Tissue Distribution of a Coliphage and Escherichia coli in Mussels after Contamination and Depuration

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Experiments were undertaken to determine the tissue distribution of Escherichia coli and a coliphage after contamination of the common mussel (Mytilus edulis). Mussels were contaminated with high levels of feces-associated E. coli and a 22-nm icosahedral coliphage over a 2-day period in a flowing-seawater facility. After contamination, individual tissues were carefully dissected and assayed for E. coli and the coliphage.

Contaminated mussels were also analyzed to determine the tissue distribution of the contaminants after 24- and 48-h depuration periods. The majority of each contaminant was located in the digestive tract (94 and 89% of E. coli and coliphage, respectively). Decreasing concentrations were found in the gills and labial palps, foot and muscles, mantle lobes, and hemolymph. Our results indicate that contamination above levels in water occurred only in the digestive tract. Contaminated mussels were depurated in a commercial-scale recirculating UV depuration system over a 48-h period. The percent reductions of E. coli occurred in the following order: digestive tract, hemolymph, foot and muscles, mantle lobes, and gills and labial palps. The percent reductions of the coliphage were different, occurring in the following order: hemolymph, foot and muscles, gills and labial palps, mantle lobes, and digestive tract. Our results clearly demonstrate that E. coli and the coliphage are differentially eliminated from the digestive tract. The two microorganisms are eliminated at similar rates from the remaining tissues. Our results also clearly show that the most significant coliphage retention after depuration for 48 h is in the digestive tract. Thus, conventional depuration practices are inappropriate for efficient virus elimination from mussels.

The ability of most species of commercially exploited shellfish to accumulate bacteria and viruses during feeding in contaminated waters has been documented (19). Tissue localization of the contaminants has been determined for a number of species (2, 5, 7, 8, 10–13, 15; A. F. Meinhold and M. D. Sobsey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, abstract no. N 19, p. 181). Invariably, the highest contaminant concentrations have been in the digestive tracts of all of the species studied, as well as in the siphons of clams. Thus, it has been deduced that contaminant ingestion is a function of shellfish feeding physiology. Depuration exploits the feeding physiology of shellfish as a means of reversing contaminant ingestion. Shellfish are placed in tanks of clean seawater to allow them to purge themselves of their bacterial and viral loads. Work in our laboratory (16, 17) and in other laboratories (3, 6, 21) has indicated that bacteria and viruses are differentially eliminated from shellfish during depuration. Bacteria are generally eliminated rapidly and efficiently, whereas viruses are eliminated at considerably slower rates under conditions suitable for shellfish activity. Low-level virus retention over long depuration periods has been documented (3, 10, 21).

Since the majority of ingested bacteria and viruses are located in the digestive tract and since Liu et al. (7) have shown that viruses in clams are not intracellular or chemically bound to any type of cell, some authors (4, 5, 7, 10) have speculated that digestive-tract-sequestered bacteria and viruses may be successfully eliminated during depuration. Indeed, Canzoneri (4) suggested that the viral load intimately associated with the gut contents should be eliminated together with the bacterial load. Furthermore, some researchers (4, 5, 10) have suggested that unsuccessful virus elimination is likely to be a product of that portion of the viral load sequestered in non-digestive-tract tissues. Indeed, Liu et al. (9) found that poliovirus is depurated more readily from the digestive tract than from the hemolymph of hard-shell clams. In a more recent communication (Meinhold and Sobsey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982), however, viruses were reported to be lost more readily from gill and mantle tissues than from the digestive tracts of oysters.

This study was undertaken to determine the distribution of Escherichia coli and a coliphage throughout the tissues of mussels after contamination, as this has been hitherto unreported. It was also designed to obtain experimental evidence of the effect of depuration on the tissue concentrations of both microorganisms under commercially operated depuration conditions.

