Shell disease syndrome in the edible crab, *Cancer pagurus* – isolation, characterization and pathogenicity of chitinolytic bacteria

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Chitinolytic bacteria are believed to be the primary aetiological agents of shell disease syndrome in marine crustaceans. The disease principally results from the breakdown of their chitinous exoskeletons by the shell disease pathogens, but pathogenicity may also manifest internally should a breach of the carapace occur. The current study looks at the pathogenicity of a number of bacteria (predominantly from the genus *Vibrio*) isolated from the edible crab, *Cancer pagurus*. All chitinase-producing bacteria investigated were capable of growth in a minimal medium consisting of chitin powder from crab shells, but differed in their speed of growth and nature of chitinolytic activity, suggesting that they have different roles within the lesion community. Two isolates (designated I4 and I7) were chosen for studies on internal pathogenicity, which included the effect of the pathogen on crab tissues, the ability of the host to remove the bacteria from circulation and the antibacterial activity of crab blood. Initially, I4 was rapidly removed from circulation, but began to reappear in the blood after 24 h. By 100 h, 100% of crabs were moribund. The septicaemic effects of the isolate were reflected in the low levels of its killing by blood-cell lysate and serum. By contrast, I7 was only slowly removed from circulation and caused the rapid mortality of all crabs in <3 h. A large decline in the number of circulating blood cells following injection of I7 was mirrored by an accumulation of these cells in the gills. Initial experiments suggest that the death of the crabs following injection with I7 may be caused by toxic extracellular bacterial products that exert their effects on the blood cells and nervous system of the crabs.

Keywords: enzymic activity, histopathology, bacterial extracellular products

INTRODUCTION

Chitin is an abundant polymer within the marine environment, thus chitinolytic bacteria are both common and vital to nutrient recycling (reviewed by Keyhani & Roseman, 1999). Chitin breakdown is largely restricted to the moulted shells of marine crustaceans. In situations where chitin degradation occurs on the exoskeletons (cuticle) of living crustaceans the condition is known as shell disease or black-spot. In natural populations the disease is rarely reported in more than 10% of individuals (e.g. Ayres & Edwards, 1982; Baross & Tester, 1978). However, elevated levels of shell

disease have frequently been reported amongst crustaceans living in degraded environmental conditions, such as aquaculture systems (e.g. Delves-Broughton & Poupard, 1976; Prince *et al.*, 1995) or polluted environments (e.g. Young & Pearce, 1975; Sawyer, 1991).

The disease commences with the removal of the non-chitin-containing outer layer of the exoskeleton, called the epicuticle, and may occur by proteolytic and lipolytic microbial activities (Cipriani *et al.*, 1980), predatory or cannibalistic attacks (Dyrynda, 1998), chemical attack (Schlotfeldt, 1972) or the abrasive action of sediment and/or articulated body parts (Young, 1991; Vogan *et al.*, 1999). Once the underlying chitin-containing procuticle is exposed, shell degradation is largely attributed to the chitinolytic members of the lesion microbial community (for reviews see Getchell, 1989; Stewart, 1993).

Abbreviations: DMAB, *p*-dimethylaminobenzaldehyde; ECP, extracellular product; pNP, *p*-nitrophenol; SEM, scanning electron microscopy.

Bacteria belonging to the genera *Vibrio*, *Aeromonas*, *Pseudomonas*, *Alteromonas*, *Flavobacterium*, *Spirillum*, *Moraxella*, *Pasteurella* and *Photobacterium* are all reported as probable agents involved in the disease syndrome (Getchell, 1989). Although the disease is not believed to be fatal in its initial stages, mortality is thought to result in the later stages of the disease either from (i) unsuccessful moulting (e.g. Smolowitz *et al.*, 1992) or (ii) as a result of septicaemic infections by pathogenic bacteria originating by entry through the lesion sites (Baross & Tester, 1978; Vogan *et al.*, 2001).

Previous studies have found high prevalences of shell disease in the commercially fished edible crab, *Cancer pagurus*, from the Swansea Bay region of South Wales (Vogan *et al.*, 1999). The current study investigates the pathogenic effects of a number of exoskeletal chitinolytic isolates both in terms of their abilities to degrade cuticular chitin (i.e. external pathogenicity) and their effect on the host following penetration into blood (i.e. internal pathogenicity).

METHODS

Animals. Edible crabs, *Cancer pagurus*, were obtained from pots anchored between Oxwich Bay and Pwlldu Head, or Langland Bay, Gower, UK. All were intermoult males (100–150 mm carapace width). Initial bacterial isolation was performed immediately after return to the laboratory on crabs with variable levels of shell disease on their external body surfaces. Crabs used in bacterial pathogenicity experiments were maintained in aerated tanks in a circulating sea-water aquarium (15 °C and 35‰ salinity) for about 24 h prior to their use and had < 1% of their external surfaces covered with black-spot lesions.

Bacteria. *Vibrio alginolyticus* (NCIMB 1339), *Vibrio splendidus* (NCIMB 2251), *Vibrio vulnificus* (NCIMB 2139) and *Vibrio pelagius* (*Listonella pelagia*; NCIMB 1900) were all routinely maintained on tryptic soy agar containing 1% NaCl (TSA + 1% NaCl) or Difco Marine Agar 2216 (MA).

