Application of AFLP for taxonomic and epidemiological studies of *Photobacterium damsela* subsp. *piscicida*

A. Thyssen,¹ S. Van Eygen,² L. Hauben,² J. Goris,² J. Swings² and F. Ollevier¹

Author for correspondence: A. Thyssen. Tel: +32 16 32 39 66. Fax: +32 16 32 45 75.
e-mail: an.thyssen@student.kuleuven.ac.be

¹ Laboratory of Aquatic Ecology, K. U. Leuven, Ch. De Bériotstraat 32, B-3000 Leuven, Belgium

² Laboratory for Microbiology, R. U. Gent, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

A collection of 106 *Photobacterium damsela* subsp. *piscicida* strains and 19 *Photobacterium damsela* subsp. *damsela* strains, including reference and type strains, were genetically characterized using AFLP. The total genomic DNA of each bacterial strain was digested using restriction endonucleases *HindIII* and *TaqI*. Using numerical analysis, six clusters were recognized. The largest cluster ($n = 106$) contained the majority of the strains tested and consisted exclusively of *Photobacterium damsela* subsp. *piscicida*. The *Photobacterium damsela* subsp. *damsela* strains fell outside this cluster. DNA–DNA hybridization experiments showed 77% DNA binding between the two subspecies, indicating a close genetic relationship. This clearly demonstrates the applicability of AFLP in studying the taxonomic position of *Photobacterium damsela* subsp. *piscicida*. In addition, AFLP proved to be a useful genotypic technique for epidemiological surveys of the pathogen, since it was able to discriminate between Mediterranean and Japanese *Photobacterium damsela* subsp. *piscicida* isolates.

Keywords: AFLP, taxonomy, epidemiology, *Photobacterium damsela* subsp. *piscicida*

INTRODUCTION

Photobacterium damsela subsp. *piscicida* (formerly *Pasteurella piscicida*) is the aetiological agent of fish pasteurellosis. This disease was first observed in the USA in populations of wild striped bass (*Morone saxatilis*) and white perch (*Morone americanus*) (Snieszko *et al.*, 1964). In 1969, the pathogen became a serious problem in Japan, causing great economic losses in cultured yellowtail (Kusuda & Yamaoka, 1972). In 1990, *Pasteurella piscicida*, for the first time, became a threat to the Southern European fish farm industry. In several countries of the Mediterranean area, including France (Baudin Laurencin *et al.*, 1991), Italy (Ceschia *et al.*, 1991), Spain (Toranzo *et al.*, 1991), Greece (Bakopoulos *et al.*, 1995), Portugal (Baptista *et al.*, 1996), Turkey (Candan *et al.*, 1996), Malta (Bakopoulos *et al.*, 1997), Israel (Bakopoulos *et al.*, 1997b) and Croatia (Oraic *et al.*, 1998), the

pathogen was responsible for severe outbreaks of pasteurellosis in cultured populations of sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*).

The taxonomic position of the pathogen has been controversial. The organism was first placed in the genus *Pasteurella* and described as *Pasteurella piscicida* (Janssen & Surgalla, 1968), although the bacterium was clearly distinguishable from all other species within this genus by a number of morphological and biochemical properties. In 1995, the pathogen was classified in the genus *Photobacterium* as a subspecies of *Photobacterium damsela* on the basis of rRNA sequence and DNA–DNA hybridization data (Gauthier *et al.*, 1995). Recent morphological and biochemical characterization tests however, found no phenotypic evidence to include the pathogen as a subspecies of *Photobacterium damsela* (Thyssen *et al.*, 1998). Various phenotypic and genotypic methods, including morphological and biochemical identification tests, serotyping, lipopolysaccharide and outer-membrane protein profiling, fatty acid methyl ester analysis, plasmid profiling, RFLP of chromo-

Abbreviation: UPGMA, unweighted pair group method with averages.

somal DNA and ribotyping have previously been used to characterize *Photobacterium damsela* subsp. *piscicida* (Magariños *et al.*, 1997; Thyssen *et al.*, 1998). Most of these techniques proved to be of limited value in discriminating between *Photobacterium damsela* subsp. *piscicida* strains, implying that the species was very homogeneous (Toranzo *et al.*, 1983; Magariños *et al.*, 1992; Romaldo *et al.*, 1995).

