A. hydrophila* with different virulence potential in fish: PPD134/91, defined as virulent, and PPD35/85, defined as avirulent on the basis of their different LD_{50} values. 115 clones with insert were selected as specific for the virulent strain. 69 of them (60%) were finally determined as real positives after southern hybridisation analysis. 23 fragments showed high homology to known proteins of other bacteria but the remaining 46 fragments were identified as new proteins of *A. hydrophila*. Interestingly, the paper includes a southern hybridisation-based screening to survey the distribution of the specific fragments among eight virulent and seven avirulent strains of *A. hydrophila*. The authors established two groups of clones on the basis of its hybridisation patterns. Group 1 included presumable universal virulence-associated genes whilst group 2 included genes which were not even conserved among different strains. Group 1 included some known virulence factors identified in *A. hydrophila,* such as a haemolysin, a protease, an outer-membrane protein, a multidrug-resistance protein and a histone-like protein (Zhang *et al.* 2000). In group 2 there were also included several known virulence determinants such as genes for the synthesis of O-antigen and type II restriction/modification system (Zhang *et al.* 2000). In summary, the SSH technique proved to be efficient for identifying genetic differences between virulent and avirulent strains of *A. hydrophila.*

However, this subtraction library was considered far from complete by the authors since just four clones were found to be duplicated.

For that reason, some years later, a more extensive study was carried out by Yu *et al.* (2005). They carried out two rounds of genomic subtraction between *A. hydrophila* PPD134/91 as the tester (virulent strain) and PPD35/85 or PPD64/90 as the driver (avirulent strains). This led to the identification of 22 unique DNA fragments encoding 19 putative virulence factors and 7 new ORFs, which were commonly present in eight virulent strains examined in the study (Yu *et al*. 2005). In addition, four genomic islands were found, including O-antigen, capsule, phage-associated, and TTSS gene clusters. Further *in vivo* study of insertion and deletion mutants showed that the TTSS may be one of the important virulence factors in *A. hydrophila* pathogenesis (Yu *et al.* 2005).

Flavobacterium psychrophilum and *F. columnare*

F. psychrophilum is, at present, one of the most important pathogens of salmonids, causing cold water disease (CWD), which affects mainly fingerlings and is characterized by skin lesions and the development of a bacteraemia. Soule *et al.* (2005) performed SSH experiments to identify genetic differences between two strains of *F. psychrophilum*, one virulent (CSF 259-93) and the other avirulent (ATCC 49418) in a trout challenge model. Reciprocal SSH libraries were prepared for the two strains and a total of 103 unique clones were obtained as a result. 52 clones were confirmed to be unique to the avirulent strain and 51 to the virulent strain. Among these, five putative virulence genes were identified, basing the identification on the homologies between their products and an immunogenic cell surface-associated protein, an outer membrane component of the type II secretory pathway, a putative outer membrane ferric siderophore receptor, a putative pathogenesis-related protein and a toxin secretion ATP-binding protein (Soule *at al*. 2005). In addition, the presence/absence of the 103 unique clones was screened in 34 *F. psychrophilum* isolates. Based on these data, the authors constructed a dendrogram which suggests the existence of two large lineages associated with the host species and the geographic origin of the isolates. The screening also led the authors to propose one of the five putative virulence genes (homologous to a gene encoding a cell surface antigen) as a target for later virulence experiments since it was present in three highly virulent strains (Soule *et al*. 2005).

Columnaris disease is distributed in a wide range of freshwater fish and it is cause by *F. columnare*. Fish infection with this bacterium may result in gill damage, muscle lesions and important erosion of the dorsal fin with the appearance of a saddle (saddleback). Olivares-Fuster and Arias (2008) applied the SSH technique to the identification of specific sequences in a highly virulent strain of the fish pathogen *F. columnare* not shared by the type strain of *Flavobacterium johnsoniae*. After the method was conducted, a total of 192 clones were selected and analyzed. Among them, 110 sequences (59%) were confirmed as *F. columnare*-specific sequences by dot blot hybridisation. All functional categories were represented in the selected clone pool with one exception, no sequences related to cell motility and secretion were identified, which suggests that these processes are highly conserved between the two species. Several putative virulence factors were identified in *F. columnare*, such as a collagenase, a chondroitinase, proteases, as well as drug resistance and iron transport-related genes (Olivares-Fuster and Arias 2008). This study led to the identification of putative virulence-associated factors in *F. columnare*, the etiological agent of columnaris disease in fish and it increased the amount of genetic information available from this bacterium.

