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Microbiology of Shellfish

Bacteriological Study of the Natural Flora of Pacific Oysters (*Crassostrea gigas*)^{1,2}

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There is an extensive literature on the public health aspects of shellfish bacteriology (Dodgson, 1928). A great deal of excellent work has been carried out on the incidence and survival of such groups as the enteric pathogens (*Salmonella*, *Shigella*) and related coliform indicator organisms in shellfish grown under various conditions (Foote, 1895; Fabre-Domergue, 1912; Kelly and Arcisz, 1954). As a result of this work, the practical conditions necessary for prevention and control of shellfish-borne infection are now well established. However, there is an almost complete lack of information concerning the bacterial types not derived from sewage associated with shellfish.

A comparison of the number of colonies obtained on count plates prepared from shellfish incubated at room temperature (*circa* 20 to 25 C) and at 37 C indicates that nonmesophilic bacteria probably comprise the bulk of the bacterial population of shellfish. One type of noncoliform microorganism said to be peculiar to shellfish belongs to the group of large *Spirochaetae*. This type of microorganism was described by Fantham (1907) from mussels, *Spirochaeta anodontae*, and by Dimitroff (1926) from oysters, *Saprospira* and *Cristispira*. In some published reports concerning the presence of coliform organisms in shellfish, casual reference has been made to the presence of other bacteria. Thus Joseph (1914) described the occurrence of spore bearing, asporogenous, pigmented, and non-pigmented bacteria in market oysters, Berry (1916), and Geiger *et al.* (1926) noted the presence of *Proteus*, *Alcaligenes*, and *Pseudomonas fluorescens* together with

other common "water bacteria," also in market oysters. Eliot (1926) found that the green fluorescent, yellow pigmented, nonpigmented, and "vibrio" groups of microorganisms rapidly increased in number during the spoilage of market oysters at 20 C. Tanikawa (1937) found that typical water bacteria of the genera *Achromobacter*, *Pseudomonas*, *Flavobacterium*, and *Micrococcus* were of greatest importance in the spoilage of market oysters held at 0 C. The results of these spoilage studies are remarkably similar to the bacteriological findings for fin fishes held at similar temperatures (Shewan and Liston, 1956), and, in the latter case, it has been quite well established that the spoilage organisms are derived from the flora of the living fish which is predominantly composed of asporogenous gram-negative rods (Georgala, 1958). By analogy it seems not unreasonable to suspect that the spoilage bacteria in oysters are related to the normal bacterial population present in the living animal.

The purpose of this study was to determine the composition of the natural bacterial flora of oysters held under controlled natural conditions in various areas of Washington. Coliform counts were carried out to obtain some information concerning the degree of pollution of the environment, but the major portion of the investigation was concerned with noncoliform bacteria.

MATERIALS AND METHODS

Yearling Pacific (that is, Japanese) oysters, *Crassostrea gigas*, were obtained from Purdy, Washington, and were placed in floating trays in three different areas of Washington: Hood Canal, Oyster Bay, and Willapa Bay; a control group was maintained in the salt-water aquarium at the College of Fisheries. Samples of three oysters and 150 ml of seawater were taken from the aquarium weekly and from the floats every

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third week. Most probable number (MPN) of coliforms and counts of *Escherichia coli* were established according to the procedures described in *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes* (APHA, 1955). The study extended over a 4½-month period, from February to July 1959.

Plating media and methods employed in the quantitative determinations were as follows:

MacLeod's maintenance medium (basal) from MacLeod *et al.* (1954) containing yeast extract, 0.5 per cent; nutrient broth, 0.8 per cent; and Bacto-agar³ 1.5 per cent in 1 L seawater.

Basal medium plus glucose (basal + 1.0 per cent glucose).

Oyster agar (OA) modification of the medium of Eyre (1923) consisting of 500 g of minced oyster meat extracted at 100 C for 30 min in 1 L sterile seawater, filtered, adjusted to pH 7.4, 15 g of Bacto-agar added, and sterilized 15 min at 120 C (15 pounds pressure).

Tryptone glucose extract (TGE).

Nutrient agar (NA).

Nutrient agar plus 0.5 per cent sodium chloride (salt NA).

Except where stated otherwise, the media were obtained from Difco Laboratories in dehydrated form and made in accordance with the manufacturer's directions. The initial quantitative determinations for selection of the most appropriate medium of those listed above were done by means of surface plate counts. The basal medium plus glucose appeared to give the maximum count, but the colonies on the plates were very mucoid and tended to fuse. The standard plate counts were thus carried out on the basal medium alone.

