AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology Spectrum

RESEARCH ARTICLE

Growth Rates of *Vibrio parahaemolyticus* Sequence Type 36 Strains in Live Oysters and in Culture Medium

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ABSTRACT The pathogenic marine bacterium *Vibrio parahaemolyticus* can cause seafood-related gastroenteritis via the consumption of raw or undercooked seafood. Infections originating from relatively cool waters in the northeast United States are typically rare, but recently, this region has shown an increase in infections attributed to the ecological introduction of pathogenic sequence type 36 (ST36) strains, which are endemic to the cool waters of the Pacific Northwest. A 2005 risk assessment performed by the Food and Drug Administration (FDA) modeled the postharvest growth of *V. parahaemolyticus* in oysters as a function of air temperature and the length of time the oysters remained unrefrigerated. This model, while useful, has raised questions about strain growth differences in oyster tissue and whether invasive pathogenic strains exhibit different growth rates than nonclinical strains, particularly at lower temperatures. To investigate this question, live eastern oysters were injected with ST36 clinical strains and non-ST36 nonclinical strains, and growth rates were measured using the most probable number (MPN) enumeration. The presence of *V. parahaemolyticus* was confirmed using PCR by targeting the thermolabile hemolysin gene (*tlh*), thermostable direct hemolysin (*tdh*), *tdh*-related hemolysin (*trh*), and a pathogenesis-related protein (*prp*). The growth rates of the ST36 strains were compared to the FDA model and several other data sets of *V. parahaemolyticus* growth in naturally inoculated oysters harvested from the Chesapeake Bay. Our data indicate that the growth rates from most studies fall within the mean of the FDA model, but with slightly higher growth at lower temperatures for ST36 strains injected into live oysters. These data suggest that further investigations of ST36 growth capability in oysters at temperatures previously thought unsuitably low for *Vibrio* growth are warranted.

IMPORTANCE *Vibrio parahaemolyticus* is the leading cause of seafood-related gastroenteritis in the United States, with an estimated 45,000 cases per year. Most individuals who suffer from vibriosis consume raw or undercooked seafood, including oysters. While gastroenteritis vibriosis is usually self-limiting and treatable, *V. parahaemolyticus* infections are a stressor on the growing aquaculture industry. Much effort has been placed on modeling the growth of *Vibrio* cells in oysters in order to aid oyster growers in designing harvesting best practices and ultimately, to protect the consumer. However, ecological invasions of nonnative bacterial strains make modeling their growth complicated, as these strains are not accounted for in current models. The National Shellfish Sanitation Program (NSSP) considers 10°C (50°F) a temperature too low to enable *Vibrio* growth, where 15°C is considered a cutoff temperature for optimal *Vibrio* growth, with temperatures approaching 20°C supporting higher growth rates. However, invasive strains may be native to cooler waters. This research aimed to understand strain growth in live oysters by measuring growth rates when oysters containing ST36 strains, which may be endemic to the U.S. Pacific Northwest, were exposed to multiple temperatures postharvest. Our results will be used to aid future model development and harvesting best practices for the aquaculture industry.

KEYWORDS ST36, *Vibrio*, *Vibrio parahaemolyticus*, growth, oyster

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The authors declare no conflict of interest.

Received 7 June 2022
Accepted 11 November 2022
Published 29 November 2022

Vibrio parahaemolyticus is a naturally occurring halophilic marine bacterium that is capable of causing gastroenteritis via the consumption of raw or undercooked seafood (1). The Centers for Disease Control and Prevention (CDC) estimates that *V. parahaemolyticus* causes around 45,000 cases of vibriosis each year in the United States (2). While infections are often self-limiting in nature, outbreaks can lead to significant negative impacts on the aquaculture industry, including oyster growers, restaurants, and consumers. *V. parahaemolyticus* is commonly found in warm brackish water, typically from May to October, and is naturally found in the gut contents of eastern oysters (*Crassostrea virginica*) (3). Because it naturally occurs in marine ecosystems, eradication of the bacteria is not possible, and controlling infection is instead focused on mitigating risk.

Vibrio bacteria, both *V. parahaemolyticus* and *V. cholerae*, are the only pathogenic marine bacteria that have spread globally in a pandemic manner (4, 5). Prior to 2012, the only known incidence of transcontinental migration by a *V. parahaemolyticus* strain was the spread of the ST3 O3:K6 pandemic clonal complex from India to nearly every major continent in the world (5–10). However, in 2012, the U.S. Northeast and Spain experienced an increased number of infections that were attributed to the migration of sequence type 36 (ST36) (serotype O4:K12) strains that are endemic to the cooler waters of the U.S. Pacific Northwest (11, 12). These strains have been characterized by multilocus sequence typing (MLST) analysis of seven housekeeping genes and are remarkably similar in terms of chromosomal content (13). Additionally, these ST36 strains, deemed the Pacific Northwest complex, have proven to have a lower infective dose than other strains, making them potentially more virulent (14). Genomic investigation of the strains isolated from the North American East Coast suggests that while the Pacific Northwest strains had been introduced to other areas of the country prior to 1995, a diversification event in 1995 led to a newer lineage of ST36 strains (15). Investigations of the ST36 lineages also indicate that this modern lineage was introduced to the East Coast multiple times before becoming a resident sequence type of the region and that this modern lineage has undergone multiple diversification events since the introduction and incorporation into resident strain populations of the U.S. East Coast (15). After their 2012 introduction to the East Coast, these strains contributed to outbreaks in these regions and subsequently sustained residency in local waters, continuing to infect oyster consumers (16, 17). More recently, it was reported that several ST36 strains of the Pacific Northwest lineage migrated into Lima, Peru, between the years 2011 and 2016 and have now been detected in New Zealand, adding to the questions about whether or not these strains have pandemic potential similar to the aforementioned ST3 O3:K6 complex (18, 19).

