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Bacterial Flora of the Sea Urchin *Echinus esculentus*

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A total of 85 isolates of mesophilic, aerobic, heterotrophic bacteria were isolated from the gut, peristomial membrane, and coelomic fluid from specimens of the sea urchin *Echinus esculentus* from the Clyde Sea area of Scotland. These isolates were compared with 26 isolates from sand and seawater in the same locality. Overall, strains of *Pseudomonas* and *Vibrio* predominated. Gut (with an average bacterial viable count of 2×10^7 per 3-cm section) and coelomic fluid (which was often sterile and rarely had more than 40 bacteria per ml) had similar distributions of genera, with *Vibrio* predominating and *Pseudomonas* and *Aeromonas* next in abundance. In contrast, the flora of the peristomial membrane (with an average count of detachable bacteria of 2.5×10^5 per membrane) resembled that of sand/seawater in having *Pseudomonas* predominating, gram-positive forms or *Vibrio* next in abundance, and smaller numbers of *Aeromonas, Flavobacterium, Acinetobacter,* and *Moraxella*.

In contrast to the extensive literature on bacteria associated with man and domestic laboratory animals, the information available on the normal bacterial flora of marine invertebrates is limited. However, although only a few species have been investigated, these appear to have characteristic bacterial floras (1–3, 5). For example, one of the best studied species, *Crassostrea gigas* (Pacific oyster), is colonized by *Pseudomonas, Vibrio, Flavobacterium, Achromobacter,* and some gram-positive bacteria (2).

In a previous study (S. E. Unkles and A. C. Wardlaw, Soc. Gen. Microbiol. Proc. 3:182, 1976), the common British sea urchin, *Echinus esculentus,* was shown to possess in its coelomic fluid an antibacterial mechanism capable of killing a marine pseudomonad used as a target organism. The present paper is a preliminary survey of the normal bacterial flora of *E. esculentus*, which hitherto has not been investigated.

MATERIALS AND METHODS

E. esculentus. Healthy specimens of *E. esculentus* were carefully collected by divers or at low tide with hand nets from the vicinity of the University Marine Biological Station, Millport, Scotland, and maintained in aquarium tanks with flowing natural seawater.

Bacteriological sampling. Three anatomical sites were sampled for their bacterial flora, the gut, the coelomic fluid, and the peristomial membrane.

Sections of gut were obtained by opening the urchin with scissors, initially avoiding perforating the gut. The gut, including contents, was cut into 3-cm pieces, each of which was put into 10 ml of sterile 3.2% NaCl. The mixture was shaken vigorously for 20 to 30 s, serial dilutions (10-fold) were made in sterile 3.2% NaCl, and 0.1-ml amounts of dilutions 10^{-2} to 10^{-5} were spread on plates of 2216E marine agar (Difco Laboratories, Detroit, Mich.).

The coelomic fluid was sampled by inserting a sterile, disposable, 26-gauge, 0.5-inch (1.27 cm) needle through the peristomial membrane and withdrawing 1.0 ml of fluid into a sterile, plastic, disposable syringe. Fluid (0.1 ml) was dispensed onto the surface of a marine agar plate and evenly spread with a bent glass rod.

Bacteria were harvested from the peristomial membrane by repeatedly scrubbing the surface of the membrane with a toothbrush that had been sterilized by immersion in 70% (vol/vol) ethanol and rinsed in sterile distilled water. The toothbrush, after each scrubbing, was rotated in 5 ml of sterile 3.2% NaCl to transfer the detached bacteria. Tenfold dilutions of this suspension were made in marine broth (Difco), and 0.1-ml amounts were spread on the surface of marine agar plates.

All marine agar plates, after inoculation, were incubated for at least 7 days at 20 to 22°C, when colonies were counted and subcultures made.

Isolation of representative bacterial strains. Marine agar was used throughout for the isolation and propagation of bacterial cultures. Plates with not more than 30 well-separated colonies were chosen from those inoculated above, and representative colonies were selected. Sometimes, every colony on a plate was taken. Pure cultures were obtained by plating out and incubating at 20 to 22°C for 2 to 3 days, or until growth appeared. Stock cultures on marine agar slopes were made and stored at 4°C.

Identification of cultures. Cultures were Gram stained, and the gram-negative isolates were iden-

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tified, to generic level where possible, by the scheme of Shewan et al. (6).

