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Uptake and depuration of the C- and E-genotypes of *Vibrio vulnificus* by the Eastern Oyster (*Crassostrea virginica*)

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Summary

The human pathogen *Vibrio vulnificus* is a Gram-negative estuarine bacterium that infect via wounds and ingestion, and is the leading cause of seafood-borne death in the United States. *Vibrio vulnificus* is part of the naturally occurring flora of both estuaries and estuarine mollusks (especially oysters). *Vibrio vulnificus* is divided into two genotypes, including a clinically associated C-type, and an environmentally associated E-type that is more rarely involved in septicemia. These two genotypes are found in a nearly even ratio in the aquatic environment, but oysters harvested from those very environments show a *V. vulnificus* genotype ratio disparity, with 87% of the species being that of the E-genotype. To determine if oysters selectively incorporate E-types over C-types, we placed oysters in water inoculated with either C- or E-type *V. vulnificus* strains that were phenotypically different from the normal flora and measured the uptake and depuration over a course of 6 days. We found significantly greater uptake, but equally effective depuration, of C-type *V. vulnificus* in oyster gill tissue, mantle tissue and whole oyster homogenates. Because uptake of the C-genotype was generally greater than the E-genotype, it appears unlikely that simple selective uptake is the cause of E-type *V. vulnificus* predominating in oysters.

Vibrio vulnificus is a Gram-negative, halophilic bacterium capable of causing gastroenteritis, wound infections and fatal septicemia in humans (Johnston *et al.*, 1985; Oliver, 1989; 2006). This organism is routinely found in oysters and waters of estuarine environments as part of the normal flora, and is the leading cause of seafood-borne deaths in the United States, usually resulting from the consumption of raw or undercooked oysters (Oliver, 2006).

Vibrio vulnificus can be divided into three biotypes (1–3), all of which are able to cause human infection, but biotype 1 is of greatest import to oyster producers and consumers (Tison *et al.*, 1982; Oliver, 1989; Bisharat *et al.*, 1999). Biotype 1 strains of *V. vulnificus* can be further divided into a C- and an E-genotype, with the former genotype most frequently isolated from clinical sources and the latter predominately occurring in environmental samples (Rosche *et al.*, 2005). Oysters feed by filtering out and sorting particles, including bacteria, suspended in the water column using gills and labial palps (Langdon and Newell, 1996; Newell and Langdon, 1996). Oddly, while the ratio of C- to E-genotype strains in the aquatic environment is nearly even, population studies have shown that in oysters the E-genotype strains are overwhelmingly dominant, averaging 87% of the total *V. vulnificus* population (Warner and Oliver, 2007).

The purpose of the study was to compare the uptake and depuration rates of the two genotypes of *V. vulnificus* biotype 1 in North Carolina oysters, *Crassostrea virginica*. It was hypothesized that the disparity between the genotype ratios in water versus oysters might be due to selective uptake of E-type strains and/or increased elimination/depuration of C-type strains. For these studies, we used 'marked' *V. vulnificus* strains that were easily recoverable once added to oysters in a manner similar to Murphy and Oliver (1992). Previous oyster uptake studies of *V. vulnificus* by other researchers have shown that the cells are not multiplying in the oysters, simplifying the recovery and enumeration of inoculated strains (Srivastava *et al.*, 2009). These strains included *V. vulnificus* CVD713 (a C-type strain modified by the inclusion of a TnphoA transposon that confers kanamycin resistance and alkaline phosphatase activity), *V. vulnificus* pGTR-Env1 (an E-type strain with a pGTR plasmid that confers kanamycin resistance), and *V. vulnificus* VVL1 (a naturally occurring E-type strain that is distinguishable from background via inherent bioluminescence) (Oliver *et al.*, 1986; Morris *et al.*, 1987; Wright *et al.*, 1990; Murphy and Oliver, 1992; Starks *et al.*, 2006). In addition to testing the whole-organism uptake and depuration of *V. vulnificus* in

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oysters, we also determined distribution of the introduced bacterial strains in different tissues of oysters, including the gills (which serve not only as a gas exchange site, but also as an organ for acquiring food), the digestive gland and the mantle tissue, which covers the internal organs of oysters and secretes shell materials (Carriker, 1996; Eble and Scro, 1996; Langdon and Newell, 1996; Newell and Langdon, 1996), in order to test for the potential tissue-specific differences in uptake and/or depuration rates of these bacteria.

Unsurprisingly, in all tissues examined, including the whole oyster homogenates, there was significant uptake of the marked strains of *V. vulnificus* into the oysters after 24 h of incubation (Fig. 1). Contrary to our hypothesis, however, the E-type strains did not have increased uptake when compared with the C-type strains, and remarkably, the opposite was true in all tissue types save for the digestive gland, where C-type cells did not show significantly greater uptake (Fig. 1). Following this observation, it seems unlikely that simple selective uptake and/or colonization would generate the high internal levels of E-type strains in oysters while the external, environmental levels retain a nearly equal ratio of C- and E-type.

In all cases, 48 h of depuration in clean ASW was able to reduce the number of marked cells in the artificially infected oysters to the levels close to those of the non-inoculated control oysters (Fig. 1), suggesting that neither genotype experiences greater (or less) depuration than the other. This is, again, a phenomenon unlikely to create the overabundance of E-types within the oysters.