The oyster aquaculture industry is consistently growing in the U.S. Northeast and mid-Atlantic regions. Nationally, *V. parahaemolyticus* infections impact aquaculture industry revenue and burden the health care system, with health care costs estimated at around $20 million per year (20). To mitigate illnesses caused by *V. parahaemolyticus*, the U.S. Food and Drug Administration (FDA) released a risk assessment in 2005 that modeled *V. parahaemolyticus* growth in live oysters postharvest as a function of air temperature at the time of harvest and the length of time harvested oysters remain unrefrigerated (21). Subsequently, other agencies such as the National Oceanic and Atmospheric Administration's National Centers for Coastal Ocean Science (NCCOS) have used this FDA model to create models for specific regions (https://products.coastalscience.noaa.gov/vibrioforecast/). These models assist the aquaculture industry, including oyster growers, by conveying information to assist with understanding *Vibrio* growth in their product based on the time of harvest, air temperature at the time of harvest, and the different cooling strategies used. However, there is a lack of sufficient data on growth rates of the nonnative ST36 *V. parahaemolyticus* strains that are causing infections and seemingly outcompeting local strains, especially at lower temperatures, which are typically suboptimal for *Vibrio* growth. These strains are native to the cooler waters of the Pacific Northwest, and it is expected that they may grow well at temperatures typically considered too low for optimal *Vibrio* growth (<15°C). Furthermore, the FDA risk assessment study was performed prior to the 2012 outbreaks, in which these strains took up residence in East Coast waters, which could potentially leave gaps in our knowledge of *Vibrio* growth in scenarios beyond what was explored in the risk assessment.

States that have historically had *V. parahaemolyticus* infections, or states where illness is reasonably likely to occur based on environmental metrics, are required to have control plans that are overseen by the states in coordination with FDA. The National Shellfish Sanitation Program (NSSP) keeps record of the rules and regulations implemented by these state authorities (22). These control plans require harvesters to adhere to harvest time restrictions during warmer months and require harvested product to be cooled to 10°C within a certain amount of time. These control plans are incredibly effective at maintaining lower temperatures in harvested oysters to control the growth of *Vibrio* species but do not account for the potential behavior of these invasive strains at lower temperatures. The goals of this study were to (i) calculate the growth rates of ST36 and non-ST36 *V. parahaemolyticus* strains in live *C. virginica* oyster tissue harvested in Maryland, USA; (ii) calculate the bacterial growth rates in naturally inoculated *Crassostrea gigas* oysters harvested in Washington, USA; (iii) compare the growth rate data obtained from these experiments to previously published growth rate data of *V. parahaemolyticus* in *C. virginica* oysters and the 2005 FDA model; (iv) calculate the growth rates of ST36 and non-ST36 strains in culture medium. The data obtained from this work will assist in evaluating the strength of the FDA model predictions and will help health organizations and the aquaculture industry better understand the potential risks of harvest practices in the presence of invasive strains that persistently cause infections throughout the U.S. East Coast.

RESULTS

Growth rates in culture were calculated for individual *Vibrio parahaemolyticus* strains, including the same strains used for the oyster injection trials, as well as additional ST36 strains obtained from the FDA (Tables 1 and 2). Growth rate replicates for each strain were averaged and plotted (Fig. 1). A one-way analysis of variance (ANOVA) and Tukey's procedure correcting for multiple comparisons indicated that growth rates were not significantly different among strains for both ST36 and non-ST36 ($P > 0.0001$).

Qualitatively, the ST36 strain cocktail injected into live oysters exhibited a higher growth rate per hour (0.091 $\log_{10}$/h) at 15°C incubation than the non-ST36 strains (0.043 $\log_{10}$/h) (Table 3; Fig. 2), but the statistical significance of this result cannot be quantified based on methodology. Additionally, the ST36 strain cocktail showed a higher growth rate per hour at 15°C than in all other trials compared for this study, including the natural infection of *C. gigas* oysters (0.05 $\log_{10}$/h), previous studies done on naturally inoculated *C. virginica* in Maryland waters in 2005 and 2006 (0.054 and 0.022 $\log_{10}$/h, respectively), and the 2005 FDA

TABLE 1 *V. parahaemolyticus* strains used in this study for both the culture and live oyster methods