Bacterial isolation. Bacteria were cultured from the blood (haemolymph) and exoskeleton of healthy and shell-diseased specimens of *C. pagurus*. Bacteria from the blood of crabs were isolated on MA as described by Vogan *et al.* (2001). Exoskeletal bacterial isolates were obtained by abrasion of shell-disease lesion and non-lesion areas of both dorsal and ventral surfaces (see Fig. 1) using a sterile scalpel. Each scraping was transferred into 600 µl sterile 3.2% NaCl solution, homogenized briefly and spread-plated in triplicate onto MA and chitin agar (comprising an underlay containing 20 g Difco agar l⁻¹, 1 g NH₄Cl l⁻¹, 75 mg K₂HPO₄·3H₂O l⁻¹, 25 mg FeSO₄·7H₂O l⁻¹, 50 ml 1 M Tris/HCl, 500 ml sea water and 450 ml H₂O, pH 7.5, plus an overlay containing 10 g Difco Noble agar, 75 mg K₂HPO₄·3H₂O l⁻¹, 25 mg FeSO₄·7H₂O l⁻¹, 50 ml 1 M Tris/HCl, 500 ml colloidal chitin and 450 ml H₂O, pH 7.5). Plates were incubated at 10, 20 or 25 °C for up to 10 days. Pure cultures from randomly chosen colonies were placed on MA slopes and stored at 4 °C until use.

Characterization of chitinolytic bacterial isolates. Based on preliminary characterization (colony morphology, chitinase production on chitin agar, bacterial morphology and Gram-staining), nine chitinolytic isolates were chosen for initial phenotypic profiling. Each of the chosen isolates, designated I1–I9, were distinctive in terms of these tests. Known strains of

V. alginolyticus (NCIMB 1339 and a further strain courtesy of DRIM Laboratories, Montpellier, France) and *V. splendidus* (courtesy of DRIM Laboratories) were run in parallel. Isolates were taken from slopes and subcultured twice on MA plates before determining each test characteristic.

Temperature tolerance, the level and requirement of sodium ions for growth, susceptibility towards the vibriostatic agent V0129 (10 and 150 µg), motility and catalase activity were all investigated using standard techniques. API 20E and 20NE isolate profiles were obtained according to the instructions of the manufacturer (bioMérieux) with the following recommended modifications for use with marine bacteria. Suspension medium (AUX, supplied with the bioMérieux API 20NE kit) used for assimilation tests in API 20NE was supplemented with 200 µl saturated NaCl solution. All other bacterial suspensions for strip inoculations were made using 3.2% NaCl. Biolog GN Microplates (Don Whitely Scientific) were used to assess utilization of 95 different carbon sources by the isolates. The plating protocol of the supplier for use with marine bacteria was followed using a marine cation supplement (2.5% NaCl, 0.8% MgCl₂, 0.05% KCl) as the suspension medium. All plates were incubated at 25 °C for 72 h and positive reaction wells were recorded.

Bacteria were also tested for their ability to produce caseinase, lipase, phospholipase and gelatinase on agar plates as described by Zhang & Austin (2000). Two selective agars were used for the partial differentiation of *Vibrio* spp. Cellobiose–colistin agar (CC agar) and *Vibrio vulnificus* medium (VVM agar) were prepared and isolate-inoculated plates were incubated as described by Høi & Dalsgaard (2000) and Cerdà-Cuellar *et al.* (2000), respectively. Presumptive *V. vulnificus* isolates produce yellow colonies on CC agar and bright yellow colonies with yellow diffusion haloes on VVM agar. According to Cerdà-Cuellar *et al.* (2000), on VVM agar *Pseudomonas aeruginosa*, *Vibrio* (*Listonella*) *anguillarum*, *Vibrio cholerae*, *Vibrio mediterranei*, *Vibrio mimicus* and *Vibrio proteolyticus* appear as green colonies, whereas *Vibrio campbellii*, *Vibrio carchariae* (*harveyi*) and *Vibrio navarrensis* produce yellow colonies.

Extracellular chitinase activity of bacterial isolates. The patterns of chitinase production by bacterial isolates were determined in a minimal chitin medium which consisted of 4% (w/v) Sigma sea salts, 5% (v/v) 1 M Tris/HCl, pH 7.5, and 1% (w/v) chitin powder (from crab shells; Sigma). Isolates used in chitinase production experiments were initially grown for 24 h in tryptic soy broth (TSB) supplemented with 1% NaCl and washed twice in sterile Tris-buffered sea-salt solution (4%, w/v, Sigma sea salts, 5%, v/v, 1 M Tris/HCl, pH 7.5) prior to inoculation into the minimal chitinase medium. Bacterial counts were adjusted to give a final concentration of 2 × 10⁶ bacteria ml⁻¹ in inoculated minimal medium. Both test and control flasks (containing sterile chitinase medium) were incubated in triplicate at 25 °C and aliquots were removed aseptically at a number of time points. Initial time series chitinase profiles (using I3; see Table 1) were determined from 50 ml cultures with aliquots (6 ml) taken at 0, 48 and 96 h, and thereafter at every 24 h until the bacteria had been growing for 408 h. All other chitinase incubation experiments were measured in cultures (25 ml) with 4 ml aliquots taken after 0, 192 and 408 h. Each aliquot was enumerated on a Thoma bacterial counting chamber and the remainder was centrifuged (1800 g, 5 min, room temperature) before filter-sterilization. Aliquots of each crude enzyme supernatant were heat-inactivated (100 °C, 5 min). All enzyme and heat-inactivated supernatant samples were stored at –20 °C until analysis of their chitinolytic abilities.