Colonies were picked at random from the count plates. The cultures obtained were subjected to purification procedures and the pure cultures were tested by a number of determinative methods. One hundred fifty-two cultures were maintained in seawater + 1 per cent peptone or on basal agar slopes depending upon how fastidious the organism was. Selective media (Difco, dehydrated) including the Enterococci Presumptive, Ethyl Azide Violet Broth, Eosin-Methylene Blue Agar, S S Agar, Brilliant Green Bile Broth, and Triple Sugar Iron Agar, were used to assist in the identification of possible members of the *Enterobacteriaceae*. Tests for identification and classification were carried out according to the *Manual of Microbiological Methods* (SAB, 1957). Pure cultures of all the organisms isolated in this study were streaked on basal agar plates for determination of colonial morphology and for tests of sensitivity to 0/129 vibriostat compound (Shewan *et al.*, 1954) and to 2, 5, 10 unit Difco penicillin discs. Other tests and media used were as follows: litmus milk; seawater nutrient gelatin;

lead acetate agar slopes; methyl red; Voges-Proskauer; nitrate broth; indole; urea agar slopes; Koser's citrate broth; Hugh and Liefson oxidative and fermentative medium (Hugh and Liefson, 1953); lactose, glucose, maltose, mannitol, and sucrose fermentation tubes; and ammonia production. Temperature growth tests at 0, 25, and 37 C were carried out in a medium consisting of 0.5 per cent sodium chloride and 1 per cent peptone water. Routine tests and identification media were inoculated and incubated at 25 C (RT), but selective media for enterobacteria were incubated at 37 C.

RESULTS

There was no significant difference between the counts obtained in basal + 1 per cent glucose, basal, and oyster agar, which were always higher than those in the other media. Basal medium seemed to support

TABLE 1
Standard plate counts on several media*

Medium	Experiment 1		Experiment 2		Experiment 3	
	25 C	37 C	25 C	37 C	25 C	37 C
	Total count per ml†					
Basal agar....	7,320	1,990	12,900	2,895	13,500	2,000
Basal + 1% glucose agar.....	10,500	1,395	14,000	2,895	14,500	3,395
Oyster agar...	7,870	2,150	11,900	3,035	14,000	3,700
Tryptone glu- cose extract agar.....	5,240	700	5,300	365	5,500	500
Nutrient agar.	1,170	400	1,170	395	1,500	500
Nutrient agar + 0.5% NaCl.....	3,325	20	3,225	40	3,125	30

* See text for description.

† Avg of duplicate platings.

TABLE 2
Coliform content of oysters and seawater examined at 3-week intervals

	Time (weeks)				
	0	3	6	9	12
	MPN coliforms per 100 ml sample				
Aquarium oysters.....	0	0	0	0	0
Aquarium seawater (control)...	0	0	0	0	0
Hood Canal oysters.....	—	450	450	200	200
Hood Canal seawater.....	—	0	0	2	2
Oyster Bay oysters.....	—	0	200	0	0
Oyster Bay seawater.....	—	0	0	0	0
Willapa Bay oysters.....	—	450	1100	20	20
Willapa Bay seawater.....	—	0	200	2	2

³ Difco Laboratories, Inc., Detroit, Michigan.

growth of marine and other bacteria as well as either basal + glucose or oyster agar and was by far the simplest to prepare (table 1). It was therefore chosen for subsequent work.

The MPN of coliforms in the seawater and oysters, sampled at 3-week intervals throughout the period of the study, is given in table 2. Pollution as indicated by MPN counts was light except during one brief period in the Willapa Bay area. Our results confirmed that oysters tend to show higher coliform counts than the surrounding seawater. Figures 1 and 2 show the

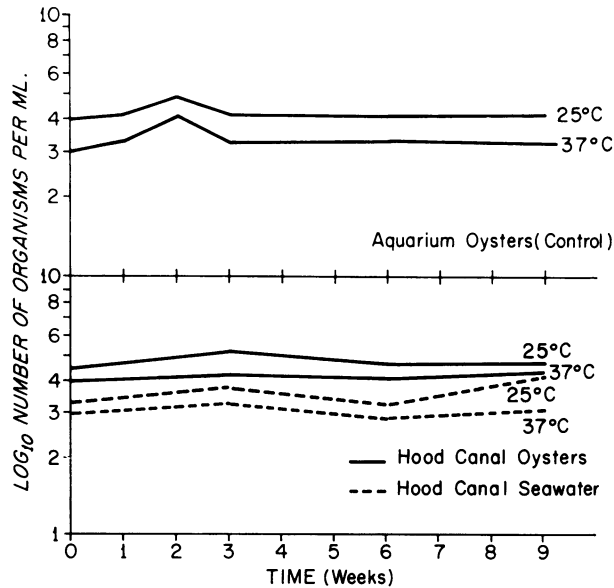


Figure 1. Total viable count of bacteria per ml oyster fluid and per ml seawater for aquarium-held oysters (control) and Hood Canal oysters.