Crassostrea virginica has been reported to retain only 5% of planktonic bacteria, which are about 1 μm diameter

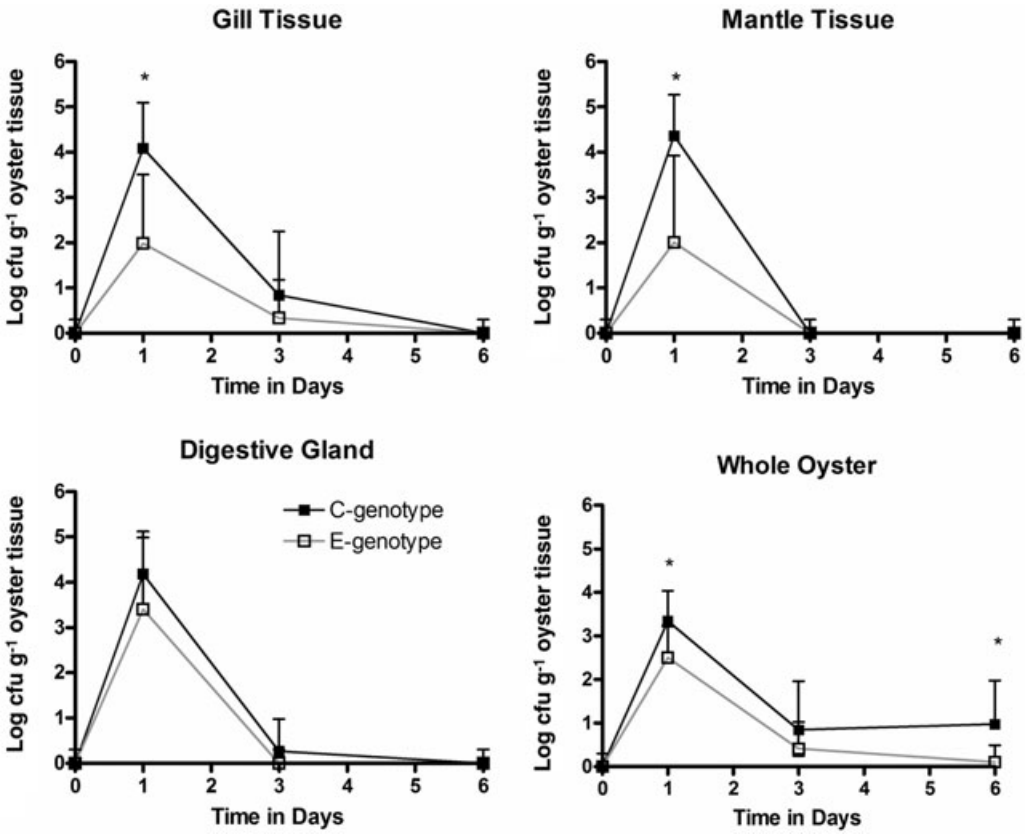

Fig. 1. Geometric means of recovered C-genotype (closed squares) and E-genotype (open squares) strains of *V. vulnificus* from dissected oyster tissues and whole oyster homogenates. Asterisks indicate significant ($P < 0.05$) differences between C- and E-genotype values derived by two-way ANOVA followed by *post hoc* tests with Bonferroni corrections (Sokal and Rohlf, 1995) using SigmaStat statistical analysis software (Version 2.0, Access Softek). Bars represent standard error of the mean. Oysters were kept in aerated tanks of ASW (20‰ salinity). The zero time points represent oysters sampled just before inoculation with *c.* 7500 bacterial cells per millilitre of tank water. Oyster tissue samples were aseptically dissected, diluted with 20‰ ASW, homogenized and plated onto Luria–Bertani medium (LB) with 1.5% agar (w/v), along with strain-specific modifications described herein. Media from experiments using C-genotype strain CVD713 were supplemented with 2 g l^{-1} glucose, 0.04 g l^{-1} 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt and 0.2 g l^{-1} kanamycin, and *V. vulnificus* was identified by the formation of blue colonies. Samples from experiments using E-genotype strain VVL1 were plated onto LB agar plates without additional ingredients, and VVL1 colonies were identified by natural luminescence. LB agar plates for experiments using strain pGTR-ENV1 were supplemented with 10 g l^{-1} L-arabinose and 0.3 g l^{-1} kanamycin. Each data point represents the means of two separate experiments using five oysters per sample per experiment. For C-genotype data, strain CVD713 was used in both experiments. The E-genotype data resulted from experiments using strain pGTR-ENV1 or VVL1, which had statistically similar results (data not shown).

(Langdon and Newell, 1990). Calculating this retention percentage versus the number of *V. vulnificus* cells inoculated in our experiments, we would expectan uptake of *c.* 10^5 cfu g^{-1} of tissue. This is consistent with our observed uptake of $> 10^4$ cfu g^{-1} uptake after 24 h in most tissues (Fig. 1). These numbers could vary greatly by allowing the bacteria to become incorporated into marine aggregates (naturally occurring clumps of bacteria and phytoplankton sometimes known as 'marine snow'), which have been shown to significantly increase retention rates of particles less than 1 μm in size (Riley, 1963; Ward and Kach, 2009).

Our laboratory has recently observed, using *in situ* experimentation, that both genotypes of *V. vulnificus* downregulate a gene required for capsule production when the cells are incubated in estuarine waters (T. Williams and J. Oliver, unpublished). Although this loss of capsule has been reported to increase biofilm formation by *V. vulnificus,* it appears to reduce uptake of free-living cells in certain oyster tissues (Joseph and Wright, 2004; Srivastava *et al.*, 2009). It thus seems reasonable that biofilm-forming, non-encapsulated *V. vulnificus* strains might be better able to attach to phytoplankton and marine snow, facilitating their entry into oysters. Perhaps this ability differs by *V. vulnificus* genotype. Future studies will examine the ability of both genotypes of *V. vulnificus* to colonize marine aggregates, using both the encapsulated and non-encapsulated forms, to determine if differential oyster uptake is due to genotype-specific aggregation with marine snow particles.

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