Fig. 1. (a) The ventral surfaces of *C. pagurus* displaying the characteristic black-spot lesions of shell disease. (b) Low power scanning electron micrograph of a ventral carapace lesion. Boxed regions are enlarged in (c) and (d). Note the columnar pattern of degradation in the central regions of the lesion (c) which contrasts with the lamellar cleavage planes at lesion peripheries (d). Bars: 5 cm, (a); 100 μ m (b); 10 μ m (c, d).

Total chitinase activity was assessed by the production of *N*-acetylglucosamine when the crude enzyme supernatants were incubated with colloidal chitin using a method adapted from Yanai *et al.* (1992). Briefly, enzyme supernatant samples (250 μ l) were incubated with 125 μ l colloidal chitin and 125 μ l 0.1 M Tris/HCl, pH 7.5, containing 0.08% sodium azide, for 46 h at 25 °C. After centrifugation (21000 g, 2 min, 25 °C),

125 μ l supernatant was added to 25 μ l 0.8 M boric acid (adjusted to pH 10.2 using KOH) and the mixture was heated in a boiling water bath for 3 min. The sample was cooled (30 min, 4 °C) and 750 μ l *p*-dimethylaminobenzaldehyde (DMAB) solution [1%, w/v, *p*-dimethylaminobenzaldehyde (Sigma), 99%, v/v, glacial acetic acid, 1%, v/v, concentrated HCl] was added. After incubation at 37 °C for 20 min, each sample

Table 1. Summary of the principal test characteristics for chitinolytic bacterial isolates 1–9 (I1–I9)

All isolates were found to be Gram-negative rods which were motile in liquid medium. Cluster groups are based on the isolates displaying 85 % simple matching coefficients to each other (see Fig. 2). +, Positive activity; –, negative enzyme activity; NG, strain did not grow on medium.

Test	Cluster group								
	A				D		E	F	G
	I1	I2	I6	I3	I4	I8	I5	I7	I9
Na ⁺ requirement	+	+	+	+	–	–	+	+	+
Max. Na ⁺ where growth observed (%)	8	8	8	10	3	3	3	3	10
Oxidase	+	+	+	+	–	–	+	+	+
Catalase	+	+	+	+	+	+	+	–	+
V0129 sensitivity	+	+	+	+	–	–	+	+	–
Water-soluble pigment	–	–	–	–	–	+	–	–	–
D-Mannitol utilization	+	+	+	+	–	–	+	+	+
Caseinase	–	+	–	–	+	+	–	+	–
Gelatinase*	– (+)	NG (+)	– (+)	NG (+)	+	+	NG (–)	NG (+)	–
Phospholipase	+	+	+	+	+	+	–	–	NG
Lipase	–	–	–	–	+	+	+	+	NG
Colony colour on VVM agar	NG	NG	NG	Green/yellow	NG	NG	NG	NG	NG
Colony colour on CC agar		Green/yellow			Green	Yellow	Yellow	NG	NG
Closest genus† (% S _{SM})	(100)	<i>Vibrio</i>		(100)	(80)	<i>Xanthomonas</i>		(88)	(82)
Closest species† (% S _{SM})		<i>V. splendidus</i> b1		<i>V. anguillarum</i>	<i>X. maltophilia</i>		<i>V. splendidus</i> b2	<i>V. pelagius</i> b1/ <i>V. vulnificus</i>	<i>V. splendidus</i> b2
	(93)	(87)	(93)	(82)	(86)	(86)	(80)	(71)	(80)

* Values in parentheses indicate differing results detected using API commercial test kits.

† Compared to those defined in Holt *et al.* (1994).

was plated in triplicate onto a 96-well microtitre plate and the OD₅₅₀ was measured. Sample blanks (containing 250 µl heat-inactivated supernatants) and negative controls (consisting of 250 µl supernatant from sterile control culture flasks) were run in parallel. A standard curve, for the linear range of the assay, was produced using serial dilutions of *N*-acetylglucosamine (Sigma) and DMAB detection. One unit of enzyme activity was defined as the amount of enzyme required to produce sugars equivalent to 1 µmol *N*-acetylglucosamine min^{–1} under the above conditions.

‘Endo-acting’ chitinase activity (i.e. that principally acting on the central portions of the chitin polymer) of each supernatant was detected in a turbidometric microplate assay using colloidal chitin (adapted from Tronsmo & Harman, 1993). Aliquots (150 µl) of crude enzyme supernatants (test samples) and heat-inactivated supernatants (sample blanks) were added in triplicate to wells that contained 50 µl colloidal chitin. Negative controls that contained 150 µl of supernatants from sterile control culture flasks were also included on each plate. After a 10 s shake, a time zero OD₄₉₂ reading was taken on a microplate reader. The plates were sealed and incubated at 25 °C for 14 days. Endochitinase activity for each bacterial supernatant was defined as a reduction in the sample OD₄₉₂ between time zero and 14 days compared with its sample blank.

