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Classification of isolates of *Vibrio harveyi* virulent to *Penaeus monodon* larvae by protein profile analysis and M13 DNA fingerprinting

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ABSTRACT: A total of 17 *Vibrio harveyi* isolates were examined for virulence to *Penaeus monodon* larvae and classified by total soluble protein profiles generated by sodium-dodecyl-sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) under reducing conditions. Two isolates out of 17 proved to be virulent. Most isolates fell within 2 protein groups. Group I was characterised by a 42 kDa protein and contained 8 isolates including both virulent isolates. Group II was characterised by a 40 kDa polypeptide and contained 7 isolates. A further 2 isolates could not be assigned to either group. Isolates were further characterised by M13 DNA fingerprinting. *V. harveyi* isolates were compared to *V. parahaemolyticus* isolates and found to be substantially more diverse in genotype. This suggested high genetic diversity within *V. harveyi*. The separation of isolates into Groups I and II by SDS-PAGE was shown to be genetically based, however the 2 virulent isolates classified within Group I did not demonstrate a high genetic association. Based on the present results, it is suggested that virulent isolates within *V. harveyi* are rare and that virulence may be explained by genetic transfer of virulence factors.

KEY WORDS: *Vibrio harveyi* · *Penaeus monodon* · M13 DNA fingerprinting · *Vibrio* virulence · *Vibrio* classification

INTRODUCTION

Vibrio harveyi is commonly isolated from warm near-shore marine waters, but only recently has the virulence of this species been recognised. There is a small but growing list of marine animals found to be susceptible to infection. These include common snook *Centropomus unidecimalis* (Kraxenberger-Beatty et al. 1990), pearl oysters *Pinctada maxima* (Pass et al. 1987) and penaeid prawns (Lavilla-Pitogo et al. 1990, Muir 1991).

The losses experienced by the tiger prawn *Penaeus monodon* industry have been the most economically important to date. Larval prawns are particularly susceptible to *Vibrio harveyi* succumbing to what has been termed luminescent bacterial disease (Lavilla-Pitogo et al. 1990). This disease has been identified as a major problem in the Philippines, causing severe losses of juvenile prawns in several hatcheries (Lavilla-Pitogo et al. 1992).

Current research is aimed at identifying the source of *Vibrio harveyi* in prawn hatcheries (Lavilla-Pitogo et al. 1992). However, *V. harveyi* is a commonly occurring and generally non-pathogenic marine bacterium, which appears to be rarely virulent to *Penaeus monodon* larvae. Virulent isolates from the Philippines are 100% lethal to *P. monodon* larvae at challenge doses of approximately 100 cells ml^{-1} seawater (Lavilla-Pitogo et al. 1990). Similarly a northern Australian isolate of *V. harveyi* also has been shown to be virulent at approximately 100 cells ml^{-1} seawater (P. Muir pers. comm.). This contrasts with other isolates which are not virulent at a challenge dose of 10^6 cells ml^{-1} seawater (Muir 1991).

Attempts to explain the differences in virulence among isolates of *Vibrio harveyi* required an investigation into the relationship between virulent and avirulent isolates. Accordingly we investigated 2 virulent isolates and determined their relationship to other avirulent *V. harveyi* isolates obtained from diverse

ecological sources. Subtyping of isolates was carried out using 2 different techniques. Bacterial proteins were analysed on the basis of differences seen on sodium-dodecyl-sulphate polyacrylamide gels under reducing conditions, which has been used successfully to subdivide other bacterial groups (Krech et al. 1988). The relationship among isolates was investigated further by comparing DNA profiles generated using an M13 probe, which has also been used successfully to subgroup bacterial species (Ryshov et al. 1988, Huey & Hall 1989). Importantly, both of these methods provide information on the phylogenetic relationship among strains of bacteria.

