APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1992, p. 1039–1042 Vol. 58, No. 3
0099-2240/92/031039-04$02.00/0
Copyright © 1992, American Society for Microbiology

# DNA Probe for *Aeromonas salmonicida*

MAURA HINEY,[1] MICHAEL T. DAWSON,[2] DAVID M. HEERY,[2] PETER R. SMITH,[3] FRANK GANNON,[1,2] AND RICHARD POWELL[2]*

*Department of Microbiology,[2] National Diagnostics Centre, BioResearch Ireland,[1] and Fish Disease Laboratory,[3] University College, Galway, Ireland*

Received 14 August 1991/Accepted 10 December 1991

**A DNA fragment that is specific to *Aeromonas salmonicida* has been isolated from a genomic DNA library by differential hybridization. The specificity of this fragment as a DNA probe for *A. salmonicida* was shown by hybridization against reference strains and clinical isolates of *A. salmonicida*, related aeromonads, and species from several other bacterial genera. The sensitivity of detection by a polymerase chain reaction test, based on this fragment, was approximately two *A. salmonicida* cells.**

*Aeromonas salmonicida* is the causal agent of furunculosis, a disease which affects many species of fish and is of major importance in salmonid farming (1). The absence of an efficient selective medium and the poor plating efficiency of the organism in mixed cultures (9) have severely hampered the study of the ecology of this organism and the epidemiology of furunculosis (1). The assumption that the organism is an obligate pathogen of fish (11, 12) may be an artifact of the limited ability of classical bacteriological techniques to detect the organism in natural environments (1). Studies based on DNA hybridization (7) and restriction fragment length polymorphisms (5) have shown that *A. salmonicida* is a highly homogeneous taxon in the genus *Aeromonas* which is classified as a member of the family *Vibrionaceae*. More recently, molecular genetic evidence suggesting that the species of the *Aeromonas* genus show sufficient differences from members both of the *Vibrionaceae* and of the family *Enterobacteriaceae* that a new family, the *Aeromonadaceae*, should be created (4) has been presented.

This paper reports the isolation of a DNA fragment from an *A. salmonicida* genomic DNA library which is specific for all strains of *A. salmonicida* tested to date. Along with the specificity tests, sensitivity tests using the polymerase chain reaction (PCR) (13) allow the detection of 2.4 *A. salmonicida* genome equivalents. The use of this DNA fragment as a specific DNA probe now facilitates a comprehensive study of the ecology of *A. salmonicida* and the etiology of furunculosis.

*Aeromonas* strains used in this study were grown on or in tryptone soya agar or broth. Total DNAs from *Aeromonas* species were prepared as follows. Overnight cultures (10 ml) (18 to 22°C for *A. salmonicida* strains and 30°C for motile aeromonads) were centrifuged at 5,000 rpm (Heraeus Sepatech Biofuge A) for 5 min, and the supernatant was discarded. Cells were resuspended in 0.4-ml of solution 1 (50 mM Tris-HCl [pH 8.0], 50 mM EDTA, 25% sucrose). A 0.1-ml volume of lysozyme (10 mg/ml in solution 1) was added, and the suspension was incubated at 37°C for 15 min. A 0.4-ml volume of solution 2 (10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 1% sodium dodecyl sulfate [SDS]) was mixed gently with the cell lysate, 0.1 ml of proteinase K (4 mg/ml in solution 2) was added, and the mixture was incubated at 55°C for 4 h, and then RNase A (10 mg/ml) was added and the solution was incubated at 37°C overnight. The DNA was then extracted with phenol-chloroform and precipitated with 2 volumes of isopropanol before resuspension in 0.1 ml of sterile $H_2O$. Chemicals were purchased from BDH Ltd. (Poole, England), and the enzymes were purchased from Sigma Chemical Co. (St. Louis, Mo.).