MATERIALS AND METHODS

E. coli and coliphage. The origins and relevant characteristics of the E. coli and coliphage strains used in this study have been described previously (18). Briefly, the E. coli strain was a rifampin-resistant, broad-spectrum coliphage-resistant strain that was isolated from shellfish and designated E. coli 4A. The coliphage strain was a 22-nm icosahedral sewage effluent isolate designated φA1-5a. E. coli A-19 (Hfr) (kindly provided by R. E. Stettler, U.S. Environmental Protection Agency), which exhibits broad-spectrum bacteriophage sensitivity, was used as the host bacterial strain. φA1-5a was chosen as a viral simulant because its size and shape closely resembled the size and shape of enteroviruses, it was isolated from sewage effluent and hence was likely to simulate the mode of enteric virus transmission in the environment, and it was readily taken up by and recovered from test mussels. The maintenance and assay procedures used for both E. coli 4A and φA1-5a have also been described previously (18).

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Mussels and seawater. The common mussel, *Mytilus edulis*, was used throughout this study. Mussels were harvested in Cork Harbor, Ireland, and transported to the experimental facility within 1 h. Before use the shells were thoroughly cleaned to remove detritus and fouling organisms. A total of 120 mussels were used in each experiment. Seawater was collected from the same location as the mussels to minimize environmentally induced stress during experimentation.

Contamination and depuration. Mussels were contaminated over a 2-day period with high levels of sewage-effluent-associated $\phi$A1-5a and *E. coli* 4A in a system near the harvesting site. This system consisted of two plastic tanks with a combined capacity of approximately 400 liters. Seawater was circulated between the tanks by using a model H2 pump (Homo, Pumps Ltd., Seelscheid, Federal Republic of Germany) at a flow rate of approximately 6 liters/min. The mussels were elevated from the bottom of one tank on a perforated plastic tray to curtail recontamination with feces and pseudofeces. The contaminated effluent was prepared as described previously (18), except that 30 ml of an overnight *E. coli* 4A culture and 3 ml of $\phi$A1-5a stock were used. Briefly, contaminated effluent preparation consisted of adding the *E. coli* 4A and $\phi$A1-5a to heat-treated (85°C, 1 h) sewage effluent and stirring the mixture overnight at 4°C. The maximum contaminant levels were on the order of 2.5 x 10^6 CFU/ml for *E. coli* 4A and 2.5 x 10^7 PFU/ml for $\phi$A1-5a.

Depuration was performed over a 48-h period in a commercial-scale recirculating UV system which has been described previously (18). Samples of mussels were taken at 0, 24, and 48 h. Triplicate samples of 10 mussels were used for tissue dissection. Whole-mussel samples consisted of four mussels that were pooled.

Assay procedures. After sampling, the mussels used for tissue dissection were immersed in a solution containing 7% MgCl$_2$ and seawater (1:1, vol/vol) for 30 min to anesthetize them. Hemolymph was extracted from posterior adductor muscle sinuses by using a 2-ml syringe (Becton Dickinson Ltd., Dublin, Ireland) and a 23-gauge Stericon needle (Becton Dickinson Ltd.). The hemolymph specimens from all individuals in each sample were pooled for analysis. Subsequently, the mussels were dissected, and the specimens of each tissue from each sample were pooled. The following tissues were dissected: digestive tract, mantle lobes, gills and labial palps, and foot and muscles.

The protocols used for *E. coli* 4A and $\phi$A1-5a extraction and enumeration from the pooled tissues and whole mussels have been described previously (18). Briefly, initial extraction involved macerating the tissue and diluting the macerated tissue 1:2 (wt/vol) in 0.25 M Ringer buffer (Oxoid Ltd., London, England). The resulting homogenate was used directly for *E. coli* 4A enumeration by using Rif$^+$-selective plating techniques. The homogenate was further diluted with sterile distilled, deionized H$_2$O to give a final dilution of 1:5 (wt/vol). After vigorous shaking, this homogenate was centrifuged at 10,000 x g for 15 min, and the supernatant was retained as the coliphage extract. $\phi$A1-5a was enumerated by using a double-agar-overlay technique with *E. coli* A-19 (Hfr) as the host bacterium.