MATERIALS AND METHODS

Source of micro-organisms. The study group consisted of 17 *Vibrio harveyi* isolates listed in Table 1. Isolate PM47666-1 had previously been shown to be highly virulent to prawn larvae (P. Muir pers. comm.). The remaining isolates derived from both clinical and environmental sources. Cultures were generously supplied by the Sir George Fisher Centre for Tropical Marine Studies, James Cook University of North Queensland, Townsville, Australia, with several belonging to the Australian Collection of Marine Micro-organisms (ACMM). Isolates PM91 and 9056681 were obtained from the Oonoonba Veterinary Laboratories, Queensland Department of Primary Industry, Townsville, Australia. Isolate 301 was isolated from the haemolymph of a diseased adult *Penaeus monodon* prawn. Four *V. parahaemolyticus* isolates also used in this study are listed in Table 2.

Table 1 Sources of *Vibrio harveyi* isolates used in this study

Isolate	Source
1, 8, 12, 20, 45, ACMM 645, ACMM 652	Environmental: seawater
PM91, 301, ACMM 648	Septicaemic adult tiger prawn *Penaeus monodon*
PM47666-1, ACMM 642	*P. monodon* larvae
ACMM 644, ACMM 650, ACMM 656	Septicaemic adult banana prawn *Penaeus esculentis*
9056681, ACMM 643	Mornbund barramundi *Lates calcarifer*

Note: ACMM (Australian Culture of Marine Micro-organisms) isolates and isolates 47666-1, 1, 8, 12 and 20 courtesy of Sir George Fisher Centre for Tropical Marine Studies, James Cook University, Townsville, Australia. Isolates 9056681 and PM91 courtesy of Oonoonba Veterinary Laboratories, Queensland Department of Primary Industry, Australia

Table 2. Sources of *Vibrio parahaemolyticus* isolates used in this study

Isolate	Source
ACMM 102	Human infection
ACMM 220	Crown of thorns starfish *Acanthaster plancii*
ACMM 666, ACMM 667	Environmental: seawater

Virulence tests. Tests were carried out according to the method of Muir (1991). The virulence of each *Vibrio harveyi* isolate was tested in 3 replicate acid-washed conical flasks containing 150 ml of autoclaved seawater which had been previously filtered (3 µm) and UV-treated (wavelength 254 nm) by the Australian Institute of Marine Sciences (Townsville, Australia). The flasks were supplemented with between 5×10^4 and 1×10^5 *Chaetoceros gracilis* diatoms ml^{-1} seawater as a food source for the larvae. Actively swimming, 3-d-old *Penaeus monodon* larvae were used in the experiment. Fifteen larvae were counted into each flask before the addition of challenge bacteria then placed on a rotary shaker operating at 100 rpm and 28°C for the duration of the experiment. Bacteria were grown at 28°C in Tryptone Soya-broth (Oxoid, West Heidelberg, Australia) supplemented to 2% total NaCl [TSB(2% NaCl)] to late log phase. The cell concentration was determined from the absorbance of the broth culture at 640 nm according to Muir (1991), and 1.5×10^8 cells were removed, pelleted at $2500 \times g$, and washed once in sterile seawater before being added to the flasks containing the prawn larvae. This resulted in a challenge dose of 1×10^6 cells ml^{-1} seawater. The experiment was allowed to proceed for 40 h before numbers of surviving larvae were counted. Student's *t*-test was used to test for significant differences between the treated and control flasks at $p = 0.05$.

SDS-PAGE profiling. Bacterial proteins were made soluble by the method of Krech et al. (1988) with minor modifications and separated by sodium-dodecyl-sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) under reducing conditions. Bacteria were grown overnight at 28°C in TSB(2% NaCl). One ml of the suspension was transferred to a microcentrifuge tube and the bacteria were pelleted at $12\,000 \times g$ for 30 s. The bacterial pellet was washed once in PBS and then resuspended in 200 µl of sample buffer containing 0.5% sodium dodecyl sulphate (SDS) (SIGMA-Aldrich, Castle Hill, Australia), 1.25% 2-b-mercaptoethanol, 0.03% bromophenol blue, 2.5% glycerol in 15 mM Tris Cl at pH 6.8. The suspension was then boiled for 15 min and centrifuged at $12\,000 \times g$ to remove cell debris, then 10 µl of the sample was used for electrophoresis. Electrophoresis was carried out using a 7.5 cm

minigel (Bio-Rad Laboratories, North Ryde, Australia), with a 12.0% separating gel in SDS-running buffer (1 M glycine, 17 mM SDS (SIGMA-Aldrich), 124 mM Tris pH 8.3) at 200 V. Proteins were stained in Coomassie blue (0.2% Coomassie blue, 40% methanol, 10% acetic acid); excess stain was removed by washing in a 10% ethanol, 10% acetic acid solution.