Amplified fragment length polymorphism (AFLP) is a new genomic fingerprinting method which has been proven to be very useful in epidemiological and evolutionary studies, since it was able to differentiate between highly related bacterial strains belonging to the same species or biovar (Vos *et al.*, 1995; Janssen *et al.*, 1996). In addition, studies have shown that the AFLP technique is a powerful DNA fingerprinting method for bacterial taxonomy (Huys *et al.*, 1996; Janssen *et al.*, 1996).

The aim of this study was to investigate the applicability of the AFLP technique for epidemiological studies of *Photobacterium damsela* subsp. *piscicida*. In addition, we determined whether or not AFLP could be used in showing the taxonomic position of this pathogen.

METHODS

Strains and culture conditions. One hundred and twenty eight strains were examined, the diagnosis was as received and verified as earlier described by using morphological and biochemical identification tests (Thyssen *et al.*, 1998). A total of 106 *Photobacterium damsela* subsp. *piscicida* strains were used in this study. The collection contained isolates from Europe ($n = 61$), Japan ($n = 43$) and the USA ($n = 2$), of which 33 were isolated from sea bass, 21 from sea bream, 33 from yellowtail, 4 from Japanese flounder, 4 from striped bass, 2 from mullet and 1 each from red sea bream, black scraper, striped jack, greater amberjack, red spotted grouper, amberjack and white perch. Information concerning the host species was not available for two isolates. The reference strains ATCC 17911, ATCC 29690, ATCC 29689 and ATCC 29688 and the type strain NCMB 2058^T of *Photobacterium damsela* subsp. *piscicida* were also included in this study. For taxonomic purposes, the type strains of other *Photobacterium* species were used: NCIMB 1895^T (*Photobacterium angustum*), LMG 4228^T (*Photobacterium leiognathi*) and ATCC 7744^T (*Photobacterium fischeri*). Finally, 19 *Photobacterium damsela* subsp. *damsela* strains, including two copies of the type strain ATCC 33539^T and NCIMB 2184^T, were analysed. These strains were isolated from various sources, such as sea water, shrimp, oyster, brown shark, European eel, damsel fish and sea bream.

Bacteria were grown on brain–heart infusion agar (Difco) to which 1% (w/v) NaCl was added and incubated for 48 h at 26 °C.

AFLP

Isolation and purification of DNA. Genomic DNA was prepared according to Huys *et al.* (1996). The DNA concentrations were measured with a fluorimeter (TK 100, Hoeffer). The integrity of the DNA samples were checked on a 1% agarose

gel containing a final concentration of 0.5 µg ethidium bromide ml⁻¹ in TAE buffer (40 mM Tris-acetate, 1 mM EDTA; pH 8.0). Final DNA preparations were stored at -20 °C.

Preparation of template DNA. All protocols concerning the preparations of template DNA for PCR were performed essentially as described by Vos *et al.* (1995) and Huys *et al.* (1996), with minor modifications. In general, 1 µg DNA was digested with 15 U *TaqI* and *HindIII* in a final volume of 30 µl. After restriction, adapters were added at a concentration of 1 µM for the *HindIII* adapter and 20 µM for the *TaqI* adapter (5'-AGCTGGTACGCAGTC-3' and 5'-GACGATGAGTCCTGAC-3', respectively) and ligated to the restriction fragments. The *TaqI*–*HindIII* templates were precipitated with 1.25 M ammonium acetate and 50% (v/v) 2-propanol. Finally, the DNA pellet was washed once with 70% (v/v) ethanol and dissolved in 100 µl T 0.1 × E buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0). Template DNAs were stored at -20 °C.