Strain	Sequence type	Presence of genes[a]				Source	Isolation yr	State
		tlh	*trh*	*tdh*	*prp*			
PHL-3[b]	36	+	+	+	+	Human stool	2012	WA
PHL-4[b]	36	+	+	+	+	Human stool	2012	WA
EN9701173[b]	36	+	+	+	+	Human stool	1997	WA
12315[b]	36	+	+	+	+	Human stool	2006	WA
43[c]	322	+	−	−	−	Oyster	2007	WA
204[c]	3	+	+	−	−	Oyster	2007	WA
930[c]	3	+	−	+	−	Oyster	2007	WA
2012V-1076[d]	36	+	+	+	+	Human stool	2012	MO
2012V-1103[d]	36	+	+	+	+	Human stool	2012	WA
2012V-1108[d]	36	+	+	+	+	Human stool	2012	MA
2012V-1109[d]	36	+	+	+	+	Human stool	2012	MA
2012V-1131[d]	36	+	+	+	+	Human stool	2012	CA
2012V-1134[d]	36	+	+	+	+	Human stool	2012	CA
CDC_K4639[d]	36	+	+	+	+	Human stool	2012	NY

[a]*tlh*, thermolabile hemolysin; *tdh*, thermostable direct hemolysin; *trh*, *tdh*-related hemolysin; *prp*, pathogenesis-related protein.
[b]Isolated by the Washington Department of Health; provided by the NOAA Northwest Fisheries Science Center.
[c]Provided by the NOAA Northwest Fisheries Science Center.
[d]Provided by the FDA, Division of Seafood Science and Technology, Gulf Coast Seafood Laboratory, Dauphin Island, AL.

TABLE 2 Growth rates of all strains in broth at 15°C[a]

Strain	Growth rate (log_{10}/h)
12315	0.163
EN9701173	0.141
PHL-3	0.181
PHL-4	0.188
2012V-1076	0.167
2012V-1103	0.161
2012V-1108	0.155
2012V-1109	0.168
2012V-1131	0.169
2012V-1134	0.161
CDC_K4639	0.151
204	0.171
43	0.149
930	0.157

[a]Growth rates were calculated using the Growthcurver package in R (36).

model predictions for *V. parahaemolyticus* growth at 15°C (0.0381 log_{10}/h) (Table 3; Fig. 2) (21, 23). All oyster trials showed expected patterns in growth rate per hour, where the growth rates increased from 20 to 30°C, except in the ST36 oyster trial, in which the growth rate per hour at 15°C was slightly higher than that at 20°C but followed the expected growth rate trajectory at temperatures above 20°C (Fig. 2). The standard error could not be calculated for growth rates in live oyster tissue due to the nature of the study, because each time point estimate was measured for independent groups of oysters. However, the standard error was evaluated for the replicates averaged for each measurement time point to ensure that there were no significant differences between replicates. All aforementioned live oyster studies were averaged as a way to compare the collective results with the 2005 FDA model predictions, and the results of this comparison indicated that the FDA model falls within the calculated mean and standard error of the averaged studies (Fig. 3).

The growth rates at 15°C for the ST36 and non-ST36 strains differed when cells were grown in broth versus the rates observed in live oysters. For the ST36 strains injected into oysters, the observed growth rate at 15°C was only slightly lower than that observed in broth culture trials. The difference between the live oyster and broth culture growth rates was more pronounced for the non-ST36 strains used in this study, where the observed growth rate for the non-ST36 strains injected into oysters was nearly half of the growth rates seen for these strains in broth culture.

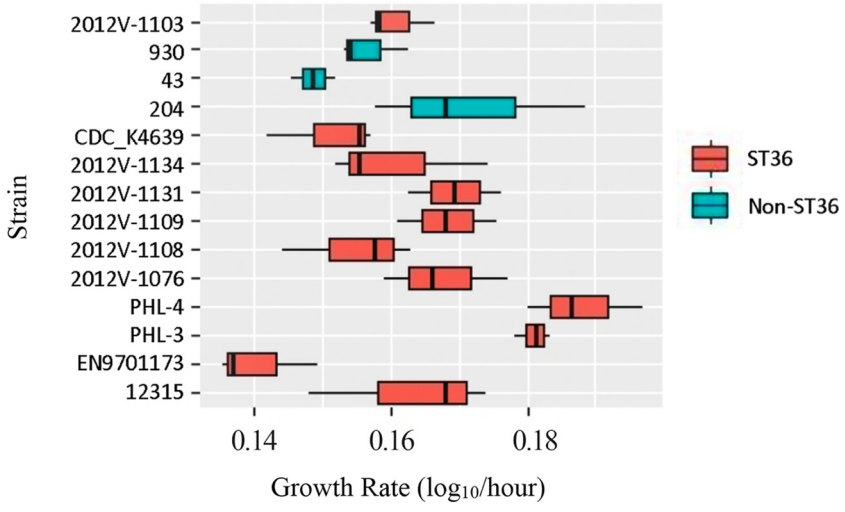

FIG 1 Growth rates of each strain per hour were calculated using the Growthcurver package in R (37). The calculated growth rates include the median, indicated by a vertical black line within each box, and the range of values per strain replicate, indicated by the horizontal line in each box.

TABLE 3 Comparison of the growth rates of ST36 and non-ST36 strains in naturally inoculated *C. gigas* in this study with the rates in other studies

Trial/study	Incubation temp (°C)[a]	Growth rate (log_{10}/h)	Source
ST36 injection	15	0.091	This study
	20	0.062	
	25	0.188	
	30	0.263	
Non-ST36 injection	15	0.043	This study
	20	0.057	
	25	0.300	
	30	0.352	
C. gigas natural uptake	15	0.050	This study
	20	0.080	
	25	0.120	
C. virginica natural uptake (2005)	15	0.054	32
	20	0.107	
	25	0.28	
	30	0.264	
C. virginica natural uptake (2006)	15	0.022	32
	20	0.058	
	25	0.177	
	30	0.175	
2005 FDA model	15	0.038	21
	20	0.088	
	25	0.158	
	30	0.249	

[a]Growth rates were calculated for temperatures of 15, 20, 25, and 30°C, except for the naturally inoculated *C. gigas* trial, which included incubation temperatures of 15, 20, and 25°C.