M13 DNA fingerprinting. Bacterial DNA was extracted by a modification of the plasmid-miniprep method (Maniatis et al. 1982) and the high-salt DNA extraction method (Miller et al. 1988). This method resulted in total genomic DNA being isolated which included both chromosomal and plasmid DNA. Approximately 10 ml of a bacterial culture, grown overnight in TSB(2%NaCl) at 28°C, was transferred to a 10 ml screw-capped polypropylene tube and the bacterial cells pelleted at 2500 × *g* for 10 min. The cells were resuspended in 600 µl of 55 mM glucose, 10 mM EDTA, 25 mM Tris at pH 8.0, to which had been added 0.3 mg ml^{-1} of lysozyme, and held on ice for 15 min. A further 1.2 ml of 1% (w/v) SDS was added and the cells held on ice for 5 min to facilitate cell lysis. A 1 ml volume of saturated sodium chloride solution was added and the tube shaken for 30 s. The solution was separated into 2 microcentrifuge tubes and centrifuged at 12 000 × *g* for 5 min at 4°C to pellet precipitated proteins. The clear supernatants were removed to a 10 ml polypropylene tube and the DNA precipitated by the addition of at least 2.5 volumes of ice-cold ethanol. The DNA was removed and washed twice in 1 ml volumes of 70% and 100% ethanol, the ethanol was evaporated and the DNA resuspended in approximately 150 µl of dH_20. The quality of DNA was determined by agarose gel electrophoresis and spectroscopy. Samples with undegraded DNA and $OD_{260/280}$ ratios greater than 2.0 were used in enzyme digests.

Approximately 5.8 µg of genomic DNA was digested to completion with the restriction endonuclease *Hin*d III (Promega, Rozelle, Australia) and separated by electrophoresis through a 25 cm, 0.65% agarose gel in tris-borate buffer (75 mM boric acid, 2.5 mM EDTA, 130mM Tris, pH 8.0) at 40 V for 40 h. Following electrophoresis the DNA gel was depurinated (0.25 N HCl for 10 min), denatured (0.5 M NaOH, 1.5 M NaCl for 60 min) and neutralised (1.5 M NaCl, 1 M Tris pH 8.0 for 60 min) before being transferred onto HYBOND-N nylon membrane (Amersham Australia, North Ryde, Australia) by the method of Southern (1975) in 20× SSC (3 M NaCl, 0.3 M sodium citrate at pH 10) and cross-linked by baking at 80°C for 2 h. The membrane was blocked for several hours at 45°C in a hybridisation oven by prehybridisation in a solution consisting of 125 mg skim milk powder, 7.5 ml SSPE (3.1 M NaCl, 0.20 M sodium phosphate, 25 mM EDTA, pH 7.4), 7.5 ml distilled formamide and 7% SDS to a total volume of 25 ml. Hybridisation was carried out overnight at 40°C in 20 ml of prehybridization solution with 400 ng of M13mp8 (Amersham, Australia) radiolabelled with [α-32P]dATP (Bresatec, Adelaide, Australia) by the primer extension method (Wells 1988). Second strand synthesis was carried out from the forward 17-mer universal sequencing primer (Promega) and was allowed to proceed for 15 min at 37°C before being terminated by the addition of 0.3% SSC thereby leaving the variable number tandem repeat (VNTR) region of the genome single-stranded, which is the active probe site of the genome (Vassart et al. 1987). After hybridisation membranes were washed under low stringency conditions in 2× SSC and 0.1% SDS at 40°C for 15 min then visualised by autoradiography with intensifying screens.