The chitinolytic activities of ‘exo-acting’ chitinases (acting on terminal locations on the chitin polymer) were quantified in two separate assays by the release of *p*-nitrophenol (pNP) from *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose (Sigma), respectively. Stock solutions of each substrate were prepared at a concentration of 0.275 mM in 1 M Tris/HCl (0.02 % sodium azide) and frozen at –20 °C until use in the microtitre assay. Crude enzyme supernatant (10 µl) was added in triplicate to wells containing 90 µl pNP substrate stock solutions. Sample

blanks (10 µl heat-inactivated supernatants) and negative controls (sterile control culture supernatants) were run in parallel. The pNP substrate microplates were sealed and incubated at 25 °C for 46 h. Reactions were terminated by the addition of 10 µl 1 M NaOH to each well and the OD₄₀₅ was measured. One unit of enzyme activity was defined as the amount of enzyme activity that liberated 1 µmol pNP min^{–1} under the above conditions. According to dal Soglio *et al.* (1998), 1 mM pNP is equivalent to an OD₄₀₅ increase of 6.367.

Clearance of chitinolytic isolates in crabs. I4 and I7 (see Table 1) were grown overnight in TSB + 1 % NaCl at 25 °C with shaking. The bacteria were washed twice in sterile marine saline [MS; 0.58 M NaCl, 20 mM CaCl₂, 12 mM KCl, 0.56 mM disodium phosphate, 0.05 M Tris(hydroxymethyl)-methylamine, pH 7.4] and adjusted to 2 × 10⁹ bacteria ml^{–1}. All injections into crabs were made through the surface-sterilized soft membrane where the fifth pereopod (fourth walking limb) joins the body. Blood samples were removed for blood-cell (haemocyte) and bacterial counts from the soft membrane on the cheliped (fighting limb) as described in Vogan *et al.* (2001).

Total blood volume in groups of *C. pagurus* was estimated using the Amaranth dye dilution technique (Smith & Ratcliffe, 1980) and the volume of bacterial suspension (2 × 10⁹ bacteria ml^{–1}) to be injected was calculated so as to give a circulating blood dose of 2 × 10⁶ bacteria (ml blood)^{–1}. Prior to bacterial injection into the crabs, a blood sample was removed from each animal for blood-cell counts and bacterial sterility checks. Control crabs received a dose of sterile MS in place of the bacterial suspension. After 1, 6, 24 and 48 h, a 200 µl blood sample was removed from each crab and serially diluted in sterile sea salts solution (Sigma). A 100 µl aliquot was spread in triplicate onto TSA + 1 % NaCl plates and incubated for about 24 h to allow for bacterial growth. The original bacterial suspension (2 × 10⁹ cell ml^{–1}) was also plated. Total blood-cell

counts were conducted between 5 min and 48 h post-injection using a haemocytometer.

Experiments involving injection of extracellular products (ECPs) from I7 (see Table 1) followed the same protocol, except for the substitution of filter-sterilized (0.22 µm) aliquots from 48 h TSB + 1 % NaCl cultures in test animals and filter-sterilized un-inoculated TSB + 1 % NaCl for control animals.

Crabs were considered dead when no ventilatory or sensory movements were detected. Crabs that died during the experimental period were immediately fixed for histology. Control crabs were sacrificed for histology at the end of the experimental period (48 h).

Histopathology and scanning electron microscopy (SEM). All crabs that died during the experimental period were immediately injected with about 50 ml Bouin's sea water fixative and dissected to remove the main organs. Tissues were stored and processed as detailed in Vogan *et al.* (2001). Wax sections (about 10 µm thick) were cut and stained using Cole's haematoxylin and eosin. Photographs were taken using a Zeiss Photomicroscope II. For SEM, small pieces (about 2 mm²) of lesion and non-lesion crab cuticle were fixed for about 12 h in ethanol and transferred into acetone (30 min) prior to air-drying and coating with gold. All samples were examined with a JEOL scanning electron microscope.

Antibacterial activity assay. The *in vitro* antibacterial activity of crab blood towards I4 and I7 was assessed using a method described by Chisholm & Smith (1992) adapted for use in a turbidometric growth microplate assay. Test samples containing 225 µl of either clotted whole blood (serum; $6.4 \times 10^4 \pm 0.8 \times 10^4$ µg protein ml⁻¹; SEM ± 1 , $n = 6$) or a lysate of the blood cells ($1.0 \times 10^2 \pm 0.2 \times 10^2$ µg protein ml⁻¹; SEM ± 1 , $n = 6$), prepared as described by Vogan & Rowley (2002), were incubated with 25 µl I4 or I7 in MS (4×10^6 bacteria ml⁻¹). For controls, test samples were substituted with equal volumes (225 µl) of sterile MS. Sample blanks contained 225 µl of each particular test sample and 25 µl sterile MS. All samples, for a given bacterial isolate, were incubated at room temperature with constant shaking for 30 min. They were then rapidly flooded with 2.25 ml TSB + 1 % NaCl and 200 µl aliquots were plated in triplicate into a 96-well microtitre plate. The OD₅₅₀ of each plate was measured on a microplate reader, after incubation at 25 °C for differing lengths of time so as to give an OD₅₅₀ of control wells that lies in the middle of the exponential growth phase for each bacterial isolate.

Protein concentrations in serum and blood-cell lysate were determined using a BCA (bicinchoninic acid) assay kit in accordance with the manufacturer's instructions for use in a microtitre plate (Pierce & Warriner). All samples were replicated in triplicate and calibrated against a bovine serum albumin (BSA) standard curve (100–1000 µg ml⁻¹) run on the same plate.