AFLP reactions. The *TaqI*–*HindIII* restriction fragments tagged with specific adapters were used as template DNA for selective PCR directed by a tetraprimer (5'-CGATGAGT-CCTGACCGAA-3') and a hexaprimer (5'-GACTGCGT-ACCAGCTTA-3') (selective bases at the 3' ends of the primers are underlined). The hexaprimer was labelled with ³²P. Selective amplification was performed according to Janssen *et al.* (1996).

Electrophoresis, autoradiography and data acquisition. All protocols concerning the electrophoresis were done according to Janssen *et al.* (1996). After electrophoresis, the gel was vacuum-dried with a gel dryer (model 583; Bio-Rad) for 55 min at 80 °C as described by Huys *et al.* (1996). The AFLP fingerprints were visualized autoradiographically by exposing the dried gels to Hyperfilm-MP (Amersham International) for 48–76 h depending on the amount of radiation determined with a Geiger–Müller counter. After exposure, the films were developed using a Fuji RG II X-Ray film processor. Resulting autoradiograms were scanned by using a high-resolution densitoscanner (X-Ray Scanner). Images were maintained as TIFF files and were further processed using GelCompar version 4.0 software (Applied Maths). The resulting dimension of each gel was 680 by 2683 data points. The size of the TIFF files were decreased by reducing the track resolution (Yres) in the GelCompar conversion program to 1500 data points.

Numerical analysis of banding patterns. Gels were normalized by the alignment of reference tracks which were included at regular intervals in each gel. Background was subtracted using the rolling-disk method which makes part of the GelCompar software package. Gels were combined by assigning one particular reference track as standard and alignment of all other reference tracks against this standard (Janssen *et al.*, 1996). The analysis window was set at 154–1363, excluding the primer and external front. The level of similarity between AFLP fingerprints was calculated using the Pearson product–moment correlation coefficient, in order to avoid subjective interpretations of band positions due to the high number of bands and unequal intensity of bands within a single pattern. The different patterns were clustered by using the unweighted pair group method with average linkage (UPGMA) (Sneath & Sokal, 1973; Vauterin & Vauterin, 1992).

DNA–DNA hybridization and DNA base composition. Genomic DNA was extracted and purified by a scaled-up

variant of the protocol of Pitcher *et al.* (1989). For analysis of DNA base composition, the DNA was enzymically degraded into nucleosides according to Mesbah *et al.* (1989). The nucleoside mixture was separated by HPLC using a Waters SymmetryShield C8 column which was maintained at 37 °C. Ammonium acid phosphate (0.02 M $\text{NH}_4\text{H}_2\text{PO}_4$; pH 4.0) to which 1.5% acetonitrile was added, was used as the solvent. Nonmethylated lambda phage DNA (Sigma) was used as the calibration reference. DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989). A HTS7000 Bio Assay Reader (Perkin Elmer) was used for the fluorescence measurements. The hybridization temperature was 37 °C.

RESULTS

DNA base composition

The DNA base composition was determined by testing four *Photobacterium damsela* subsp. *piscicida* and five *Photobacterium damsela* subsp. *damsela* isolates, including the type strains NCIMB 2058^T, ATCC 33539^T and NCIMB 2184^T. The G+C content of *Photobacterium damsela* subsp. *piscicida* was measured to be 41.1–41.3 mol%, whereas the G+C content of *Photobacterium damsela* subsp. *damsela* was 40.6–41.4 mol% (Table 1). The G+C content of the type strains of *Photobacterium leiognathi*, *Photobacterium angustum* and *Photobacterium fischeri* are also given in Table 1.