RESULTS

The physicochemical parameters determined during this study are shown in Table 1. Each parameter reflected ambient conditions at the harvest site.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Temp (°C)</th>
<th>Dissolved oxygen (% saturation)</th>
<th>Salinity (%)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contamination</td>
<td>12.6</td>
<td>59</td>
<td>22.4</td>
<td>7.5</td>
</tr>
<tr>
<td>Depuration</td>
<td>10</td>
<td>67</td>
<td>18.1</td>
<td>7.5</td>
</tr>
</tbody>
</table>

The relative distributions of $\phi$A1-5a and *E. coli* 4A throughout the tissues of the mussels after contamination are shown in Fig. 1 and Table 2. The vast majorities of the ingested coliphage and *E. coli* were located in the digestive tract (Fig. 1). The concentrating effect within the digestive tract is evident from the fact that the levels of both microorganisms per gram of tissue were higher in the digestive tract than in the whole mussels (Table 2). The relative concentrations of $\phi$A1-5a within the tissues were as follows: digestive tract > gills and labial palps > foot and muscles > mantle lobes > blood. The relative concentrations of *E. coli* 4A were similar to those of $\phi$A1-5a except that the concentrations associated with the gills and labial palps and with the foot and muscle tissues were similar.

The effects of depuration on the tissue concentrations of both *E. coli* 4A and $\phi$A1-5a are shown in Fig. 2. The most rapid *E. coli* 4A reduction occurred in the digestive tract. More than 99% of the original concentration was eliminated within 24 h. By 48 h, almost 99.9% was eliminated. In contrast to *E. coli*, $\phi$A1-5a was inefficiently eliminated from...
the digestive tract. Levels that were approximately 25% of the initial titers were detected after 24 h, and levels that were 10% of the initial titers were detected after 48 h of depuration.

Substantial reductions in E. coli and coliphage levels were also evident in the hemolymph samples during depuration. The initial levels of both microorganisms in the hemolymph were reduced by approximately 99% within 48 h. However, the initial levels of both E. coli 4A and MA1-5a detected in hemolymph samples were considerably lower than the initial levels detected in the other tissues. The rates of E. coli 4A and MA1-5a elimination from the remaining tissues were relatively constant throughout the depuration period and were very similar to one another. E. coli 4A concentrations were reduced by approximately 90, 94, and 96% from the gill and labial palp, mantle lobe, and foot and muscle tissue samples, respectively, within 48 h; MA1-5a concentrations were reduced by approximately 95, 93, and 96% from the same tissues, respectively. As with hemolymph, however, the initial coliphage titers in the mantle lobe tissue samples were relatively low.

The order of E. coli 4A percent reductions over 48 h of depuration was as follows: digestive tract > hemolymph > foot and muscles > mantle lobes > gills and labial palps. The order of MA1-5a percent reductions over 48 h of depuration was as follows: hemolymph > foot and muscles > gills and labial palps > mantle lobes > digestive tract.

**DISCUSSION**

This study was undertaken to determine the relative distributions of E. coli 4A and MA1-5a within the tissues of mussels after exposure to high levels of the contaminants. It was also designed to determine the effects of depuration on the tissue concentrations of the two contaminants.

The sequestering of the majority of E. coli 4A and MA1-5a within the digestive tract of mussels is clear evidence that contaminant uptake is a function of the feeding physiology of mussels. This correlates with the results of Minet et al. (13). Other shellfish species artificially contaminated in flowing seawater systems have also been shown to sequester the majority of the contaminants in their digestive tracts (2, 5, 8, 10, 15; Meinhold and Sobsey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982).

The levels of E. coli 4A and MA1-5a detected in the gill and labial palp, mantle lobe, and foot and muscle tissue samples probably reflected the concentrations of the microorganisms in the surrounding water. Compared with the digestive tract concentrations, concentrations of the contaminants on or within these tissues were low. This correlates with results reported by Perkins et al. (15) for oysters. Thus, contamination is likely to have resulted from passive contact with the microorganisms during the contamination phase.

The detection of both E. coli 4A and MA1-5a in hemolymph implies that the contaminants may be distributed and sequestered in any tissue of the body. However, the relatively low concentrations detected in the hemolymph...
suggest that bacterial or viral contamination of the circulatory system would be an insignificant problem in mussels derived from approved cultivation sites. This correlates with the results obtained by Liu et al. (8) with hard clams.