A genomic DNA library was prepared from *Eco*RI-digested DNA from *A. salmonicida* 7222V (see Table 1) in the vector λgt11 (6, 8). A library of $10^6$ recombinant phage was generated in *Escherichia coli* Y1090 (6). A total of $10^4$ phage, which is representative of approximately five *A. salmonicida* genome equivalents, was screened in situ with [α-$^{32}$P]dCTP-labelled total DNAs from *A. salmonicida* 7222V and *Aeromonas hydrophila* AE120 (see Table 1). *A. hydrophila* was chosen for the differential hybridization because we had noted a high degree of homology between the 16S rRNA sequences of *A. Salmonicida* and *A. hydrophila* when we had examined this approach to obtaining unique DNA sequences for use as specific DNA probes (2). This analysis was also supported by evidence from DNA homology studies which revealed a close genetic relationship between both *Aeromonas* species (3, 7, 10). Successful differential hybridization was achieved only when the total DNA was reduced to a small molecular size (less than 2 kb) with a 4-bp recognition restriction enzyme (e.g., *Sau*3A or *Hae*III) and when 1 μg of radiolabelled probe was applied per 132-mm-diameter nitrocellulose filter. The results show that under stringent hybridization conditions (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 65°C for 8 h) and washes (0.2× SSC at 65°C for 30 min), 50% of the plaques hybridized strongly with the *A. salmonicida* 7222V probe, whereas the intensities of the hybridization signals with the *A. hydrophila* AE120 probe were less easy to discriminate (Fig. 1). This presumably is a consequence of sequence divergence, even among conserved genes of the related aeromonads.

Two clones were chosen for further analysis: AS15, which showed a strong hybridization signal with the *A. salmonicida* 7222V probe and no signal with the *A. hydrophila* AE120 probe, and AS7, which hybridized with both probes. The inserts from AS15 and AS7 were amplified by PCR and primers complementary to sequences flanking the *Eco*RI cloning site of λgt11 (15). The amplification reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM deoxynucleoside triphosphates (dNTPs), 200 pmol of each primer (λgt11 forward- and reverse-sequencing primers; Boehringer GmbH, Mannheim, Germany), and 1.5 U of *Taq* polymerase (Promega Corp., Madison,

* Corresponding author.

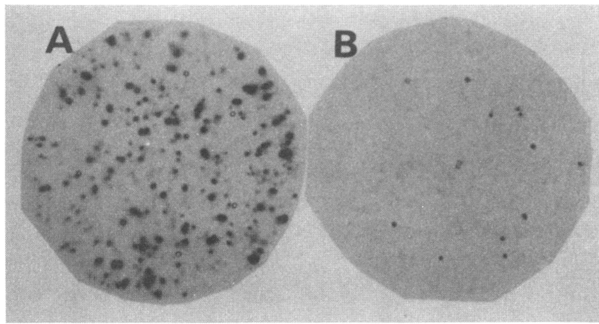


FIG. 1. Differential hybridization. Shown is *A. salmonicida* 7222V library probed with *A. salmonicida* 7222V radiolabelled total DNA (A) and with *A. hydrophila* AE120 radiolabelled total DNA (B).

Wis.). The reaction volume was 100 μl, and cycling conditions were 94°C for 1 min (denaturation), 50°C for 1 min (annealing), and 72°C for 1 min (extension); 30 cycles were performed. The results show that clones AS7 and AS15 contain inserts of 0.7 and 0.5 kb, respectively (Fig. 2A). A secondary differential screening of Southern blots of both fragments, performed under hybridization and washing conditions identical to those of the in situ screening described above, confirmed that AS7 hybridized with total DNAs from both *A. salmonicida* 7222V and *A. hydrophila* AE120, whereas AS15 was positive only with *A. salmonicida* 7222V (Fig. 2B and C).

To further analyze its specificity, the AS15 fragment was used as an [α-$^{32}$P]dCTP-radiolabelled probe in a slot blot hybridization with genomic DNAs prepared from typed and clinical isolates of *A. salmonicida*, related aeromonads, and several other bacterial genera (Table 1). These included strains of *E. coli*, *Pseudomonas aeruginosa*, *Corynebacterium glutamicum*, *Clostridium perfringens*, *Serratia marcescens*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis* and marine and salmon isolates identified as *Pseudomonas* and *Vibrio* species, respectively. The aeromonads analyzed included different strains of *A. hydrophila*, *Aeromonas caviae*, *Aeromonas sobria*, and *Aeromonas media*. *A. salmonicida* strains included typed strains; clinical disease isolates from Ireland, England, Scotland, and the United States; and *A. salmonicida* subsp. *achromogens* from Iceland.