DISCUSSION

The main purpose of this study was to investigate the growth rate differences exhibited by ST36 and non-ST36 *V. parahaemolyticus* strains both inside live oyster tissue as well as in broth cultures, as there are currently no available data on the growth characteristics of these sequence type strains. The results of the broth culture study indicated that the strain growth rates per hour were generally consistent and do not appear to be related to ST36. While all strains used in the oyster injection studies were isolated in Washington State, the strains tested in broth culture came from various states, including Washington, Missouri, Massachusetts, California, Maryland, and New York (Table 1). A next step in investigating the growth rate differences at lower temperatures is to perform similar oyster injection studies using ST36 isolated from other regions as a means of investigating the impact of sequence type versus isolation source on bacterial growth characteristics.

When considering the year that each strain used in the broth study was isolated, most were isolated between the years 2006 and 2012, with one strain isolated prior to 2000 (strain EN9701173, 1997; Table 1). Throughout all trials of both the broth and live oyster experiments, this strain typically showed slower growth than the other strains (Fig. 1). While strain EN9701173 was included in the strain dose cocktail for the live oyster experiments, this strain was likely outcompeted by the other three strains that showed faster growth at lower temperatures.

Genetic analysis of the genus *Vibrio* has indicated that many species have gained more genes than they have lost over the course of their evolutionary history (23). For instance, several species of *Vibrio* have shown an increase in genes related to metabolism, allowing them to be one of the most successful and fastest-replicating marine bacteria (23). Lin et al. suggest that this evolutionary history is evident of vibrios' ability to gain and lose genes as needed to remain competitive and inhabit new niches (23). As previously mentioned, a recently discovered genetic diversification event in 1995 led to a divergent population of ST36 strains that invaded the U.S. East Coast (15). The newer ST36 lineage that resides on the East Coast and

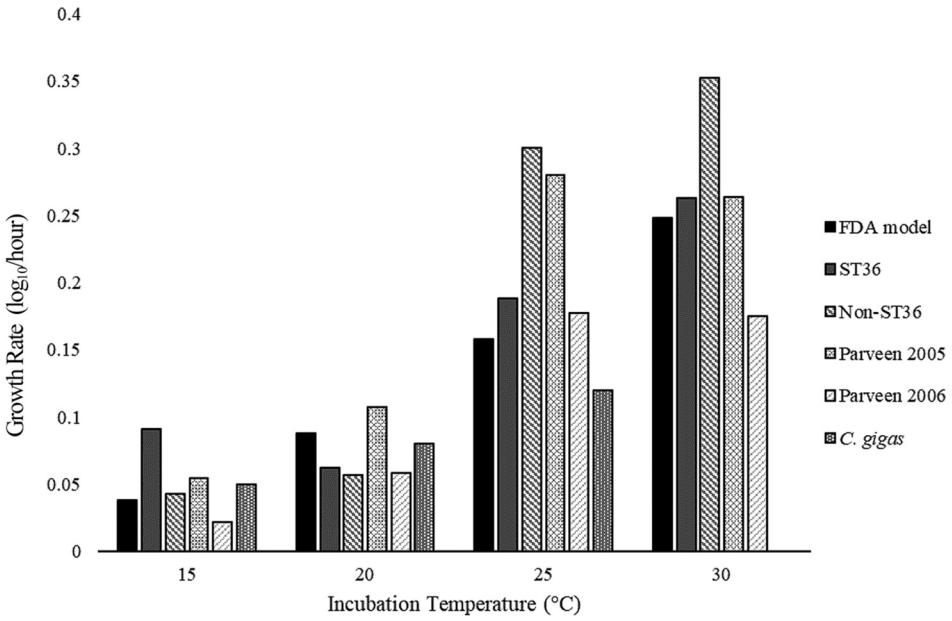

FIG 2 Growth rates of *V. parahaemolyticus* ST36 and non-ST36 strains injected into *Crassostrea virginica* oyster tissue at temperatures of 15 to 30°C and of the strains in two growth rate studies previously conducted on naturally inoculated *Crassostrea virginica* Chesapeake Bay oysters (25), a study conducted on naturally inoculated *Crassostrea gigas* in the Pacific Northwest at temperatures of 15 to 25°C, and the 2005 FDA risk assessment model (21).

in the Pacific Northwest is genetically similar to the old Pacific Northwest lineage but is still considered distinct. Additionally, investigation of the ST36 clade evolution over its introduction to new regions indicated that the strains that now reside on the East Coast show signs of genomic simplification, or smaller genomes, compared to those of the old Pacific Northwest complex and even those that were isolated from Spain in 2012 (15). There has additionally been some research on bacteria and gene loss indicating that some bacteria that have experienced gene loss, and therefore contain smaller genomes, show higher rates of mutation and evolution (24). The results of these studies suggest that it is possible that