Photobacterium damselae subsp. *piscicida*

P. damselae subsp. *piscicida* causes a septicaemia named pasterurelosis. This disease is spread to different fish species causing significant losses in farming of yellowtail, red sea bream and sole (*Solea senegalensis*). The SSH technique was carried out by Juiz-Río *et al.* (2005) to identify DNA fragments present specifically in the *P. damselae* virulent strain PC554.2, but absent in the avirulent strain EPOY 8803-II. After two rounds of hybridisation 21 clones from the total obtained were proved to be real positives by dot-blot hybridisation analysis. These genomic regions included twenty-six distinct putative ORFs. Among them, twenty ORFs encoded proteins with homology to proteins in other bacteria, including four homologues involved in siderophore biosynthesis, and four homologues related to mobile elements; three of these were putative transposases and one was a protein related to the *Vibrio cholerae* SXT element (Beaber *et al*. 2002). The authors proved that an STX-like conjugative element exists in the genome *of P. damselae* PC554.2 and its sequence was shown to be integrated into a *prfC* gene homologue (Hochhut and Waldor 1999; McGrath and Pembroke 2004). Six ORFs showed no significant homology to known bacterial proteins (Juiz-Río *et al.* 2005).

Vibrio anguillarum

V. anguillarum is responsible for a haemorrhagic septicaemia in a wide range of fish species. The disease is characterised by the presence in the fish of red necrotic lesions in abdominal muscle and erythema. Usually the infection leads to high mortalities. More recently, SSH has been carried out by Wang *et al.* (2009) between two *V. anguillarum* strains with the aim of isolating potential virulence genes. The strains were defined as highly virulent (VIB72) and low virulent (CW1) strains on the basis of their LD_{50} values in zebra fish. 59 clones were dot-blot-confirmed as distinct VIB72-specific fragments and 55 of them were successfully sequenced. 30 out of the 55 selected fragments presented homologies to known or hypothetical proteins of other bacteria and the remaining 25 fragments showed no significant homologies to any database. The authors consider some of the fragments as good candidates to be potential virulence genes in *V. anguillarum* since their hits matched with proteins proposed as having a role in the virulence mechanisms of other bacteria. Some of these proteins are: toxic proteins, transposases, mobilization proteins, soluble lytic murein transglycosylases and resistance-related proteins (Wang *et al.* 2009).

E. ictaluri

Recently, an interesting report showed the application of the SSH technique in *E. ictaluri* to the identification of differentially expressed genes in an attenuated strain used as a vaccine and in the corresponding parental strain under *in vitro* conditions (Pridgeon *et al.* 2010). In this case, the study focuses on differences in gene expression in bacteria, not on difference in genes present in the bacteria. The strategy relies on the construction of subtractive libraries using total mRNA expression. In this way, 41 sequences were isolated from the attenuated strain that were *in vitro* upregulated in comparison to the parental strain. These included genes involved in protective immunity, adhesion, cell growth and survival and also a group of about 20% of the genes identified, which encoded putative or hypothetical proteins (Pridgeon *et al.* 2010). This study represents a new, powerful and interesting approach to the analysis of differential gene expression in fish-pathogenic bacteria.

CONCLUSIONS

The power of IVET, STM and SSH as screening and selection systems must be analyzed prior to being used since each one has its advantages and limitations well described

in different reviews (Rediers *et al.* 2005; Dudley 2008; Hsiao and Zhu 2009). As has been shown in this article, these approaches provide good alternatives for the identification and further analysis of genes involved in the virulence mechanism of fish pathogenic bacteria. However, comparatively, few publications describe the use of these techniques to understand the molecular interactions between bacteria and fish. This is particularly clear in IVET. Nevertheless, the results obtained in the study of the *Y. ruckeri*-rainbow trout interaction indicate the usefulness of this system for selecting virulence determinants as shown by the attenuated phenotype of mutants generated in the *ivi* genes.

STM has been confirmed as a good technique for giving a preliminary view of the pathogenicity of various bacterial fish pathogens. However, once genes are identified by STM, it is possible to investigate in depth the virulence mechanisms of the bacteria and the processes they control, either by analysing the biochemical properties of the gene products or by studying the physiological behaviour of mutant strains.

The ability to detect and analyze species-specific sequences within a genome provides valuable insights into the physiology and evolution of bacteria. Furthermore, comparison of the genome sequences between strains with different virulence potential represents a good strategy for the discovery of strain-specific unknown virulence-associated factors which can facilitate the establishment of the nature and severity of disease. In the case of fish bacterial pathogens, the examples cited in this review, although few, demonstrate how SSH represents an effective approach to a better understanding of the virulence of fish bacterial pathogens which is, as increasingly observed, a complex and multifactor mechanism. The SH methods alone may be unable to provide a full understanding of the differences between two bacterial genomes and their implications, but they constitute an excellent first choice for the identification of those genetic factors that account for differences in virulence between closely related strains (Winstanley 2002).

Due to the high effectiveness and usefulness of these techniques it is sure that in the coming years an increasing number of papers based on the application of IVET, STM, SSH and their variants to fish pathogenic bacteria will be published. This will contribute to a wide increase in the knowledge of bacteria-fish interactions. Furthermore, the information obtained through the application of these techniques may provide helpful evidence to try to develop attenuated vaccines and some of the proteins encoded by virulence genes may be suitable targets for the design of future antimicrobial drugs.

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