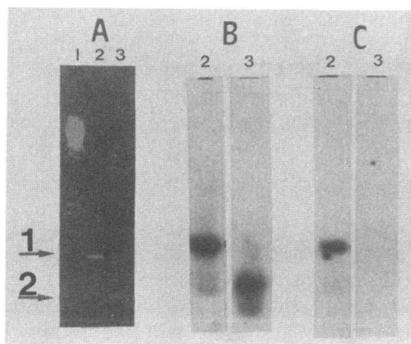


FIG. 2. Southern blot analysis of AS7 and AS15. Shown are PCR-amplified AS7 (lanes 2) and AS15 (lanes 3) inserts separated on a 1% agarose gel, along with λ*Hin*dIII molecular weight size markers (lane 1) (A), and Southern blots of the gel probed with *A. salmonicida* 7222V radiolabelled total DNA (B) and with *A. hydrophila* AE120 radiolabelled total DNA (C). The AS7 (arrow 1) and AS15 (arrow 2) amplified DNA fragments are indicated.

TABLE 1. Bacterial strains

| FDG code[a] | Origin[b] | Species | Signal with AS15[c] |
|---|---|---|---|
| — | Marine isolate | *Pseudomonas* spp. | − |
| — | Marine isolate | *Pseudomonas* spp. | − |
| — | Laboratory strain | *P. aeruginosa* | − |
| — | Laboratory strain | *E. coli* | − |
| — | Laboratory strain | *C. glutamicum* | − |
| — | Laboratory strain | *C. perfringens* | − |
| — | Laboratory strain | *B. subtilis* | − |
| — | Laboratory strain | *S. marcescens* | − |
| — | Laboratory strain | *E. faecalis* | − |
| — | Laboratory strain | *S. aureus* | − |
| — | Salmon isolate | *Vibrio* spp. | − |
| 7222V | Ireland | *A. salmonicida* | + |
| AE120 | United States | *A. hydrophila* | − |
| F265 | Iceland | *A. salmonicida* | + |
| 8112 | Ireland | *A. salmonicida* | + |
| 1102 | NCIMB | *A. salmonicida* | + |
| CP91 | Ireland | *A. salmonicida* | + |
| FWTSA | Ireland | *A. salmonicida* | + |
| ZS-28 | England | *A. salmonicida* | + |
| AE205 | Switzerland | *A. hydrophila* | − |
| AE250 | Peru | *A. hydrophila* | − |
| AE203 | Bangladesh | *A. caviae* | − |
| AE130 | United States | *A. hydrophila* | − |
| 910L/19 | Ireland | *A. salmonicida* | + |
| AE254 | Peru | *A. hydrophila* | − |
| AE230 | Spain | *A. hydrophila* | − |
| A20 | Ireland | *A. salmonicida* | + |
| AE10 | Italy | *A. hydrophila* | − |
| AE67 | Italy | *A. caviae* | − |
| 028 | United States | *A. salmonicida* | + |
| NPT | Ireland | *A. salmonicida* | + |
| ESB13 | Ireland | *A. salmonicida* | + |
| MT206 | Scotland | *A. salmonicida* | + |
| LP90/2 | Ireland | *A. salmonicida* | + |
| SRT480 | Ireland | *A. salmonicida* | + |
| A475 | Ireland | *A. salmonicida* | + |
| 81P/P | Ireland | *A. salmonicida* | + |
| LP50 | Ireland | *A. salmonicida* | + |
| 7222vA$^-$ | Ireland | *A. salmonicida* | + |
| 495 | Canada | *A. salmonicida* | + |
| A8 | Scotland | *A. salmonicida* | + |
| MT030 | Scotland | *A. salmonicida* | + |
| MT206 | Scotland | *A. salmonicida* | + |
| LPT2 | Ireland | *A. salmonicida* | + |
| AE20 | Italy | *A. caviae* | − |
| AE215 | Switzerland | *A. sobria* | − |
| AE236 | United States | *A. media* | − |
| AE188 | Spain | *A. hydrophila* | − |
| AE184 | United States | *A. sobria* | − |
| 8112Hq | Ireland | *A. salmonicida* | + |

[a] FDG, Fish Disease Group; —, no FDG code.
[b] NCIMB, National Centre for Industrial and Marine Bacteria.
[c] By slot blot hybridization: − negative reaction; +, positive reaction.