Colony appearance and Gram reaction were determined after growth of the organisms on marine agar at 20 to 22°C for 24 to 48 h. Phase-contrast microscopy was used to observe motility and morphology of 24- to 48-h cultures grown in marine broth. To detect pigment formation, 2216E agar incorporating 30% skim milk was used (6). This was streak-inoculated and incubated for 48 h.

The oxidase test was carried out by the method of J. Anderson (Ph.D. thesis, University of Glasgow, 1962). A small strip of Whatman no. 1 filter paper was impregnated with a 1% solution (wt/vol) of oxidase reagent (BDH Chemicals Ltd., Poole, Dorset, England) and laid on the surface of a colony for a few seconds. The paper was then removed, and the colony, according to its nature, either adhered to the paper or remained on the agar. A positive reaction was recorded if the colony turned deep purple within 15 s.

Oxidative or fermentative attack on glucose was determined using the marine oxidation-fermentation medium of Leifson (4). The strains were stab-inoculated into duplicate tubes containing 5 ml of medium. One set of cultures was incubated aerobically, and one was incubated anaerobically in a McIntosh and Fildes jar. All cultures were incubated at room temperature for 3 days before examination. If only the aerobic culture produced acid, this was designated an oxidative attack, whereas, if both the aerobic and the anaerobic cultures produced acid, this was designated a fermentative attack. If neither culture produced acid but growth was seen, it was recorded that the strain produced no change.

Susceptibility to penicillin, 2 U, was examined by heavily inoculating a marine agar plate, adding a penicillin disk, 2 U, and incubating the plate for 24 to 48 h at room temperature. Susceptibility was recorded if there was a zone of inhibition around the disk.

Susceptibility to the vibriostatic compound 0/129 (BDH) (7) was determined by inoculating a marine agar plate as above. A Whatman antibiotic disk was impregnated with 0.14% aqueous solution (wt/vol) of 0/129, dried, sterilized (121°C for 15 min), and placed on the plate. After incubating the plate at room temperature for 24 to 48 h, a positive result was recorded if a zone of inhibition was seen around the disk.

RESULTS

Total bacterial counts. Approximately 200 specimens of *E. esculentus* were examined in this survey, the animals being collected over an 18-month period at approximately monthly intervals. Gut isolates were obtained from 27 urchins, peristomial membrane isolates were obtained from 17, and coelomic fluid was obtained from 188, with some of the animals sampled at two or three sites. Histograms showing the total bacterial viable counts from the three sites are presented in Fig. 1 through 3. Gut counts ranged from 1.2×10^6 to 6.3×10^7 per 3-cm section, with a modal value around 1.5×10^7. Washings from the peristomial membrane (Fig. 2) showed a range of 1×10^4 to 60×10^4 organisms released by scrubbing the membrane in sterile 3.2% NaCl. Here the modal value was about 2.5×10^5 per membrane. In contrast to these heavily colonized sites on the animal, coelomic fluid (Fig. 3) usually either was sterile (i.e., no growth from 0.1 ml) or yielded only a few colonies. Only 7 sea urchins out of 188 yielded coelomic fluid that gave confluent growth from 0.1 ml.

Aquarium tank water usually yielded around 400 colonies from 0.1 ml, but, occasionally, confluent growth was obtained. The lowest count on any occasion was 176 colonies per 0.1 ml.

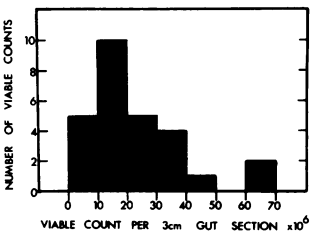

FIG. 1. *Distribution of viable counts of bacteria from sea urchin gut.*

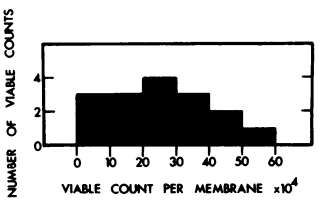

FIG. 2. *Distribution of viable counts of bacteria from sea urchin peristomial membrane.*