Data analyses. All data used for numerical taxonomic comparisons were converted into binary figures, scoring '1' for positive reactions and '0' for negative reactions. Inconclusive results were omitted from all comparisons. A positive test result (1) was recorded when 75 % of the strains tested were positive, a negative test result (0) was recorded when 25 % of the strains tested were positive and tests with results between 25 and 75 % were taken as being inconclusive (omitted from comparison), as described by Holt *et al.* (1994). The isolate test results were then compared to each other and to data published in Holt *et al.* (1994) using the simple matching coefficient of similarity. The resultant similarities (0–100 %) were then plotted onto dendrograms using the

UPGMA (unweighted pair group method using arithmetic averages) clustering method in the Genstat software package.

Bacterial diversity frequencies were assessed using contingency tables followed by either χ^2 test (for tables with < two rows or columns) or Fisher's exact test (for tables with $\leq$ two rows or columns). For mean values $\pm$ SE the appropriate parametric test (*t*-test or ANOVA) was employed. For ANOVA, the Tukey's, Bonferroni and Dunnett post-tests were used as recommended by Instat (GraphPad).

RESULTS AND DISCUSSION

Bacterial diversity in shell disease lesions and diseased crabs

Crabs with shell disease syndrome show characteristic darkened lesions through the cuticle (Fig. 1a). Such lesions have previously been shown to occur on all regions of the exoskeleton (Vogan *et al.*, 1999). SEM examination of these lesions revealed the extensive nature of the cuticular erosion into the chitinous procuticle (Fig. 1b–d). In all regions of the lesion the epicuticle was absent as indicated by the appearance of small pore canals (about 1 µm in diameter) that permeate through the underlying procuticle. Exoskeletal degradation was most severe towards the centre of the lesion, which tended to display severe pitting with a columnar pattern of degradation (Fig. 1c). Towards the peripheries of the lesion the degree of pitting decreased and displayed areas of exposed procuticle which had broken along the lamellar cleavage planes that run parallel to the epidermis (Fig. 1d).

The majority of bacteria cultured from the blood ($n = 19$) and exoskeleton ($n = 67$) of shell-diseased *C. pagurus* were Gram-negative rods (57.9 and 89.6 % respectively). The proportion of Gram-positive eubacteria was significantly higher amongst the blood-derived isolates (33 %) than those found in the exoskeleton (7.7 %; χ^2 test, $P = 0.0009$). Chitinase-producing bacteria were isolated from the blood (21.1 % of total culturable isolates) and from both lesioned and non-lesioned portions of the exoskeleton (26.9 % of total culturable isolates). No significant differences were found in the percentages of culturable chitinolytic bacteria residing in lesion (31 %) and non-lesion (15 %) areas (Fisher's exact test, $P = 0.2289$).

From the 22 bacterial colony types that were positive for chitinolytic activity, 9 isolates (designated I1–I9) were selected for further phenotypic analysis. Selection was based on their distinct behaviour in several preliminary tests, including Gram staining, colony and bacterial morphology. I1 and I8 were from blood of shell-diseased individuals. I2, I4, I5, I6, I7 and I9 were from exoskeletal lesions, while I3 was from a non-lesion area. All were motile, Gram-negative rods that generally varied in length between 0.6 and 2 µm. However, the rods of I4 were elongate (1.5–5 µm) and formed long filamentous chains, whereas those of I9 were almost spherical in appearance. These isolates were clustered based on 157

Fig. 2. UPGMA clustering of bacterial isolates based on 157 individual test results, using a simple matching coefficient of similarity. I1–I9 are the chitinolytic isolates. V1–V3 are *V. alginolyticus* (NCIMB 1339), *V. alginolyticus* ('French' strain) and *V. splendidus*, respectively. Cluster groups A–G are based on isolates displaying 85% simple matching coefficients.

Fig. 3. Total extracellular chitinase activity (---) in relation to growth of cultures (—) of I3. The bacterial isolate was grown in a minimal chitin powder medium at 25 °C with shaking for > 408 h. Mean values $\pm$ 1 SE are shown ($n = 3$).

test characteristics (Fig. 2; Table 1). Bacteria in cluster group A (I1, I2, I3 and I6) differed mainly in their ability to produce β -galactosidase, ferment amygdalin and arabinose, and in several carbon utilization tests. Cluster D contained two isolates, I4 and I8, that were distinct from all others tested and were less likely to belong within the genus *Vibrio*. Within the cluster the two strains differed in pigment production, cell form and a small number of carbon utilization tests (Tween 40, N-acetyl-D-glucosamine, acetic acid, *cis*-aconitic acid,

formic acid, glucose-1-phosphate). I5, I7 and I9 were <85% similar to all others and hence were contained within individual clusters.

According to Holt *et al.* (1994) members of the family *Vibrionaceae* (Bergey's Group 5.2) are Gram-negative, oxidase-positive, motile rods, capable of growth at 37 °C as long as there is a source of sodium ions. Although growth at 37 °C was not tested, I1, I2, I5, I6, I7 and I9 all grew at 35 °C and satisfy the other requirements for classification into *Vibrionaceae* (Table 1). Comparisons of the test isolates to genera known to contain chitinolytic strains were made using data in Holt *et al.* (1994) (Table 1). It was found that I1, I2, I3 and I6 were most likely to be *Vibrio* species (100% simple matching similarity). I5, I7 and I9 were also most likely to belong within the genus *Vibrio* (88, 82 and 82% simple matching similarities, respectively). Like the majority of members of this genus, I5 and I7 were sensitive to the vibriostatic agent V0129 (150 μ g) and utilized D-mannitol as carbon source. I9, however, was resistant to V0129 and did not utilize D-mannitol, suggesting that it may not be a *Vibrio* species. However, Holt *et al.* (1994) recorded that some strains of *V. alginolyticus*, *Vibrio cincinnatiensis* and *Vibrio parahaemolyticus* are resistant to V0129. In the current study, a lack of sensitivity to V0129 was also displayed by both *V. alginolyticus* and *V. splendidus*.