Vibrio harveyi isolates grouped by similarity of protein profile following SDS-PAGE were DNA fingerprinted. Variation within groups was calculated so that the phylogenetic relatedness of isolates grouped by protein profile could be assessed. Sharing of hybridizing *Hin*d III fragments longer than approximately 1.0 kilo base pair (kbp) was quantified as *F* (the proportion of shared fragments) (Lansman et al. 1981).

RESULTS

Virulence tests

Two isolates, 642 and PM47666-1, had a significant effect on larval mortality ($p = 0.0010$, $df = 4$), causing 100% mortality in all replicate flasks. These were the only isolates obtained from prawn hatcheries experiencing epizootics. Confirmation of these isolates' classification as *Vibrio harveyi* is provided in Table 3. Larval survival associated with other isolates did not vary significantly from the controls ($p > 0.1161$, $df = 4$).

SDS-PAGE profiles

All *Vibrio harveyi* isolates were phenotypically characterised by comparing whole-cell soluble proteins separated by SDS-PAGE. The most useful proteins for characterisation occurred in the region between 24 and 52 kDa. Most isolates could be separated into 2 main groups based on proteins within this region with little variation within each group. Group I isolates were characterised by a protein band of approximately 42 kDa and included both the virulent isolates 642 and PM47666-1 as well as isolates 650, 652, PM91, 645, 8 and 20 (Fig. 1, Group I isolates PM47666-1, 650, 652 and PM91). Isolate PM47666-1 expressed a unique protein of approximately 24 kDa (Fig. 1, lane 1). Iso-

Table 3. Characteristics used to classify isolates 642 and PM47666-1, virulent to *Penaeus monodon* larvae, into the species *Vibrio harveyi*. Characteristics of *V. harveyi* from Baumann et al. (1984)

Characteristics	*V. harveyi*	642	PM47666-1
Straight Gram -ve rods	+	+	+
Arginine dihydrolase	-	-	-
Oxidase	+	+	+
Reduction of NO_3^- to NO_2^-	+	+	+
O/F utilization of glucose	+	+	+
Luminescence	variable	+	+
Gas from D-glucose	-	-	-
Production of acetoin and/or diacetyl	-	-	-
Na^+ requirement	+	+	+
Growth at 35°C	+	+	+
Production of:			
Amylase	+	+	+
Gelatinase	+	+	-
Utilization of:			
D-mannose	+	+	+
Cellobiose	+	+	+
D-gluconate	+	+	+
D-glucuronate	+	+	+
Heptanoate	+	+	+
α-ketoglutarate	+	+	+
L-serine	+	+	+
L-glutamate	+	+	+
D,L-lactate	+	+	+
Citrate	+	+	-
L-proline	+	+	+
α-ketoglutarate	+	+	+
Pyruvate	+	+	+
D-mannitol	+	+	+
L-glutamate	+	+	+
β-hydroxybutyrate	-	-	-
D-sorbitol	-	-	+
Ethanol	-	-	-
L-leucine	-	-	-
γ-aminobutyrate	-	-	-
Putrescine	-	-	-
L-arabinose	variable	-	-
D-galactose	variable	+weak	+
Sucrose	variable	+weak	-

lates from Group II expressed a 40 kDa protein and 2 protein bands, nearly superimposed, of approximately 36 kDa. Group II included isolates 1, 301, 643, 644, 648, 656, and 9056681. The 40 kDa protein band appeared as a very strong protein band in some profiles or alternatively dissociated into 2 less intense protein bands. Both examples are evident in Fig 2a in alternating lanes 1 to 4. This polymorphism was found to result from a variation in running conditions rather than actual isolate differences as seen by separating the same protein extract from isolate 644 on different SDS-polyacrylamide gel slabs (Fig. 2b). The 2 remaining isolates, isolate 45 and 12, were polymorphic with respect to each other and to both Groups I and II in that