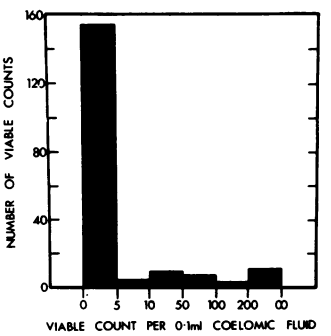

FIG. 3. *Distribution of viable counts of bacteria from sea urchin coelomic fluid.*

Distribution of bacterial types. A total of 85 bacterial isolates from the three sites and, for comparison, 26 strains from sand and seawater were identified, mostly in generic level, by the scheme of Shewan et al. (6). Figure 4 shows the numbers in each group identified, as a percentage of the total from each site. Overall, the main genera found were identified as *Pseudomonas* and *Vibrio*. From seawater and sand and from peristomial membrane there was a high percentage of gram-positive bacteria, but none was found in the gut or coelomic fluid. From the latter sites the predominant genera were *Vibrio*, followed by *Pseudomonas*, *Aeromonas*, and *Flavobacterium*, in that order. A few isolates of *Acinetobacter* and *Moraxella* (formerly classed as *Achromobacter*) were made from the peristomial membrane and from sand and seawater, but none were made from the gut or from coelomic fluid.

DISCUSSION

A few species of marine vertebrates and invertebrates, mainly creatures of economic importance, have been surveyed for their normal bacterial flora. Indeed, the identification scheme of Shewan et al. (6) was developed to assist in the investigation of bacteria involved in spoilage of fish. Among the more extensive surveys of normal bacterial flora of marine invertebrates are those of Colwell and Liston (2, 3), who studied representative species from the phyla *Porifera*, *Coelenterata*, *Mollusca*, *Arthropoda*, *Crustacea*, and *Platyhelminthes*. The predominant organisms associated with these animals were the gram-negative asporogenous rods of the genera *Pseudomonas*, *Achromobacter*, and *Flavobacterium*. In addition, *Vibrio*, enterobacteria, and gram-positive forms were found.

The present findings with *E. esculentus* follow a similar pattern to the above studies but with some indication of qualitatively different bacterial flora in the different sites. For example, the peristomial membrane, which is normally in contact with the seabed, had associated with it a mixed bacterial population similar in its generic makeup to the bacteria of sand and seawater, with the genus *Pseudomonas* predominating. This mixture differed from that found in the gut, since the latter was deficient in gram-positive forms and in *Moraxella* and *Acinetobacter* and showed a predominance of *Vibrio*. This suggests that the environment of the gut is selective for certain forms and may enable it to maintain a bacterial flora of different composition from those in sand and seawater. These findings correspond to those of Colwell and Liston (3) and Beeson and Johnson (1), who noted that the bacterial species found varied according to the organ from which they were isolated, and Leifson et al. (5), who showed that the bacterial flora of seawater consisted mainly of *Pseudomonas*, whereas, in the gut of several marine animals, *Vibrio* predominated. Relative to the gut, coelomic fluid had a very low bacterial population and, in many cases, was sterile. This would be in keeping with its antibacterial properties, described previously (Unkles and Wardlaw, Soc. Gen. Microbiol. Proc. 3:182, 1976). Those samples of coelomic fluid that contained bacteria yielded a mixture of *Pseudomonas*, *Vibrio*, and *Aeromonas* that was very similar to that of the gut. This suggests that coelomic fluid in the healthy animal is probably sterile but that bacteria from the gut may temporarily gain access from time to time. Also, the possibility, in some cases, of the gut being punctured by the syringe needle during sampling cannot be excluded.

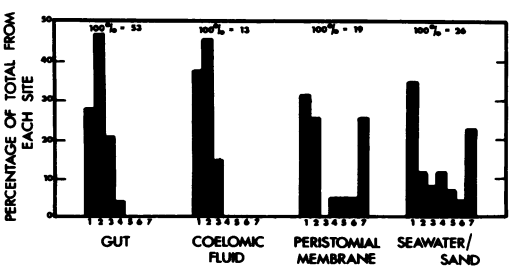

FIG. 4. *Distribution of bacterial genera from different sites of E. esculentus and from seawater and sand. The numbering on the abscissa corresponds to: 1, Pseudomonas; 2, Vibrio; 3, Aeromonas; 4, Flavobacterium; 5, Acinetobacter; 6, Moraxella; 7, gram-positive bacteria.*

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