Urakawa *et al.* (1999) found that *V. splendidus* strains were the most abundant culturable vibrios from sea water. The current study found five of the nine strains isolated from *C. pagurus* to be most closely related to *V. splendidus* (2 biovars listed in Holt *et al.*, 1994). I7 displayed joint highest similarities to *V. pelagius* (71%) and *V. vulnificus*. It was concluded that this isolate was unlikely to be a strain of *V. vulnificus*, since it did not produce the characteristic yellow diffusion haloes on VVM agar (Table 1).

The isolates of cluster group D (I4 and I8) were distinct from the other isolates in that they were oxidase-negative and capable of growth in the absence of sodium ions. Chitinolytic Gram-negative rods that are oxidase-negative either belong to the family *Enterobacteriaceae* (Bergey's Group 5.1) or within Bergey's Group 4.4A (Holt *et al.*, 1994). Although the similarity comparison calculations were based only upon seven test characteristics, I4 and I8 both seem to be most closely related to *Xanthomonas maltophilia* (as listed in Holt *et al.*, 1994). These results are in agreement with those found when comparing data to the API published profile (data not shown) and the genus identification (Table 1).

It is generally accepted that a large proportion of bacteria in the marine environment are not detected by culturing onto high nutrient media. Over the last 15 years, the existence and nature of a viable but non-culturable state has been much debated (e.g. McDougald *et al.*, 1998). Roszak & Colwell (1987) described the dynamic state of the microbial cell in its natural state as being able to readily adapt and survive fluctuations in environmental parameters such as nutrient source and

Fig. 4. Chitinase activity from culture supernatants of I3, I4, I7 and I8 grown in a minimal chitin powder medium when sampled after 192 (□) and 408 h (■) of growth. (a) Number of bacteria (ml culture)⁻¹. (b) Total chitinase activity, (c) endo-acting chitinase activity and (d, e) exo-acting chitinase against the pNP-monomer (d) and dimer (e) in culture supernatants standardized for 1×10^9 bacteria. Mean values ± 1 SE are shown ($n = 3$). Significant differences (paired *t*-test, $P < 0.05$) for individual isolates between 192 and 408 h are indicated by asterisks. Significant interisolate differences (ANOVA, $P < 0.05$; Tukey's post-test, $P < 0.05$) at either 192 or 408 h are indicated by roman or italicized letters, respectively.

availability, temperature and light. Thus, culturing onto standard laboratory media provides non-comparable conditions to those of oligotrophy that are found in the natural environment. Hence, only those organisms that are most able to adapt to the specific culture conditions will be able to grow and divide. The current study was not intended to provide an exhaustive list of all micro-organisms involved in or capable of exoskeletal degradation. Compared to the vast majority of the marine environment, shell disease lesions provide a high nutrient niche and thus, culturing onto a chitin-containing medium may not be as extreme as, for instance, culturing directly from sediments. Thus, the types of chitinolytic micro-organisms cultured on chitin agar plates might,

perhaps, be considered analogous to the primary lesion colonizers, i.e. having the ability to rapidly detect and adapt to newly exposed chitin.

Pathogenicity of chitinolytic isolates

In this study, the pathogenicity of the chitinolytic (shell disease) isolates was considered on two levels: (i) their ability to contribute to exoskeletal breakdown by the expression of chitinase activity (external pathogenicity), and (ii) should they penetrate the cuticle, their ability to cause damage to host tissues and to overwhelm the cellular and humoral defences of the host (internal pathogenicity).

Fig. 5. Response of *C. pagurus* to injection with I4, I7 and ECPs from I7. (a) Recovery of viable bacteria from blood samples taken over a 48 h post-bacterial injection period. (b) Total blood-cell counts from crabs after injection with bacteria (ANOVA, $P < 0.05$; Dunnett's post-test, $*P < 0.05$, $**P < 0.01$, compared to the control for the individual time periods). (c) Total blood-cell counts from crabs after injection with 0.5 ml ECPs (unpaired t -test, $*P < 0.05$, $***P < 0.001$). Mean values ± 1 SE are shown ($n = 4-6$). Δ , I4; $\square$, I7; $\bullet$, control.

Chitinase activity of bacterial isolates (external pathogenicity)

Preliminary experiments investigating the media required to induce chitinase production in liquid culture found chitinase activity to be present only within inoculated flasks that contained Tris-buffered sea salts and 1% chitin powder (data not shown). Inoculated flasks, run in parallel, that contained either Tris-buffered sea salts, 1% chitin powder plus 0.01% tryptone or

Tris-buffered sea salts alone, did not induce chitinase production (data not shown). The crude chitinase found in supernatant samples was stable at -20°C without a significant loss in activity for at least 6 months (data not shown).