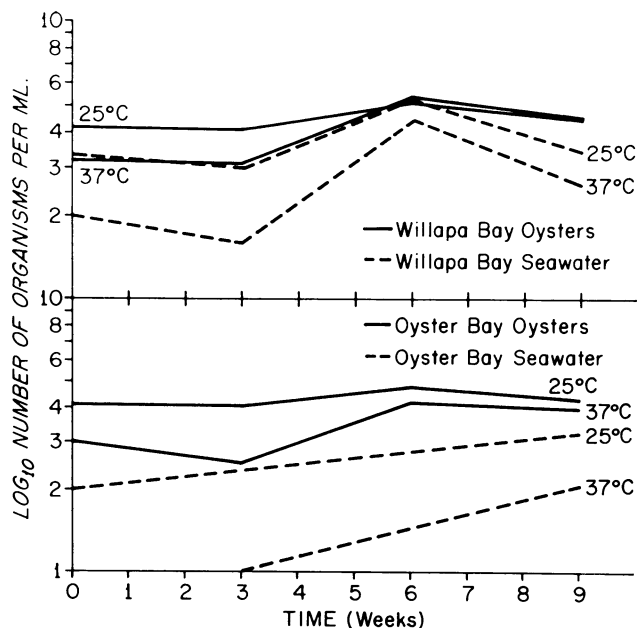


Figure 2. Total viable count of bacteria per ml oyster fluid and per ml seawater for Willapa Bay and Oyster Bay samples.

total viable counts of bacteria per ml seawater and per ml oyster body fluid sampled concurrently with the MPN determinations.

The group distribution of bacteria classified according to genera, with the *Pseudomonas* and *Vibrio* groups combined, is given in table 3. A similar distribution, but related to site of isolation in the oyster is given in table 4. Gram-negative nonsporing rods predominated in the flora with *Pseudomonas/Vibrio* contributing the major (circa 50 per cent) type and *Flavobacterium* following in importance. Gram-positive organisms constituted less than 20 per cent of the isolates. No real difference appeared to exist in generic distribution of organisms isolated from different sites in the oyster.

In table 5 are shown some of the more important biochemical properties of the organisms isolated, given as percentages of the total number tested. It can be seen that the biochemical abilities of the organisms within the flora are similar in each environmental area.

In general, the bacterial population of oysters is proteolytic in nature, with only weak saccharolytic and reductive capacity. However, nearly 50 per cent of the organisms isolated were able to ferment glucose anaerobically.

DISCUSSION

Coliform bacteria, as calculated from the total viable count and MPN values, never constituted more than 0.5 per cent of the total viable flora in our oyster samples. The failure to isolate any of these organisms from count plates by our random selection procedure indicates that this is a true measure of the numerical insignificance of these types in the oyster. Nevertheless, the results show once again the ability of the oyster to concentrate coliform bacteria from seawater and underlines the public health significance of oysters as potential agents of enteric infection.

The general composition of the bacterial flora of the oysters studied by us is similar in outline to that indicated by the studies of Eliot (1926) and Tanikawa (1937). Gram-negative rod forms predominate while gram-positive forms constitute a minor portion of the flora. The *Pseudomonas/Vibrio* group was found to be the largest single group, and members of the *Achromobacteriaceae* next largest. This distribution of generic types is very similar to that described by several workers for free swimming fish (Liston, 1957; Georgala, 1958). There is, moreover, a striking resemblance between physiological characters of the oyster flora and that of the fish flora. In both cases proteolytic activity (as measured by litmus milk digestion and liquefaction of gelatin) is high while general saccharolytic activity is low. Most of the organisms from both fish and shellfish are characteristic marine types exhibiting a partial or complete salt dependence demonstrated, in the case of the shellfish bacteria, by the growth stimulating

effect of sodium chloride added to standard media. These bacteria are also psychrophilic and will grow readily at temperatures close to 0 C and poorly or not at all at 37 C. The major point of difference between the two floras is the rather high incidence of organisms able to ferment glucose anaerobically (Hugh and Liefson test) in the oyster flora. This may be related to a greater availability of glucose in the oyster as compared with free-swimming fish.