AFLP genomic fingerprinting

Choice of restriction enzymes. Based on the DNA base composition, several restriction enzyme combinations, such as *EcoRI*/*MseI* and *ApaI*/*TaqI* and *HindIII*/*TaqI*, were tested as suggested by Huys *et al.* (1996). In our study, only restriction with *HindIII*/*TaqI* yielded more than 50 clearly separated bands and proved to be the best combination (results not shown). This combination was therefore chosen for the preparation of the DNA templates for PCR of all bacteria used in this study.

Reproducibility of AFLP. Previous studies have shown that AFLP can be considered a highly reproducible fingerprinting technique (Vos *et al.*, 1995; Janssen *et al.*, 1996). In this study, the level of reproducibility within and between gels was determined by including the AFLP pattern obtained on DNA of the type strain of *Photobacterium damsela* subsp. *damsela* (ATCC 33539^T) at regular intervals in each electrophoresis run. Following normalization and background subtraction, the similarity between these reference patterns was measured to be at least 90% within each gel and at least 87% between gels, using the Pearson product–moment correlation. The differences in the correlation values between the reference lanes were the result of slight variations in background intensities and duration of electrophoresis.

Cluster analysis of AFLP data. After numerical analysis of

Table 1. DNA base compositions and levels of DNA–DNA relatedness

Presented values are the means of three independent experiments, except where indicated otherwise.

Strain	Cluster	G + C content (mol %)	DNA–DNA reassociation with (%)											
			1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
<i>Photobacterium damsela</i> subsp. <i>piscicida</i> strains														
1. ATCC 17911	Ia	41.2	100	99	88*	87	69	75	74†	71*	73	18†	20†	18†
2. 510/96	Ia	41.3	99	100	94*	91	78	78	79†	71	79	19†	19†	20†
3. LA91-197	Ib	41.1	101*	99*	100	101	76	82	72†	85	75*	20†	22†	15†
4. NCIMB 2058 ^T	Ib	41.1	101	101	95	100	80	83	76†	76	82	20†	22†	19†
<i>Photobacterium damsela</i> subsp. <i>damsela</i> strains														
5. KUL 22	II	41.3	81	84	70	69	100	87	79†	77	80	20†	22†	24†
6. ATCC 35083	IV	40.6	87	85	69	73	85	100	73†	78	87	17†	20†	25†
7. NCIMB 2184 ^T	V	41.4	75†	84†	77†	72†	80†	92†	100†	79†	86†	20†	20†	15†
8. ATCC 33539 ^T	V	40.9	84*	85	75	68	82	85	97†	100	80	18†	19†	23†
9. 6-58	VI	41.2	82	83	63*	75	80	85	72†	74	100	19†	19†	22†
Other <i>Photobacterium</i> species														
10. <i>Photobacterium angustum</i> NCIMB 1895 ^T	II	40.6	24†	25†	23†	22†	25†	25†	30†	19†	30†	100†	43†	29†
11. <i>Photobacterium leiognathi</i> LMG 4228 ^T	–	41.6	24†	27†	23†	24†	26†	28†	27†	19†	29†	44†	100†	22†
12. <i>Photobacterium fischeri</i> ATCC 7744 ^T	–	38.8	7†	8†	8†	9†	9†	10†	9†	7†	9†	9†	9†	100†

* Results obtained from two experiments.

† Results obtained from a single experiment.

Fig. 1. Dendrogram derived from an UPGMA cluster analysis of the AFLP patterns of all strains used in this study. Levels of linkage are expressed as the Pearson product-moment correlation coefficient (r). The numbers (I–VII) on the right of the dendrogram indicate the cluster to which the strains belong.

the AFLP banding patterns, six clusters could be distinguished at a cut-off value of 57% similarity (Fig. 1).