For I3, chitinase activity in the culture supernatant was not detected before 96 h of bacterial growth (Fig. 3). Activity levels were not significantly higher than control flasks until the isolate had undergone 144 h of growth (ANOVA, $P < 0.01$; Bonferroni post-test, $P < 0.001$ from 144 to 408 h). The maximal rate of chitinase synthesis was found to occur during the late exponential and early stationary phases of growth (120–192 h). This result is consistent with patterns of chitinase production for *Bacillus* spp. (Priest, 1977; Frandberg & Schnurer, 1994) and the fungus *Metarhizium anisopliae* (Kang *et al.*, 1999). The highest rates of chitinase production between 120 and 192 h were found when the culture supernatant from I3 was tested for endo- and exo-acting activity (data not shown).

All the isolates tested (I3, I4, I7, I8) were able to grow using 1% chitin powder as their nutrient source (Fig. 4a). I3 and I4 were found to have similar numbers of bacteria at both 192 and 408 h (paired t -tests for individual isolates, $P > 0.05$), indicating that these cultures had reached, or were close to reaching the stationary phase of growth (Fig. 4a). This was further reflected when total chitinase activity was standardized for 1×10^9 bacteria with no significant differences found in the amount of extracellular chitinase activity for I3 and I4 between the two sample times (Fig. 4b). When chitinase activity was divided in terms of its activity towards the chitin polymer (i.e. endo- and exo-acting) it was found that I3 and I4 displayed low to moderate levels of both endo- and exo-activity that did not change significantly over the culture period (Fig. 4c–e; paired t -tests, $P > 0.05$).

In contrast to I3 and I4, the culture flasks for I7 and I8 were found to have significantly higher bacterial numbers at 408 h compared to 192 h, suggesting that both cultures were still in exponential growth phase. Significant increases in total chitinase activity between the two incubation times were only found for I7 (Fig. 4b; paired t -test, $P = 0.0316$). Thus, although cultures of I8 were still actively dividing, a higher rate of chitinase synthesis was reached earlier in the growth period. Indeed, I8 cultures showed the highest total chitinase activity per standard number of bacteria at both time periods. I8 produced the highest quantities of endo-acting chitinase at 192 h and the highest amount of exo-type activity by 408 h (Fig. 4d, e).

Overall within the culture system, I3 and I4, with their faster growth rates and production of moderate levels of enzymes capable of degrading all areas of the chitin polymer, are presumed to represent primary colonizers of chitin powder. By comparison, I7 and I8 grew more slowly, producing greater amounts of chitinases later in the culture period and thus may be more likely to represent secondary colonizers. However, it should be

noted that I8 initially (192 h) produced high levels of endo-acting enzyme and subsequently (408 h) released large quantities of extracellular enzymes capable of degrading the smaller chitin oligomer. Thus, I8 is potentially best adapted for the degradation of the chitin powder.

The present investigation examined the production of chitinases in bacteria in culture systems and thus is unlikely to reflect their behaviour within the natural lesion community. Pure cultures fail to consider such factors as the interactive effects of the various lesion-dwelling bacteria and predation rates (Wardell, 1988). Chitin powder used as a substrate is both deproteinized and decalcified compared to its native form within the cuticle. Both microbial attachment (Dietrich *et al.*, 1984) and chitinase production (Hood & Meyers, 1977) have been shown to be enhanced when microbial populations are exposed to native chitin. In the current study, preliminary experiments using shell pieces derived from *C. pagurus* as the growth substrate within the culture system have indicated that levels of extracellular chitinase activity increase for I3 (data not shown). This may be an approach for future studies.

C. pagurus has been shown to have a chitin:protein ratio (calculated from the percentage organic fraction; dry wt) of approximately 7:1 within the exoskeleton (Brimacombe & Webber, 1964). The outermost waterproof cuticle layer, the epicuticle, is ultrathin and composed of lipid and protein alone. Thus, although chitinolytic activity is fundamental to lesion progression, microbial proteases and lipases may also be involved in exoskeletal breakdown, particularly in the initial stages of shell disease (Cipriani *et al.*, 1980). Although not widely investigated, protease activity [specifically gelatinase (collagenase)] was found in all but one of the isolates (I9) while lipase activity amongst the exoskeletal isolates was restricted to cluster groups D, E and F (I4, I5, I7, I8) (Table 1).

Internal pathogenicity

The internal effects of only two of the isolates (I4 and I7) on the crab host were studied. These were chosen because both isolates displayed distinct morphological characteristics that would enable their identity to be rapidly ascertained on reisolation and growth following intrahaemocoelic injections (i.e. I4 had an elongate cell morphology while I7 was negative for catalase).

I4 was rapidly cleared from the blood of crabs, with 99% of c.f.u. removed by 15 min post-injection (Fig. 5a). This rapid reduction in bacterial number coincided

Fig. 6. Changes observed within *C. pagurus* gills after injection with (a) marine saline (control), (b) 2×10^6 I7 ml⁻¹ and (c) ECPs (0.5 ml) from I7 cultures after 48 h of growth. Arrows indicate the accumulation of blood cells at the base of the gills near the branchial space (BS). Bar, 200 μ m.

with a marked reduction in the number of circulating blood cells (haemocytes; Fig. 5b). By 24 h, c.f.u. numbers on spread plates increased and at 100 h mortality was seen in 80 % of individuals. All control (saline-injected) crabs survived and only showed transient changes in the total numbers of circulating blood cells (Fig. 5b).