A more minor point in which the oyster and fish floras appear to differ is in the gram-positive representation. In fish, gram-positive bacteria have been reported to occur to the extent of 3 to 10 per cent (Liston, 1957; Georgala, 1958) while for oysters the range appears to be nearer 15 to 20 per cent. Gram-positive organisms are typically terrigenous in origin and oysters, because of their nearness to the shore, are exposed to quite considerable terrigenous contamination. The higher

gram-positive representation may therefore be due to an environmental effect.

The extent of the influence of external environmental factors on the bacterial flora of oysters is obviously important. It has been assumed by many bacteriologists that the bacterial populations associated with aquatic animals and particularly with sessile aquatic animals such as oysters, are directly related to the bacterial content of the surrounding water. According to this view, the types of bacteria occurring in oysters will vary depending on the flora of the environment. This view has been challenged in the case of fin fishes where, it has been argued, a definite fish "commensal" flora exists. The "commensal" flora hypothesis is supported by the uniformity of generic distribution observed in the floras of fish belonging to quite different taxonomic groups taken in widely separated parts of the world (Liston, 1959).

TABLE 3

Generic distribution of organisms isolated from oysters in controlled and natural environments

Environment	Sample Total	<i>Pseudomonas/Vibrio</i>	<i>Achromobacter</i>	<i>Flavobacterium</i>	<i>Corynebacterium</i>	<i>Alcaligenes</i>	<i>Micrococcus</i>	<i>Bacillus</i>	Enterococci	Miscellaneous
Salt-water aquarium.....	43	20	2	6	1	2	6	4	1	1
Hood Canal.....	50	27	5	7	3	1	5	1	0	1
Willapa Bay.....	30	18	1	5	0	0	2	2	1	1
Oyster Bay.....	29	14	0	8	1	0	3	0	0	3
Total.....	152	79	8	26	5	3	16	7	2	6
% Distribution.....	100	52.0	5.3	17.1	3.3	2.0	10.5	4.6	1.3	3.9

TABLE 4

Generic distribution of organisms according to site of isolation from the oysters

Site of Isolation	Sample Total	<i>Pseudomonas/Vibrio</i>	<i>Achromobacter</i>	<i>Flavobacterium</i>	<i>Corynebacterium</i>	<i>Alcaligenes</i>	<i>Micrococcus</i>	<i>Bacillus</i>	Enterococci	Miscellaneous
Japanese seed oyster.....	20	9	1	3	1	2	4	0	0	0
Rectal area.....	32	22	3	3	0	1	2	1	0	0
Stomach.....	22	15	1	3	0	0	1	1	0	1
Gills.....	4	1	0	3	0	0	0	0	0	0
Body fluid.....	74	32	3	14	4	0	9	5	2	5
Total.....	152	79	8	26	5	3	16	7	2	6

TABLE 5

Distribution of certain biochemical characteristics among pure cultures isolated from oysters in controlled and natural environments

Environment	No. in Sample	Litmus Milk Peptonized	Gelatin Liquefied	Nitrate Reduced	H ₂ S Produced	Glucose* Fermented	Lactose Fermented	Nonsaccharolytic	Ammonia Produced	Sensitive to Penicillin	Urea Utilized
Salt-water aquarium.....	43	30.2†	67.5	14.0	18.6	46.5	23.2	44.2	65.0	34.9	18.6
Hood Canal.....	50	12.0	46.0	20.0	8.0	46.0	4.0	52.0	82.0	14.0	16.0
Willapa Bay.....	30	13.3	63.3	16.6	13.3	47.0	10.0	50.0	86.5	10.0	10.0
Oyster Bay.....	29	13.8	58.6	6.9	10.3	41.4	0	51.8	100.0	10.3	10.3
Total.....	152	17.8	59.2	15.1	12.5	46.7	9.9	49.4	84.5	18.4	14.5

* Hugh and Liefson (1953).

† Given as per cent of cultures sampled showing characteristic.

The bacterial floras of the oysters tested in this investigation showed a high degree of similarity from area to area. Moreover, the similarity extended beyond the taxonomic groupings to biochemical groupings. The same organisms with the same biochemical properties were found in all areas. This general similarity would be explained on the earlier hypothesis by the assumption that all the environments were similar. That this is not so can be seen from the coliform MPN results (table 2) which indicate that the areas were subject to different degrees of sewage pollution. Moreover, the sites for the floats were selected to provide different water conditions at each place. It may be argued that the overriding environmental effects such as salinity and so-called antibacterial effect of seawater (ZoBell, 1946) are similar in all areas, so that the bacterial flora of the water is the same in all areas. Nevertheless, the proximity of the oysters to the shore would permit quite rapid transfer of terrigenous contaminant organisms and there is evidence in the coliform counts that even the more sensitive nonmarine types can survive for some time in seawater. Thus, the external water environment cannot exert the absolute control over the bacterial flora that this theory requires.