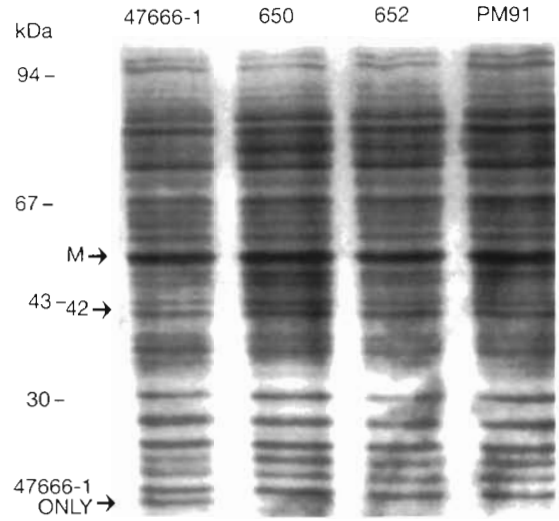

Fig. 1. *Vibrio harveyi*. SDS-PAGE profiles of whole-cell lysates of isolates designated as belonging to Group I. Numbers at the top refer to isolate numbers. The Group I specific protein band is labelled at 42 kDa. A protein of approximately 24 kDa is unique to isolate PM47666-1 M: 52 kDa major protein band. Sizes of known markers are given to the left (kDa)

they did not express the 42 kDa or 40 kDa proteins respectively (Fig. 3), and were thus not assigned to either group.

M13 DNA fingerprinting

The M13 DNA probe hybridised to between 24 and 35 DNA fragments under the conditions used (Fig. 4, Table 4) Six of the isolates classified in Group I, including the virulent isolates 642 and PM47666-1, were DNA fingerprinted (Fig. 4a) The average F was determined to be 25.34%. The average F-value calculated from 4 isolates with different protein profiles (unrelated group), consisting of isolates 656 (Group II); 642 (Group I); and 45 and 12 (unassigned isolates) (Fig. 4c), was marginally lower at 12.07%. Within Group I, all F-values were below 39.39% with the exception of the isolate pair including the virulent 642 and the avirulent PM91 which had identical DNA fingerprints (F = 100%). The virulent isolate pair of 642 and PM47666-1 had an F-value of 33.33%. In contrast to Group I, the 6 Group II isolates DNA fingerprinted (Fig. 4b) were substantially more related (average F = 47.22%) than the *Vibrio harveyi* isolates in the unrelated group (average F = 12.07).

The significance of variation present in M13 fingerprints was assessed by comparing a subset of 4 *Vibrio harveyi* isolates, 643, 644, 645 and 652 (Table 1), with 4 *V. parahaemolyticus* isolates (Table 2). The *V. harveyi* and *V. parahaemolyticus* isolates were selected to