Typically, 1 μg of total DNA was denatured by addition of a half volume of 1 M NaOH and incubation at 55°C for 5 min. Samples were then neutralized by the addition of an equal volume of 1 M HCl prior to application onto a Nytran membrane (Schleicher & Schuell) with a Schleicher & Schuell slot blot apparatus. Gel-purified radiolabelled AS15 probe (1 μg) was used in a standard hybridization (5× SSC, 5× Denhardt's solution, 0.1% SDS) for 8 h at 65°C. The

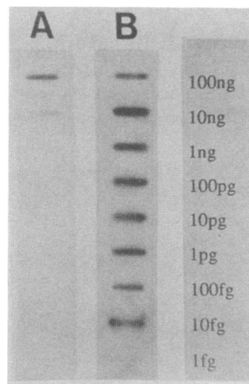


FIG. 3. Slot blot hybridization: autoradiograms of the sensitivity of the AS15 probe. (A) Hybridization against serial dilutions (100 ng to 1 fg) of *A. salmonicida* total DNA; (B) hybridization against the PCR-amplified products of the individual serial dilutions (100 ng to 1 fg) of *A. salmonicida* total DNA.

stringency of the final wash was 0.2× SSC for 30 min at 65°C. The results show that the probe hybridized strongly to DNAs from all *A. salmonicida* strains tested; however, no signal could be detected with other bacterial genera or with fellow aeromonads (Table 1).

These results demonstrated that a specific DNA probe for *A. salmonicida* had been isolated. To determine whether this DNA probe might prove to be a useful tool in the study of the ecology of *A. salmonicida* and the epidemiology of furunculosis, we decided to determine the detection limits of the DNA probe. Slot blot analysis of serial dilutions of *A. salmonicida* 7222V DNA revealed that 1 μg of radiolabelled AS15 fragment clearly detected 10 ng of total DNA after 8 h of autoradiography (Fig. 3A). This is equivalent to the DNA content of $2.4 \times 10^6$ *A. salmonicida* cells (3). As this degree of sensitivity is insufficient for an environmental study of *A. salmonicida*, we developed a PCR-based detection system. Subcloning into the vector M13mp18 (16) and sequencing with dideoxynucleotides (14) allowed the determination of the nucleotide sequence of the AS15 fragment (Fig. 4). Comparison of the DNA sequence with the GenBank sequence data library revealed no significant homologies. Comparison of inferred protein sequences from several small open reading frames found in AS15 with the translated products of the GenBank sequence data library also revealed no significant homologies.

Primers for PCR were designed from the AS15 sequence (Fig. 4; underlined sequences). These primers specifically amplify a 423-bp DNA fragment from DNA prepared from *A. salmonicida* strains, and all amplified fragments hybridize with an oligodeoxynucleotide complementary to an internal sequence in AS15 (data not shown). In a sensitivity experiment comparable to that described above, similar serial dilutions of *A. salmonicida* 7222V DNA were prepared and amplified by PCR with the designed primers. The amplification reaction mixture contained 10 mM Tris-HCl (pH 8.3), 3.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs, 200 pmol of each primer (5′-CGTTGGATATGGCTCTTCCT-3′ and 5′-CTCAAAACGGCTGCGTACCA-3′), and 1.5 U of *Taq* polymerase (Promega Corp.). The reaction volume was 100 μl, and cycling conditions were 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 1 min (extension); 30 cycles were performed. The amplification products from each serial dilution of genomic DNA were slot blotted as described above and probed with 1 μg of radiolabelled AS15 fragment under a hybridization and washing regimen similar to that stated above. The sensitivity of detection was increased by approximately $10^6$ times; i.e., 10 fg of total DNA gave a positive signal after 4 h of autoradiography (Fig. 3B). This is equivalent to the DNA content of 2.4 *A. salmonicida* cells (3).

On the basis of the specificity and sensitivity results presented and the lack of methods to specifically define and cultivate *A. salmonicida*, particularly from environmental samples, this DNA probe should now prove a very useful tool in furthering our understanding of the habitat(s) of *A. salmonicida* and the mode of transmission of furunculosis.