The dynamics of clearance and pathology of I7 was markedly different than for I4. Injection of I7 ($9 \times 10^3 \pm 6.7 \times 10^3$ c.f.u. ml⁻¹) resulted in a more modest reduction in the number of viable bacteria from circulation. For example, after 60 min bacterial numbers had only decreased to 17.8 % of the original. The number of circulating blood cells showed a rapid decline, such that after 60 min only 4.7 ± 1.1 % (mean value ± 1 SE, $n = 5$) of the cells remained in circulation. Of importance was the finding that by 3 h post-injection all crabs were moribund. Indeed, after only 15 min post-injection, crabs showed early changes in their behaviour, including retraction of eye stalks and reduced or increased gill ventilation movements. Subsequently, they all exhibited loss of limb movement by 60 min and some also showed tremors in their walking limbs. Because such changes indicated that mortality may have been caused by toxins (i.e. ECPs), crabs were injected with filter-sterilized culture filtrates taken from 48 h cultures of I7. All crabs injected were moribund 60 min after injection, while culture filtrate from un-inoculated cultures had no effect. The decline in the number of circulating blood cells seen after injection of I7 was also observed with the crude ECP preparation (Fig. 5c). Furthermore, the behaviour of crabs injected with ECPs was the same as that seen following injection of I7 bacteria. Histological examination of crabs injected with I7 bacteria or ECPs revealed a massive accumulation of blood cells in the gills (Fig. 6). Such accumulations effectively blocked the central vascular region of the gills (branchial spaces). There was also evidence in other organs that blood cells dropped out of circulation and attached to such surfaces following exposure to ECPs.

Much is known of the cellular defence responses in crustaceans to micro-organisms. Injection of heat-killed *Bacillus cereus* into shore crabs (*Carcinus maenas*) results in their rapid clearance as a result of the formation of clumps of blood cells and bacteria, termed nodules, in the gills (Smith & Ratcliffe, 1981). These observations largely accounted for the rapid reduction in the number of circulating blood cells and bacteria. In the current study with I4, following an initial rapid clearance of the bacteria, presumably by nodule formation, there was some evidence that bacterial replication occurred *in vivo*, leading to limited septicaemia and death. It must be remembered, however, that the dose injected (2×10^6 bacteria ml⁻¹; Thoma count) and the method of delivery suggest that this bacterium has limited virulence in edible crabs.

The mechanism by which I7 kills the crabs is clearly different to that employed by I4. In particular, injection of culture filtrates (containing ECPs) resulted in the same symptoms observed after the injection of washed I7 bacteria. Hence, in the case of I7, ECPs appear to be

major determinants of pathogenicity. There is surprisingly little known about the mechanisms of pathogenicity for vibrios in marine invertebrates. A number of heat-sensitive proteases produced by *V. parahaemolyticus* have been shown to be toxic for tiger prawns (*Penaeus monodon*), but the nature of these enzymes is unknown (Sudheesh & Xu, 2001) while alkaline serine proteases produced by *V. alginolyticus* have been reported to be toxic for Kuruma prawns (*Penaeus japonicus*) (Liu *et al.*, 1997). Although it is premature to speculate on the activities of the toxic ECPs from I7, the symptoms of the disease, including limb tremors and eye stalk withdrawal, suggest a neurotoxic component.

Both serum and blood-cell lysates displayed variable levels of antibacterial activity against I4 and I7. In the case of I4, 91.3 ± 3.7 % bacterial survival was found after 30 min incubation with the crab blood-cell lysate, while 61.7 ± 10.2 % bacterial survival was observed after incubation with neat whole serum (mean values $\pm$ SE, $n = 3$). I7 was more effectively killed by both blood-cell lysates and serum preparations (0.26 ± 0.17 % bacterial survival compared with 4.3 ± 2.5 % bacterial survival respectively; mean values $\pm$ SE, $n = 3$). Notably, with I7, and to some extent I4, the blood-cell lysate preparations were particularly active in bacterial killing, taking into account the difference in total protein content between this lysate and serum (the concentration of protein in the blood-cell lysate was about 1000-fold less concentrated than in serum). The limited killing of I4 by both serum and blood-cell lysates partially explains why it is able to multiply *in vivo* and cause limited mortality. The nature of this antibacterial activity was not investigated further. However, in a closely related species of crab, the shore crab (*Carcinus maenas*), Schnapp *et al.* (1996) identified four proteins with antimicrobial activity that reside in the blood cells. In the shrimp, *Penaeus vannamei*, a new family of antimicrobial peptides, termed penaeidins, have also been reported to be located in certain blood-cell types (Destoumieux *et al.*, 1997). Although there have been reports of serum antibacterial activity in crabs (e.g. Noga *et al.*, 1994) the extreme fragility of their blood cells suggests that some or all of the serum killing factors may be derived from the haemocytes by degranulation following bleeding.

In conclusion, shell disease is not a disease caused by a single pathogen and should not be considered a disease solely restricted to the exoskeleton. Numerous bacteria within the marine environment are capable of degradation of the chitin component of the crustacean cuticle and it is likely that the collective effects of the lesion community lead to further exoskeletal degradation. If breach of the cuticle occurs, infection of the body cavity of the crustacean may result, with the internal symptoms differing depending on the nature of the penetrating bacteria and this may ultimately lead to the death of the animal.

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