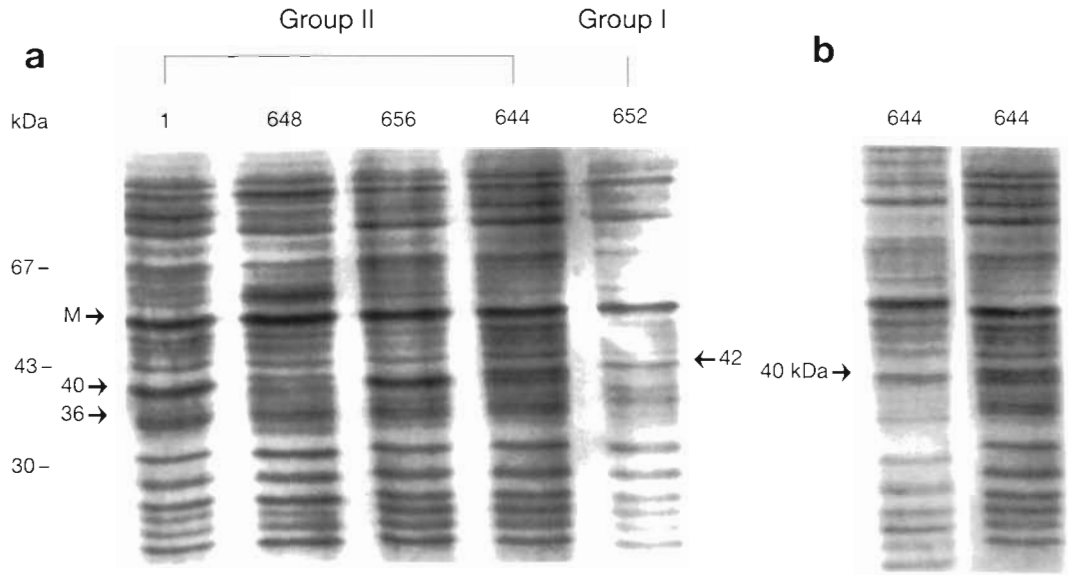

Fig. 2. *Vibrio harveyi*. (a) SDS-PAGE profiles of whole-cell lysates of isolates designated as belonging to Group II. Numbers at the top refer to isolate numbers. The Group II specific protein bands are labelled at 40 and 36 kDa. Isolate 652 demonstrating the Group I specific 42 kDa protein band is included for comparison. M: 52 kDa major protein band. Size of known markers are given to the left (kDa). (b) SDS-PAGE profile of whole cell lysate of isolate 644. Polymorphism of the 42 kDa protein band results from minor variation in the running conditions as seen by separating the same protein extract from isolate 644 on different SDS-polyacrylamide gel slabs

include isolates diverse in origin without knowledge of protein or DNA fingerprint data. *V. parahaemolyticus* is closely related to *V. harveyi* and provided an indication of the level of genetic diversity which could be expected within these *Vibrio* species. Under slightly higher conditions of stringency than used previously, the *V. harveyi* isolates demonstrated a substantially lower average association when compared with the *V. parahaemolyticus* isolates (average F = 17.22% and 61.94% respectively) (Fig. 5).

DISCUSSION

The DNA probe used in this study was derived from the bacteriophage M13. It is a multilocus probe binding to regions in DNA with homology to a 15 bp tandem repeat (Vassart et al. 1987). M13 has been used successfully to probe a wide variety of eukaryotic and prokaryotic organisms (Ryshov et al. 1988) but has only seen limited use as a phylogenetic tool with bacteria (Huey & Hall 1989).

The variation in hybridisation patterns exhibited among isolates of *Vibrio harveyi* suggests that this species is genetically diverse. *V. parahaemolyticus* is closely related to *V. harveyi* (Reichelt et al. 1976, Bang et al. 1978, Brenner et al. 1983, Lambert et al. 1983) but seems to exhibit much less intra-species variation with an average F-value of 61.94% which is substantially higher than the *V. harveyi* average F-value of 17.22%. Genetic variability amongst *V. harveyi* isolates was noted in earlier DNA hybridisation experiments (Reichelt et al. 1976). This may in part account for the high phenetic heterogeneity seen in *V. harveyi* when screened for their nutritional requirements (Reichelt & Baumann 1973).

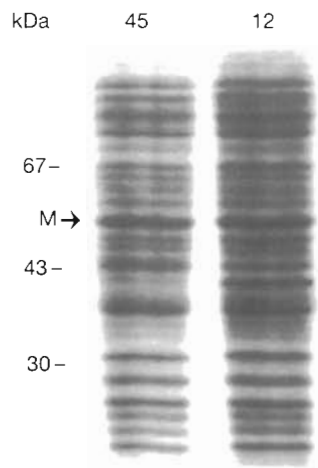

Fig. 3. *Vibrio harveyi*. SDS-PAGE profiles of whole-cell lysates of isolates not classified as belonging to either Group I or Group II. Numbers at the top refer to isolate numbers. M: 52 kDa major protein band. Sizes of known markers are given to the left (kDa)