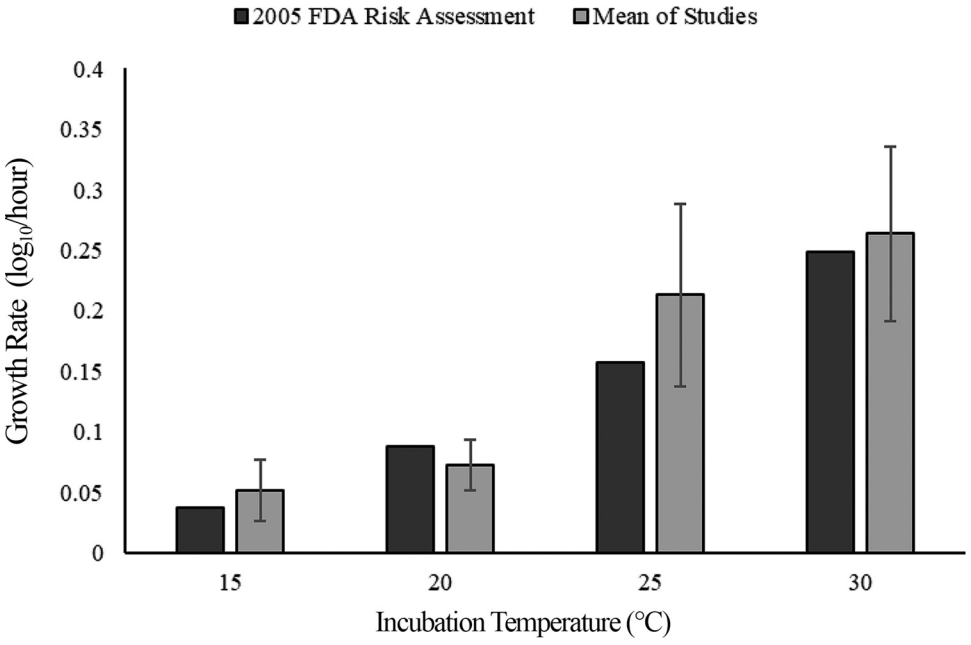

FIG 3 Growth rates at temperatures of 15 to 30°C of strains in the 2005 FDA risk assessment model and the mean of all previously referenced studies (21, 25) (Fig. 2).

the ST36 East Coast strains have been able to mutate faster with smaller genomes, so that the gene loss could have been advantageous to their survival in a new environment. Throughout this study, the two strains PHL-3 and PHL-4 consistently showed faster growth in most media and in the study at 15°C (Table 2). Though statistics correcting for multiple comparisons proved that these differences were not significant at 15°C, the phenomenon was still noted for future research. These were the only two strains included in the study that were isolated in 2012, with all other strains isolated prior to the 2012 ecological introductions across the world and North America. Furthermore, EN9701173 was the only strain used in this study that was isolated prior to 2000, and it was therefore the oldest isolated strain used in these experiments and, as previously mentioned, the slowest-growing strain throughout all stages of the experiments. More research investigating these ST36 lineages and genes gained/lost over their migration would be beneficial to understanding the strains that are now endemic to East Coast waters, especially coupling these genetic factors with growth activity.

Strain growth studies have been done by other researchers in the past. Miles et al. conducted an original study looking at growth in broth culture using isolates from patients with gastroenteritis, using the fastest growing of four available strains to create a mathematical model predicting growth (25). This study by Miles et al. did not consider pathogenicity or genotype, which has typically been incorporated in more recent studies. Additionally, work done by Gooch et al. found that the study done by Miles et al. overestimated the growth rates of *V. parahaemolyticus* by 4-fold, which is why our study included both a broth culture component and a live oyster component (26). Yoon et al. found that *tdh*$^-$ strains grew faster than *tdh*$^+$ strains in every medium tested, which included broth culture and Korean oyster slurry (27). The Yoon et al. study also observed that this phenomenon was less notable as the study temperature increased, indicating that at lower temperatures, the nonpathogenic strains typically had higher growth rates (27). Our study found no significance between pathogenic and nonpathogenic strains in broth generally, but we found the opposite effect in live oysters, in which the pathogenic ST36 strains had higher growth rates than the nonpathogenic, or *tdh*$^-$, strains at the lowest temperature tested (15°C).

When *V. parahaemolyticus* cells were injected into oysters, the ST36 strains exhibited faster growth ($\log_{10}$ per hour) in live oyster tissue than the non-ST36 strains at the same 15°C mark (0.091 and 0.043 $\log_{10}$/h, respectively) (Fig. 2). Furthermore, the ST36 trial showed fast *V. parahaemolyticus* growth at 15°C compared to the other studies referenced (Fig. 2). This phenomenon was only noted at 15°C, with other trials showing faster growth at other temperatures than in the ST36 injection trial. Although the ST36 strains exhibited faster growth than the non-ST36 strains, it is currently unknown whether this would result in increased *Vibrio*-related illnesses compared to FDA seafood safety model predictions due to the uncertainty of the infective doses. Some pathogenic *V. parahaemolyticus* strains, including ST36 strains, have shown much lower infective doses than those in previous data (28).