Cluster I ($n = 106$) contained the majority of the strains tested and consisted exclusively of *Photobacterium damsela* subsp. *piscicida* isolates. The reference strains ATCC 17911, ATCC 29690, ATCC 29689 and ATCC 29688 and the type strain of *Photobacterium damsela* subsp. *piscicida*, NCMB 2058^T, were situated in this cluster. At 66% similarity, the cluster could be further subdivided into two subclusters, Ia and Ib. Subcluster Ia ($n = 62$) was composed of isolates from the Mediterranean Sea, and also included the American reference strain ATCC 17911, which was isolated from white perch. The strains of subcluster Ib ($n = 44$) all originated from Japan, with the exception of one that came from the USA (LA91-197) and was isolated from striped bass. Subcluster Ib also included the type strain NCMB

2058^T and the three remaining (Japanese) reference strains.

Cluster II ($n = 6$) contained the type strain of *Photobacterium angustum* (NCIMB 1895^T) and five strains, including KUL 22 (see Fig. 1), which were received and identified as *Photobacterium damsela* subsp. *damsela* based on biochemical characterization tests (Thyssen *et al.*, 1998). These strains originated from sea water, sea bream, European eel and shrimp.

The strains of cluster III ($n = 2$), which formed at 66% similarity, were isolated from shrimp and identified as *Photobacterium damsela* subsp. *damsela* (Thyssen *et al.*, 1998).

Cluster IV ($n = 2$) included the reference strain of *Photobacterium damsela* subsp. *damsela*, ATCC 35083, which was isolated from brown shark. Specific information concerning the source of the other isolate

was not available. Cluster IV joined cluster III at 54 % similarity.

Cluster V ($n = 7$) contained two different cultures (ATCC 33539^T and NCIMB 2184^T) of the type strain of *Photobacterium damsela* subsp. *damsela*, which clustered together at 71 % similarity. Four strains of cluster V were from different fish species, whereas the remaining strains originated from environmental sources ($n = 1$) and shrimp ($n = 1$).

The strains of cluster VI ($n = 2$), which formed at 58 % similarity, were isolated from shrimp. At 49 % similarity another strain, isolated from shrimp, joined cluster VI. These strains were identified as *Photobacterium damsela* subsp. *damsela* (Thyssen *et al.*, 1998).

The type strains of *Photobacterium fischeri* (ATCC 7744^T) and *Photobacterium leiognathi* (LMG 4228^T), joined respectively at a similarity percentage of 12 and 15. The AFLP pattern of these isolates did not only differ from one another, but also displayed a clearly distinct pattern in comparison to all other strains tested.

DNA–DNA hybridization

DNA–DNA hybridization experiments were carried out by using DNA of four *Photobacterium damsela* subsp. *piscicida* isolates and five *Photobacterium damsela* subsp. *damsela* isolates, including the type strains NCIMB 2058^T, ATCC 33539^T and NCIMB 2184^T. Two isolates from cluster Ia and Ib were chosen to represent *Photobacterium damsela* subsp. *piscicida*, whereas representatives of *Photobacterium damsela* subsp. *damsela* were located in clusters II, IV, V and VI. DNA–DNA hybridization experiments within the subspecies *piscicida* showed a mean DNA relatedness of $97 \pm 7\%$ ($n = 32$), whereas the mean DNA relatedness between the *Photobacterium damsela* subsp. *damsela* isolates was $82 \pm 8\%$ ($n = 44$). Moreover, by DNA–DNA hybridization, the mean DNA binding between *Photobacterium damsela* subsp. *piscicida* and *Photobacterium damsela* subsp. *damsela* was calculated to be $77 \pm 10\%$ ($n = 100$) (Table 1). The DNA relatedness between *Photobacterium damsela* subsp. *piscicida* and *Photobacterium leiognathi*, *Photobacterium angustum* and *Photobacterium fischeri* on the other hand was much lower, $23 \pm 2\%$ ($n = 8$), $21 \pm 2\%$ ($n = 8$) and $13 \pm 5\%$ ($n = 8$), respectively.