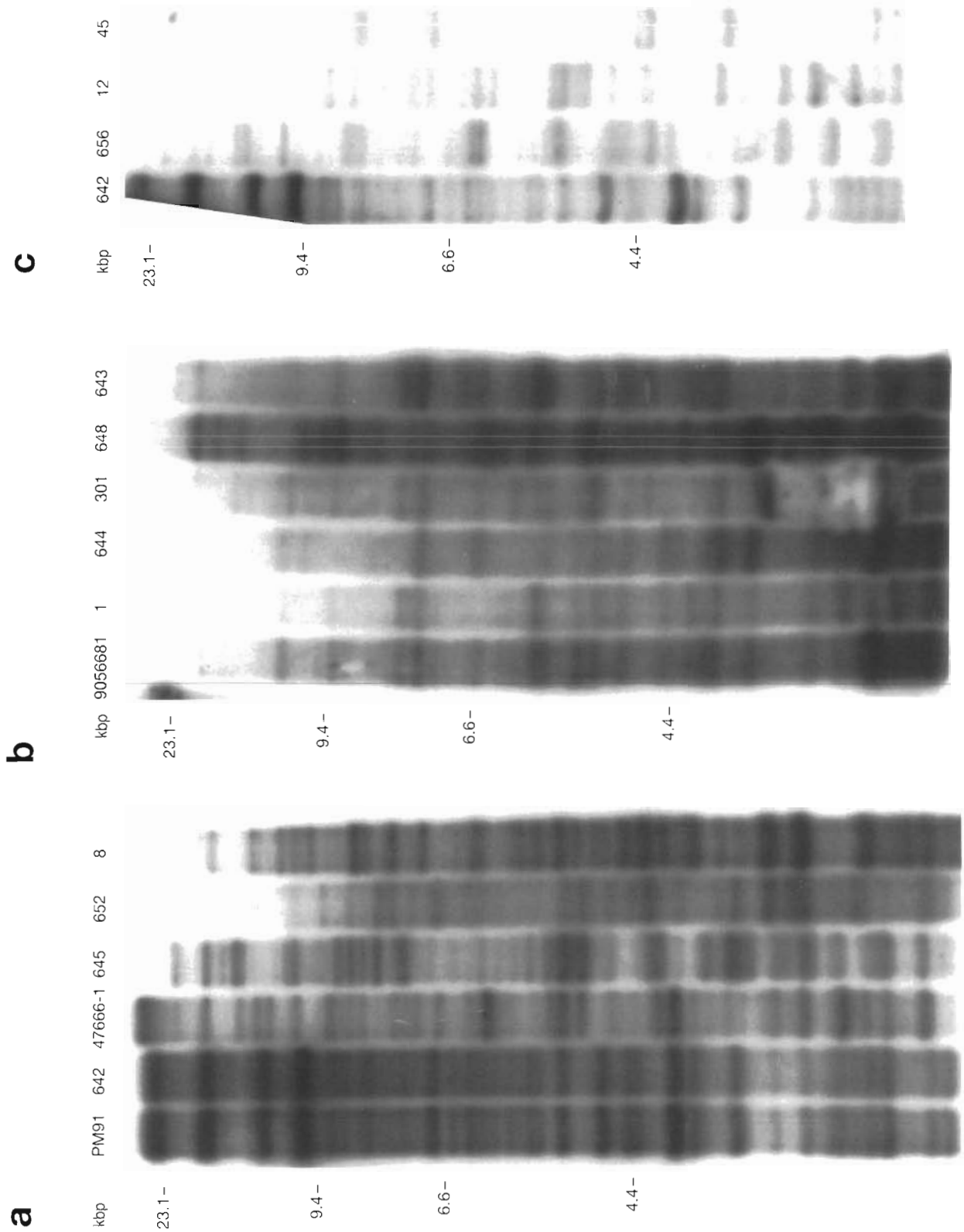

Fig. 4. *Vibrio harveyi*. DNA fingerprints of isolates. Genomic DNA digested with *Hind* III and hybridised using a radiolabelled M13 DNA probe. (a) Group I isolates; (b) Group II isolates; (c) unrelated *V. harveyi* isolates. Size of known markers are given to the left of autoradiographs in kilo base pairs (kbp)

Table 4. *Vibrio harveyi*. Number of M13 hybridising *Hind* III generated fragments in DNA fingerprints and average *F*-values

	No. of hybridising fragments	Average *F*-value (%)
Group I	24–30	25.34
Group II	25–35	47.22
Unrelated isolates	29–31	12.07

Fifteen of the 17 *Vibrio harveyi* isolates could be assigned to 2 groups based on their whole-cell protein profiles generated by SDS-PAGE under reducing conditions. These were designated Groups I and II. The phylogenetic relatedness among Group II isolates assessed by M13 DNA-fingerprinting was substantially higher compared to the unrelated isolate pairs (average F values 47.22% and 12.07% respectively). The Group I isolates appeared to be less related than the Group II isolates, even so there was a marginally higher level of relationship within this group than among the unrelated isolates (average *F*-values 25.34% and 12.07% respectively). It would seem, therefore, that the grouping by protein profile has a phylogenetic basis. Eight isolates fell within Group I, 7 isolates within Group II and 2 remained ungrouped. These proportions should reflect the relative proportions of these 2 groups found naturally in the environment, based on the wide diversity of sources of these isolates. If discrimination among most *V. harveyi* isolates can be achieved by differences in expression of the 42 and 40 kDa proteins, then the groupings proposed could be used as a means of subtyping the species.