The assumption that the observed flora is in fact typical of the oyster rather than of its environment seems to be the simplest way of explaining the observations. One cannot, of course, dismiss completely the effect of environment on the oyster flora. Obviously, all microorganisms in the immediate vicinity of the oyster may gain access to it either fortuitously or as a result of the feeding activity of the mollusc. It seems probable that only those organisms which are well adapted to the microenvironment provided by the oyster will establish themselves as a significant component of the oyster flora. Organisms such as the coliform bacillus which are, in fact, ill-adapted for active growth under these conditions may survive and even multiply to a very small extent in the oyster but will never attain a numerically significant status in the total bacterial population. The bacterial flora of the oyster which we suggest should be considered as a "commensal" flora, analogous to the "commensal" floras of fish (or for that matter of land animals), is composed essentially of typical marine psychrophilic bacteria well adapted physiologically to life within the microenvironment of the shellfish.

The large *Spirochaetes* described by Dimitroff (1926) and generally considered characteristic inhabitants of shellfish were not observed in any of the oysters examined in this survey. It is possible that our methods of examination (phase contrast, dark field, and direct illumination microscopy) were at fault. Another possibility may be that the floating tray arrangement employed in our studies in which the oysters were suspended clear of the sea bottom, provided conditions

under which the *Spirillae* described by Dimitroff could not survive.

The high incidence of proteolytic bacteria and of types capable of fermenting glucose in the natural flora may be of practical significance in post-mortem spoilage of food oysters.

Eliot (1926) divided the spoilage process into three stages: acidity increase, abundant gas production, and proteolysis. He ascribed the major part of the spoilage to the "water forms" described as green fluorescent and yellow pigmented groups. Tanikawa (1937) showed that *Achromobacter*, *Pseudomonas*, *Flavobacterium*, and *Micrococcus* were responsible for spoilage of oyster meat stored at 0 C. Thus it appears that the natural flora of the oyster, as defined above, may be the major factor in spoilage. Methods of preservation should be directed, therefore, to dealing with this flora.

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SUMMARY

The study was undertaken to determine the composition of the bacterial flora of oysters held under approximately natural conditions in three different areas of Washington, with a control held in the salt-water aquarium at the College of Fisheries. Most probable number of coliform group organisms and total viable counts for the 4½-month period of study showed little or no apparent pollution.

The group distribution of bacteria classified according to genera showed that gram-negative, asporogenous rods of *Pseudomonas/Vibrio*, and *Flavobacterium*, predominated. Gram-positive organisms constituted less than 20 per cent of the isolates. No real difference appeared to exist in generic distribution of organisms isolated from different sites in the oyster or within the flora of oysters maintained in different areas of Washington. Also, the biochemical abilities, proteolytic and weakly saccharolytic, were similar for all organisms from each environmental area.

The "commensal" flora of the oyster was compared with that found on free-swimming fish and discussed in terms of environmental factors. The relationship of a natural flora to post-mortem spoilage is also discussed.

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Survival of *Salmonella typhimurium*, *Staphylococcus aureus*, and *Streptococcus faecalis* Frozen in Simplified Food Substrates^{1,2}

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In nonsterile food products, including those preserved by freezing, the possible survival of those bacteria frequently implicated in food-borne illness has important public health implications. Borgstrom (1955) in a review of the research in the area of the microbiology of frozen foods stated that additional studies could profitably be undertaken to elucidate more clearly the relationships which may exist between bacterial survival and the substrate on which the organism exists. Recently, the concept of the unique nature and requirements of the bacterial cell which has been frozen was forwarded by Bretz and Hartsell (1959).

In the present study, data were obtained on the numbers of viable cells surviving freezing, frozen storage, and thawing of a single species of each of three bacterial genera generally acknowledged as causes of food-borne illnesses. Four substrates were prepared, each consisting of a single food ingredient dissolved or suspended in buffer. To each was added an inoculum of viable cells of a single species. Bacterial cells suspended in buffer or in physiological saline served as controls. Observations were made at three temperatures of frozen storage and at intervals over a 10-week period.

EXPERIMENTAL METHODS

Ten-milliliter quantities of the inoculated suspending media were frozen in capped test tubes (16 by 125 mm) at -11, -21, or -30 C. The tubes were subsequently stored at the same temperature as had been used for freezing. Duplicate samples were withdrawn after 24

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