DISCUSSION

AFLP

AFLP is a reproducible technique that has been previously applied in the characterization and identification of bacteria at a subgeneric level (Janssen *et al.*, 1996). In this study, all *Photobacterium damsela* subsp. *piscicida* isolates clustered together in one cluster (Fig. 1). Based on this finding, one could say that this subspecies is very homogeneous as previously

stated by Magariños *et al.* (1992), who found that Japanese, European and American strains shared very similar phenotypic, serological and genetic properties. Moreover, fatty acid methyl ester analysis showed a high degree of correlation between the different isolates (Romaldo *et al.*, 1995). In our study however, two distinct groups could be detected within this apparently homogenous subspecies. One subcluster (Ia) contained all European isolates, whereas the other one (Ib) included all Japanese strains. This indicates the existence of two clonal lineages: one Japanese and one European. This finding is in agreement with recent investigations using monoclonal antibodies and ribotyping, which have also shown that strains from the two distinct geographical areas differ from one another and seem to belong to two different clones (Bakopoulos *et al.*, 1997b; Magariños *et al.*, 1997). In contrast to Magariños *et al.* (1997), no further genetic differentiation between the European strains could be detected, although apparently a subdivision within the European subcluster at 77 % similarity could be seen (subcluster Ia, Fig. 1). This however was neither correlated with geographical origin nor with host-fish species. Kawahara *et al.* (1998) recently reported serological differences between Japanese strains isolated from yellowtail. In our study, isolates from yellowtail also displayed differences in AFLP banding patterns (results not shown).

In cluster II, the type strain of *Photobacterium angustum* was found together with five isolates which had been identified biochemically as *Photobacterium damsela* subsp. *damsela*. This finding could imply that the five field strains are mis-identified and were in fact *Photobacterium angustum*. However, according to Thyssen *et al.* (1998) the five field strains (including KUL 22) were clearly distinguishable from the type strain of *Photobacterium angustum* (NCIMB 1895^T) by a number of biochemical traits such as motility, β -haemolysis, growth at 37 and 42 °C, nitrate reduction, growth on MacConkey and thioisulfate/citrate/bile/sucrose agar, production of lipase, amylase, caseinase, gelatinase and urease, and fermentation from glycerol, saccharose and trehalose. Moreover DNA–DNA hybridization showed a low level of relatedness between KUL 22 and NCIMB 1895^T (Table 1). Also the ongoing ribotyping studies using *PvuII* as a restriction enzyme, NCIMB 1895^T clustered together with the same *Photobacterium damsela* subsp. *damsela* strains as in the present study, however, using *MluI*, the type strain fell outside the *Photobacterium damsela* subsp. *damsela* cluster (A. Thyssen, K. Pedersen, J. L. Larsen & F. Ollevier, unpublished).

DNA–DNA hybridization

The results obtained by AFLP were confirmed by results of the DNA–DNA hybridization experiments. In the present study, the DNA–DNA hybridization results showed 97 % DNA binding between the four *Photobacterium damsela* subsp. *piscicida* isolates tested. This finding is in accordance with previous

studies, carried out by Gauthier *et al.* (1995) who also found a high homogeneity within this subspecies (95% DNA relatedness). The level of relatedness between the five *Photobacterium damsela* subsp. *damsela* on the other hand was calculated to be 82%, indicating that this subspecies is more heterogeneous than *Photobacterium damsela* subsp. *piscicida*. This perfectly reflects the AFLP dendrogram (Fig. 1). Moreover, the DNA–DNA hybridization study revealed a high level of relatedness between *Photobacterium damsela* subsp. *piscicida* and *Photobacterium damsela* subsp. *damsela*, showing 77% DNA binding. The classification of the two different organisms in a single species (i.e. *Photobacterium damsela*), is therefore justified. This finding was supported by previous studies performed by Gauthier *et al.* (1995), who found a DNA binding of 80% between the two subspecies.

Final remark

Based on our results, it is concluded that AFLP is able to identify and differentiate strains within *Photobacterium damsela* subsp. *piscicida*.

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