Inoculation of oysters with specific strains by natural uptake was a method that was considered for this study in lieu of injection. There have been successful studies done on *Vibrio* growth in oysters using natural inoculation in a water bath containing *Vibrio* cells (29–31). This method of tank inoculation was attempted for our study but resulted in significant variability between the replicate groups of pooled oysters and individual oysters during the trials (unpublished data). This type of variability is common, as it is known that *Vibrio* concentrations in oysters can vary greatly from oyster to oyster. This is also evident when comparing the two studies done on naturally inoculated oysters by Parveen et al. in 2005 and 2006; the same methods were used for both of these studies, but as shown in Fig. 2, the growth rates observed varied at the same temperatures between years (32). For this reason, direct inoculation methods similar to those used by Kaysner et al. were employed in our study to ensure less variability between replicates and to provide a more precise growth rate (33).

Despite the differences between methods for different studies, when all of the studies referenced in this paper were averaged for each challenge temperature from 15 to 30°C, the mean of the studies fell within the expected growth rates modeled by the 2005 FDA risk assessment (21) (Fig. 3). After collectively investigating the various studies, including the

ST36 and non-ST36 injection trials from our study, we concluded that the FDA model still performs adequately, though it is not possible to ascertain if the model is performing under all scenarios at this time. Regardless, these results indicate that the FDA model has promising performance and the ability to provide meaningful guidance to the aquaculture industry in terms of *Vibrio* growth in live oysters. While this model is very useful, there is still observed variability in the *Vibrio* growth rates in oysters, depending on the oyster species and location, based on the compilation and comparisons of the experiments referenced in this study. Continuing to investigate the growth of *V. parahaemolyticus* in live shellfish is a way to further the industry's understanding of *Vibrio* growth tendencies and investigate possible anomalies that stray from the model expectations. For example, the ST36 strains used for the oyster tissue experiment in this study were the strains that were isolated and available to the researchers at the time, but it would be beneficial to investigate the growth characteristics of more ST36 strains, as they become available, inside live oyster tissue to further investigate the lower temperature (15°C) phenomenon noted in this study. This method of investigation in live oysters would also prove useful concerning other sequence types, such as ST631 strains, which are emerging as another lineage of interest in North America (34). The FDA risk assessment assumes that the growth rates for pathogenic and nonpathogenic strains of *V. parahaemolyticus* are similar. However, that assessment was conducted over 15 years ago and before genetic events that have led to distinct populations of pathogenic strains. As strains continue to evolve, more studies are needed to confirm whether pathogenic strains have adapted to faster growth at lower temperatures.

Due to the fact that ST36 strains have been presumed to be the collective cause of increased *V. parahaemolyticus* infections in the northeast United States, it is useful to understand how their growth may differ from that of other sequence types (35). The main vector of infection by ST36 strains is raw or undercooked seafood, so the results presented in this study have implications for the aquaculture industry in terms of preventing illness and reiterating the importance of keeping product at a temperature low enough to prevent *Vibrio* growth. Furthermore, ST36 strains are continuing to mutate and cause increasing illness throughout the world (17). Additionally, other sequence types are becoming more prominent and may undergo similar genetic events that could lead to more resilient bacteria that have capabilities of increasing the rates of infection. This study indicates a need to continue research on how growth rates differ by strain, and over time, and how strains and sequence types of interest survive and proliferate inside live oysters.

MATERIALS AND METHODS

Preparing ST36 and non-ST36 inoculums for oyster injection. For the non-ST36 strain trial, three environmentally isolated strains of *V. parahaemolyticus* were used: 43, 930, and 204 (Table 1). The strains were inoculated onto T_1N_2 agar plates (2% NaCl, 1% pancreatic digest of casein, and 2% agarose [wt/vol]) and incubated at approximately 22°C, for 24 h $\pm$ 4 h. Two milliliters of LB (Miller) (Sigma-Aldrich, MO, USA) broth was inoculated with a loopfull of each strain and incubated at 25°C in a static incubator for 24 h $\pm$ 4 h. Following incubation, 20 mL of LB (Miller) broth was inoculated with 133 μL of each strain to form a cocktail of all three cultures. This cocktail culture was incubated at 30°C with shaking (100 rpm) for 2 h. Following shaking incubation, the optical density at 600 nm (OD_{600}) was measured to estimate the bacterial concentration. One milliliter of culture was washed twice by centrifuging at 8,000 rcf (relative centrifugal force) for 1 min, discarding the supernatant, and resuspending the pellet in 1 mL phosphate buffer solution (PBS; 0.85% NaCl, 0.058% NaH_2PO_4, 0.25% Na_2HPO_4 [wt/vol]). Tenfold serial dilutions were performed from the estimated starting point, most frequently 10^6 to 10^8 (estimation made based on OD measurement) through 10^1 by inoculating 800 μL of washed culture into 7.2 mL PBS for each dilution. The 10^4 dilution was set aside as the inoculum. Replicate T_1N_2 agar spread plates were prepared for the 10^3, 10^2, and 10^1 dilutions by transferring 200 μL of the diluted culture onto the plate and spreading the culture with a flame-sterilized metal spreader. The spread plates were incubated at room temperature upside down for 24 $\pm$ 4 h, and the CFU were then counted to calculate the concentration of the inoculum.

The same protocol used to prepare the non-ST36 inoculum was used to prepare the ST36 inoculum, with minor exceptions. Four ST36 strains were used to create the dosage cocktail: PHL-3, PHL-4, 9701173, and 12315 (Table 1). When creating the combined broth cocktail, 100 μL of each strain was added to 20 mL of LB (Miller) broth.