Only 2 isolates proved to be virulent to *Penaeus monodon* larvae despite the fact that 10 of the 17 isolates were derived from moribund prawns. Most of these clinical isolates may well have been secondary invaders only. The 2 isolates shown to be virulent to prawn larvae, PM47666-1 and 642, originated from prawn hatcheries experiencing epizootics. Isolate PM47666-1 has been recognised previously as a highly virulent strain able to cause disease at doses as low as 100 organisms ml^{-1} seawater (P. Muir pers. comm.).

Even though most isolates could be subgrouped, the association between virulence and phylogeny is tenuous. Both of the virulent isolates belonged to protein Group I but they were not closely related within this group ($F = 33.33\%$). In contrast, a 100% relationship was observed between the virulent isolate 642 and the avirulent PM91, demonstrating that there would appear to be no association between virulence and the phylogenetic background of the isolates. A

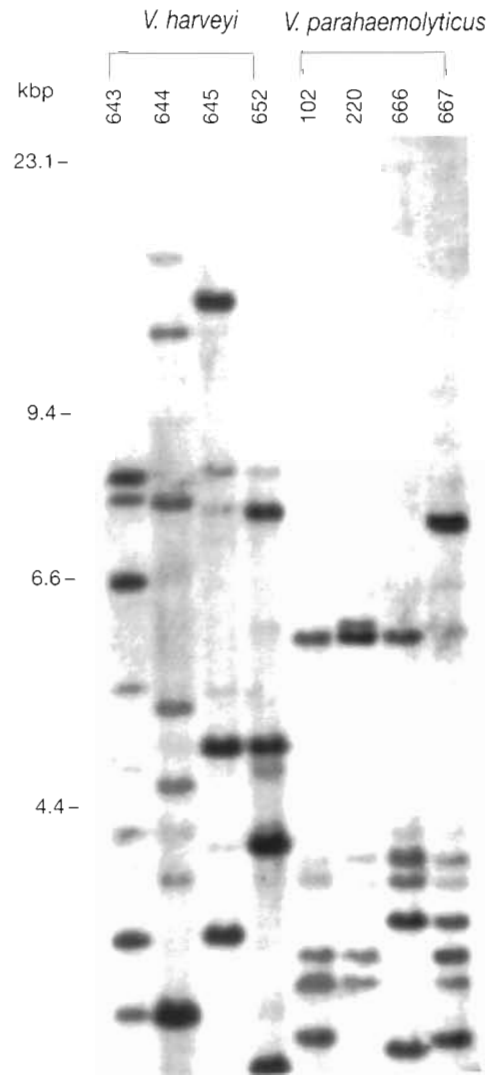

Fig. 5. *Vibrio harveyi* and *V. parahaemolyticus*. DNA fingerprints of isolates from diverse sources. Genomic DNA digested with *Hind* III and hybridised using a radiolabelled M13 DNA probe. Conditions of stringency were higher than previously used with hybridisation carried out at 45°C. Size of known markers are given to the left of the autoradiograph in kilo base pairs (kbp)

likely explanation is that isolates of *Vibrio harveyi* have acquired virulence factors associated with genetically mobile elements, most probably in the form of plasmids and/or transposons. Horizontal genetic transfer is a highly evolved process in bacteria and may explain why a few strains of *V. harveyi* such as isolates PM47666-1 and 642 have acquired virulence factors, even though similarly related or even highly related isolates (such as isolate PM91 with 642), do not appear to be virulent. Work is continuing to investigate whether virulence amongst *V. harveyi* strains could result from genetic transfer.

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