Canzonier (4) has contended that depuration effectively eliminates bacteria and viruses intimately associated with gut contents at similar rates. This was not the case with mussels in this study (Fig. 2). There was a considerable difference in the relative rates of E. coli 4A elimination and ϕA1-5a elimination from the digestive tracts of the mussels (Fig. 2b). E. coli 4A was eliminated rapidly and extensively, while ϕA1-5a was eliminated slowly.

Since most of the ingested E. coli 4A was sequestered in the digestive tract and the greatest percent reduction was achieved in this tissue, it is evident that E. coli elimination from the digestive tract dictates the efficiency of depuration in ridding the whole animal of the bacterium. Elimination from the digestive tract is primarily a function of defection or digestion or both, which in turn are functions of the physiological status of the mussels. This may explain the previously identified relationship between E. coli elimination and the physiological condition of the mussels during depuration (18).

In contrast to E. coli 4A elimination, the lowest rate of ϕA1-5a elimination was from the digestive tract (Fig. 2b). Liu et al. (7) assumed that digestive-tract-sequestered viruses were only transient and were readily removed if contaminated shellfish were placed in clean seawater under appropriate conditions. Canzonier (4) and DiGirolamo et al. (5) adopted similar opinions. Furthermore, those researchers (4, 5) suggested that inefficient virus depuration is the result of that fraction of the viral load that is sequestered in non-digestive-tract tissues and is unavailable for direct removal via defection or digestion. The data in Fig. 2 show that this is not the case. Our results show that the coliphage was as stably associated with the digestive tract during depuration as with any other tissue tested except the hemolymph. Meinhold and Sobsey (Abstr. Annu. Meet. Am. Soc. Microbiol. 1982) reported similar results for oysters contaminated with coliphage. This suggests that intracellular and extracellular digestion are not major factors in the elimination of coliphages and perhaps enteric viruses from mussels. It may also explain the relative independence of coliphage elimination from mussel activity which was observed previously (18).

While contaminant reduction in the hemolymph was of little consequence to overall contaminant elimination during depuration, the substantial E. coli 4A and ϕA1-5a reductions (Fig. 2c) suggest that mussel hemolymph is a very hostile environment for both microorganisms. The specific causes of the reductions were not investigated in this study, but the reductions might be due to phagocytosis by hemocytes followed by intracellular digestion or agglutination or both. The ability of oyster hemocytes to phagocytize both bacteria and viruses (1) supports this hypothesis.

The elimination of E. coli 4A and ϕA1-5a at similar rates from gill and labial palp, mantle lobe, and foot and muscle tissues suggests that the factors that affect elimination of both microorganisms from these tissues are similar. E. coli 4A elimination from these tissues was very slow relative to elimination from the digestive tract. However, under cultivation conditions microbiologically acceptable for shellfish harvesting, the levels of bacteria associated with the non-digestive-tract tissues are likely to be very low, considering the distribution shown in Fig. 1. Consequently, slow bacterial elimination from these tissues is unlikely to constitute a public health concern in view of the relatively high bacterial doses required to induce illness. This hypothesis is supported by the lack of depurated shellfish-borne bacterial disease outbreaks in the United Kingdom since 1965 (14).

Unlike bacteria, one infectious virus unit has been reported to be sufficient to induce illness in susceptible hosts (20). Therefore, slow virus elimination from non-digestive-tract tissues does constitute a public health concern, even when initial contamination levels are low. However, the distribution of coliphage within the tissues (Fig. 1) and the rates of elimination from the tissues (Fig. 2) indicate that the infectious hazards associated with non-digestive-tract tissues are far less than those associated with the digestive tract.

Our results suggest that virus contamination of mussels is transient. Liu et al. (7) expressed a similar opinion with regard to virus contamination of clams. However, it is clear that a commercial depuration period of 48 h is insufficient to ensure adequate virus elimination from any tissue under the depuration conditions used during this study. The retention of the largest portion of ingested viruses in the digestive tract after depuration suggests that conventional depuration theory is not directly applicable to virus elimination from mussels. This is of major significance from a public health viewpoint. Further research directed toward reversing the association between viruses and the digestive tracts of bivalve shellfish is, in our opinion, the major priority for future virus depuration studies.

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