Oyster inoculation and incubation. *C. virginica* oysters originating from Marinetics Inc. from the Choptank River (MD, USA) were purchased on the retail market from Captain's Ketch Seafood in Easton, MD. All oysters used were uniform in size (2 to 3 in.). The oysters were acclimated from refrigeration temperatures to ambient water temperatures over the course of 7 days prior to the experiments, then to 18°C for 2 h prior to dosing. They were rinsed, scrubbed with room temperature potable tap water, and allowed to dry before

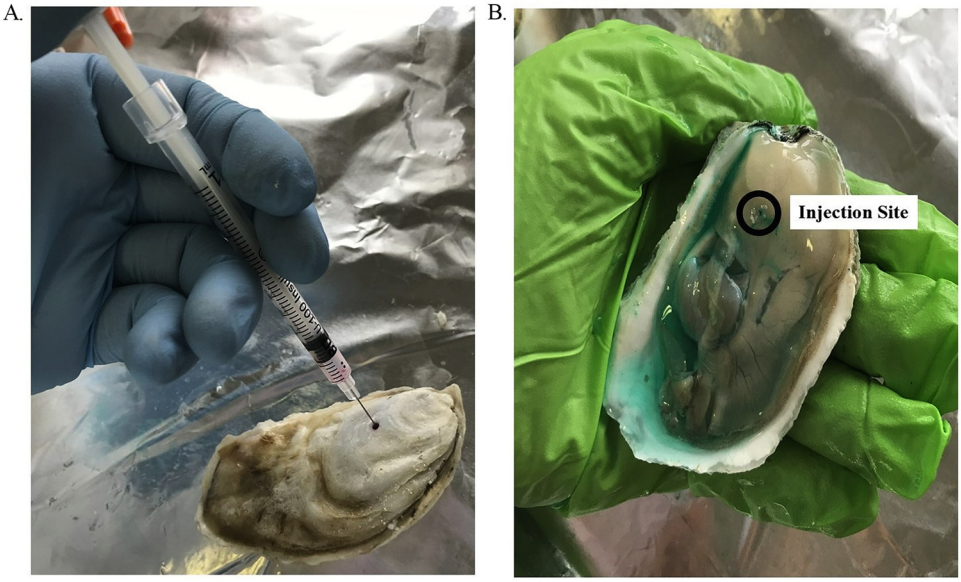

FIG 4 Depiction of the oyster drilling and injection site (A) and distribution of tissue dye (and the vibrio cocktail) after injection by syringe into the gut region of the oyster (B).

processing. Using a 1/16-in. drill bit fitted to a power drill, each oyster was drilled on the right valve approximately 1/4 distance from the hinge and slightly to the left (dorsoventral quadrant) in order to inject the gut region, as illustrated in Fig. 4. This technique was first tested on several oysters using tissue dye as the inoculum, and they were dissected to confirm that the inoculum would reach the gut tissue. One control group was tested at time point 0 for all trials, where 4 uninoculated oysters were processed to ensure that there were no prior background levels of *V. parahaemolyticus* present (the use of winter harvested oysters prevented background levels). Once drilled, each oyster was injected with 100 μL of an estimated 10^4 concentration of the *Vibrio* strain cocktail, using a separate 1 mL 27-gauge syringe for each group of 4 oysters. After injection, the drilling site was sealed with Critoseal tube sealant (Leica, Wetzlar, Germany), and the drill bit was disinfected with 70% ethanol. Each oyster was placed into a sterile plastic bag that was left open and placed level on a tray. The oysters were transferred to an incubator set to the desired temperature of the experimental trial being performed (15°C, 20°C, 25°C, or 30°C) for the duration of the experiment.

Oyster processing and MPN protocol. At each time point (0, 3, 7, and 10 h), 3 groups of 4 oysters were removed from the incubator and left on the bench prior to processing within 1 h. The oysters were shucked on a sterile metal tray with sterilized aluminum foil. Four oysters were opened using a sterile oyster shucking knife, and the entire animal, including the adductor muscle and mantle fluid, was transferred into a sterile tared blender jar. An equal amount of PBS (wt/vol) was added to the blender jar and blended on high for 90 s. A three-tube most probable number (MPN) protocol as indicated in the FDA *Bacteriological Analytical Manual* (BAM) was followed for enrichment of the oyster homogenate in alkaline peptone water (APW) (Thermo Fisher Scientific, MA, USA), and the MPN tubes were incubated at 35°C for 18 to 24 h (23). Following incubation, the MPN tubes were observed for turbidity, and 1 mL of each MPN tube culture was transferred to microcentrifuge tubes. The microtubes were boiled in a dry bath at 100°C for 10 min and stored at −80°C for PCR analysis.

PCR parameters and gel electrophoresis for detection of *Vibrio parahaemolyticus*. A multiplex PCR was performed on boiled MPN samples targeting 4 genes: thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*), *tdh*-related hemolysin (*trh*), and pathogenesis-related protein (*prp*) (Table 4). PCRs were carried out using, per sample, 1 μL of each primer, 5 μL of 5× Flexi buffer (Promega, WI, USA), 1.5 μL $MgCl_2$, 2 μL deoxynucleoside triphosphate (dNTP) solution (Epicentre, WI, USA), 7.3 μL nuclease-free PCR grade water, 0.2 μL GoTaq polymerase (Promega), and 1 μL DNA template (boiled MPN culture) for a 25-μL reaction volume. The cycling parameters included an initial denaturation at 94°C for 60 s, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 60 s, and extension at 72°C for 60 s, with a final extension step of 72°C for 10 min (1 cycle). Amplification products were stored at −20°C until further analysis. The PCR products examining the presence/absence of all four target genes were examined using double-comb ethidium bromide E-gels (Invitrogen, CA, USA). Amplicon size was confirmed with comparison to a 1 kb plus molecular weight marker (Invitrogen).