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession number X64214.

```
1     GTTTACCACGTAATCTGAATTGTTCTTTTCCGTTGGATATGGCTCTTCCT
51    ATCTCACTAGGTAAGTCTATTAGGTTCGACACAAAATTCAAATTTAACCC
101   CACATGCTTATTGTTGGCTGCCTCGATAAGCAATGGCATTTTACCTAGAT
151   CTATGCTGGCGTAATTTTCATGTGAGGTGAAAAACTCACCGCTAGCCAAC
201   TCTCTTTCCATGGTTGCAATCAAAAGTTGCAATTCTTCTTTAGTTGCCTC
251   TTGGATTGTGGTTTTTTCATCTTGGTTGGTTAGGTCGGTTTTTATATGGA
301   TGGGGGGTATATTCATAACGCCTTCCTTTTTCACATTGTCATTTCAAAAC
351   GGAATTTTGAGCATCAATTTTAAATAAAATTAAATTTTTGGGTTTTATTT
401   TTAATATCATAAATATATCACTTTCAGACATCGTGGTACGCAGCCGTTTT
451   GAGAT
```

FIG. 4. Nucleotide sequence of the AS15 DNA fragment. Both 20-base regions used for primer design are underlined.

We thank Tom Barry, Gerard Colleran, and Fidelma Boyd for helpful discussion and the provision of bacterial strains and the National Diagnostics Centre, BioResearch Ireland, for synthesis of synthetic oligodeoxynucleotides.

## REFERENCES

1. **Austin, B., and D. A. Austin.** 1987. Bacterial fish pathogens: disease in farmed and wild fish, p. 112–117. Ellis Horwood Ltd., Chichester, United Kingdom.
2. **Barry, T., R. Powell, and F. Gannon.** 1990. A general method to obtain DNA probes for microorganisms. Bio/Technology **8:**233–236.
3. **Belland, R. J., and T. J. Trust.** 1988. DNA:DNA reassociation analysis of *Aeromonas salmonicida*. J. Gen. Microbiol. **134:** 307–315.
4. **Colwell, R. R., M. T. MacDonell, and J. De Ley.** 1986. Proposal to recognize the family *Aeromonadaceae* fam. nov. Int. J. Syst. Bacteriol. **36:**473–477.
5. **Hennigan, M., L. M. Vaughan, T. J. Foster, P. Smith, and F. Gannon.** 1989. Characterization of *Aeromonas salmonicida* strains using DNA probe technology. Can. J. Fish. Aquat. Sci. **46:**877–879.
6. **Huynh, T. V., A. Young, and R. W. Davis.** 1985. Construction and screening cDNA libraries in λgt10 and λgt11, p. 49–78. *In* D. M. Glover (ed.), DNA cloning, vol. 1. IRL Press, Oxford.
7. **MacInnes, J. I., T. J. Trust, and J. H. Crossa.** 1979. Deoxyribonucleic acids relationships among the members of the genus *Aeromonas*. Can. J. Microbiol. **25:**579–586.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **McCarthy, D. H.** 1977. Some ecological aspects of the bacterial fish pathogen, *Aeromonas salmonicida*. Soc. Appl. Bacteriol. Symp. Ser. **6:**299.
10. **McCarthy, D. H.** 1978. A study of the taxonomic status of some bacteria currently assigned to the genus *Aeromonas*. Ph.D. thesis. Council of National Academic Awards, United Kingdom.
11. **Popoff, M.** 1984. Genus III. *Aeromonas* Kluyver and Van Niel 1936, 398[AL], p. 545–548. *In* N. R. Krieg and J. G. Holt (ed.),

Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.

12. **Richards, R. H., and R. J. Roberts.** 1978. The bacteriology of teleosts, p. 196. *In* R. J. Roberts (ed.), Fish pathology. Balliere Tindall, London.
13. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. F. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
14. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
15. **Trahey, M., R. Wong, B. Halenbach, G. Rubinfeld, A. Martin, M. Ladner, C. M. Long, W. J. Crosier, K. Watt, K. Koths, and F. McCormick.** 1988. Molecular cloning of two types of GAP complementary DNA from human placenta. Science **242:**1697–1700.
16. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.