***V. parahaemolyticus* ST36 and environmental strain growth in LB broth medium.** Growth rates (log_{10} per hour) were observed for all strains used in the live oyster trials, as well as for additional ST36 strains obtained from the FDA Gulf Coast Seafood Laboratory in Dauphin Island, AL (Table 1). All strains were grown from frozen glycerol stocks (OPS Diagnostics, NJ, USA) on LB (Miller) agar plates at 20°C for 24 h ± 4 h. One inoculating loop of each strain was transferred into individual conical tubes containing 5 mL of LB broth and incubated at 30°C with shaking (100 rpm) for 2 h. Assuming that each strain culture was approximately 10^8 CFU/mL, based on previous growth curves corresponding to OD measurements of the strains being used, each strain was diluted to 10^4 by 10-fold serial dilutions in LB broth. For the

TABLE 4 Primer sequences, amplicon sizes, and sources for all PCR primers used in this study

Gene/locus	Primer direction/name	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
tlh	F2	AGAACTTCATCTTGATGACACTGC	401	38
	R	GCTAC-TTTCTAGCATTTTCTCTGC		39
tdh	F	GTAAAGGTCTCTGACTTTTGGAC	269	39
	R	TGGATAGAACCTTCATCTTCACC		
trh	F	CATAACA-AACATATGCCCATTTCCG	500	39
	R	TTGGCTTCGATATTTTCAGTATCT		
ST36prp	F2	TGCGGAATCTGATCTTTATCCTC	1,028	38
	R2	AACTGTTG-GGTCTTCGTCTAACC		

final inoculum, 500 μL of each strain was inoculated into 4.5 mL of LB broth. An aliquot (150 μL) of each strain was inoculated in triplicate onto a black-walled microplate, avoiding the outer 2 wells of each row horizontally and vertically to negate the microplate edge effect throughout incubation. The microplate was transferred to a Synergy 2 microplate reader (Biotek, VT, USA) that was located in a low-temperature incubator set to 15°C. This method of low-temperature incubation was tested in the instrument prior to the experiment using a smart button temperature logger (ACR Systems, FL, USA) placed into the microplate reservoir, and consistent 15°C temperature readings were verified over the course of 72 h. The microplate reader measured the optical density (600 nm) over the course of 72 h at 30-min intervals with 5 s shaking prior to each reading.

Natural infection study on *Crassostrea gigas* oysters. *C. gigas* oysters were harvested from the south Hood Canal region (WA, USA) and transported to the Northwest Fisheries Science Center with ice packs. The oysters were placed in sterilized trays, covered with aluminum foil, and subsequently incubated at temperatures of 15, 20, and 25°C for 24 h. Three groups of six oysters were removed and processed at the 0, 5, 10, and 24-h time points. The oysters were processed using the same FDA three-tube MPN method described previously, and the *V. parahaemolyticus* concentrations were estimated by PCR analysis of the *tlh*, *tdh*, and *trh* genes (Table 4).

Statistics. For the live oyster trials, the *V. parahaemolyticus* cells per gram of oyster tissue were calculated using the FDA BAM method for the most probable number estimations (23). The MPN measurements for the three replicates at each measurement for the oyster trials were averaged. The averaged MPN data were log transformed, and a regression analysis was performed to assess the growth rates ($\log_{10}$ per hour).

For the broth study trials, optical density data were downloaded from the Biotek plate reader and sorted into separate data sets per strain. The Growthcurver package in R was used to calculate various metrics, including the lag time and growth rate per minute for each strain (24). Growthcurver uses the following logistical equation for calculating the intrinsic growth rate (r) per well over time (t):

$$Nt = \frac{K}{1 + \left(\frac{K - N_0}{N_0}\right) e - rt}$$

where N_0 is the population at the start of the growth trial, and the carrying capacity is indicated by K. The growth rates per hour of each replicate of each strain were calculated using Growthcurver, and the significance was analyzed using a one-way ANOVA and a least-square means procedure using Tukey's adjustment for multiple comparisons.

ACKNOWLEDGMENTS

We thank Suzanne Skelley, director of the Cooperative Oxford Laboratory, and Lindsay Goodwin (CSS Inc.) for the laboratory's continued support of this research. This work could not have been done without the Washington Department of Health and Jessica Jones from FDA, Division of Seafood Science and Technology, Gulf Coast Seafood Laboratory, Dauphin Island, AL, who provided the *V. parahaemolyticus* strains. We also thank Jurgen Schwarz (UMES) for his assistance with manuscript review, all Cooperative Oxford Laboratory staff for assistance with oyster processing, and Captain's Ketch Seafood Market and Taylor Shellfish for providing oysters for research purposes.

We thank the National Centers for Coastal Ocean Science within the National Oceanic and Atmospheric Administration for providing the funding for this work.

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