Journal of Food and Drug Analysis, Vol. 11, No. 3, 2003, Pages 226-232 藥物食品分析 第十一卷 第三期

Susceptibility of O3:K6 and Environmental Strains of *Vibrio parahaemolyticus* to Acid Inactivation, and Survival Competition between these Strains and *Pseudomonas fluorescens* and Indigenous Bacteria

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(Received: August 13, 2002; Accepted: July 7, 2003)

ABSTRACT

Vibrio parahaemolyticus is a common food poisoning pathogen associated with seafood. New O3:K6 strains originating from India in 1996 were the first pandemic strains and spread rapidly throughout many Asian countries. This study examines the characteristics of these new O3:K6 strains with respect to acid susceptibility and survival competition with *Pseudomonas fluorescens* and indigenous oyster spoilage microflora. Results revealed that the exponential phase cultures of new O3:K6 strains were significantly more tolerant of acid at pH 3.0 than the environmental strains. In these co-culture experiments, survival of the *V. parahaemolyticus* strains was influenced by the bacterial strains, incubation medium and incubation temperature. All *V. parahaemolyticus* strains showed similar survival ability when co-cultured with *P. fluorescens* in 1/10 tryptic soy broth. In the oyster medium, the environmental strain survived better than the new O3:K6 strains at 4 or 25°C. Both of the new O3:K6 strains (1121 and 1137) showed similar survivability in this study. These data offer hints on the survival of the new O3:K6 strains and their ability to spread.

Key words: *Vibrio parahaemolyticus*, O3:K6, *Pseudomonas fluorescens*, growth, oyster, acid susceptibility

INTRODUCTION

Vibrio parahaemolyticus, a halophilic gram-negative bacterium that causes acute gastroenteritis in humans, was first discovered in 1950 during a food poisoning outbreak in Osaka, Japan. This bacterium is one of the most prevalent food-borne pathogens in Taiwan, Japan, and other coastal countries[1,2]. Clinical manifestations of *V. parahaemolyticus* infections include diarrhea, abdominal cramps, nausea, vomiting, headaches, fever, and chills. The incubation period ranges from 4 to 96 hr[1]. Isolates of *V. parahaemolyticus* can be distinguished from each other by serotyping. Various serovars typically cause infection[3].

Since 1996, a new O3:K6 strain of this pathogen has caused widespread food poisoning outbreaks across many Asian countries, including Taiwan. It was the first pandemic strain of this pathogen[4,5]. After pulsed-field gel electrophoresis (PFGE) and cluster analysis, all the new and old O3:K6 strains were separated into two groups. The new O3:K6 strains were in one group that included eight closely related patterns, of which I1 (81%) and I5 (13%) were the most frequent patterns. Pattern I1 was prevalent in Japan, Korea and Taiwan[5]. These new O3:K6 strains are probably derived from a single or closely related clones[5,6].

All new O3:K6 strains carry the thermostable direct hemolysin (TDH) gene (*tdh*) which is the major virulence factor in this pathogen. The new O3:K6 strains do not significantly differ from the non-O3:K6 strains and the old O3:K6 strains isolated before 1996, in terms of antibiotic susceptibility and TDH level. Stationary phase cultures of new and old O3:K6 strains do not differ in their susceptibility to environmental stresses[5]. Stress susceptibility in exponential and stationary phases may differ since the stress tolerance of bacterial cells is normally acquired at the stationary phase[7,8].

V. parahaemolyticus is often present in marine substrata, and either grows on living marine organisms or exists freely in seawater [9,10]. Stronger growth competition against other indigenous microorganisms in the natural environment or in the food-processing chain would probably enhance the survival of the new O3:K6 strains and facilitate spread of this pathogen.

In order to learn more about their survival ability, the new O3:K6 strain was grown in competition with *Pseudomonas flurorescens*, one of the most common indigenous bacteria in seafood, and indigenous microflora in low nutrient and oyster media at room and low temperatures. The acid susceptibility of *V. parahaemolyticus* in the exponential growth phase was also determined.

MATERIALS AND METHODS

I. *Bacterial Strains and Culture Conditions*

Ten *V. parahaemolyticus* strains were used in this

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study (Table 1). Five were the new O3:K6 strains isolated from clinical samples[5]. Others were nonpathogenic environmental strains isolated from seafood or marine water [11]. All these strains were isolated in 1998. The *Vibrio* cultures were stored at -85˚C in tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.) supplemented with 3% NaCl that contained 20% glycerol. *P. fluorescens* CCRC10304 was obtained from the Collection and Culture Reseach Center, Hsinchu, Taiwan, frozen in TSB-1% NaCl (TSB-NaCl).

All the cultures were grown on TSB-NaCl at 25˚C, and shaken at 110 rpm.

II. *Acid Susceptibility*

V. parahaemolyticus new O3:K6 and environmental strains were grown overnight at 25˚C in TSB-NaCl media, shaken at 110 rpm, and were then transferred to a fresh medium. The bacterial cells were grown to exponential phase for approximately 5 hr and the absorbance of the culture at 600 nm was adjusted to about 1 by adding fresh medium. Cultures were acid challenged by acidifying the medium to pH 3.0 with 3N HCl[12]. Viable cells were counted at 0, 2, 5, and 10 min following acidification. Serial dilutions were made in phosphate-buffered saline (PBS)-1%NaCl, and 100 μl of each dilution was spread onto tryptic soy agar (TSA-NaCl) (TSB supplemented with 1% agar), and were incubated overnight at 25˚C. The D value (the time to cause a 90% reduction in the number of viable cells) of each strain was calculated using the curvefit function of Slide Write Plus software, version 1.10 (Advanced Graphic Software, Inc., Carlsbad, Calif.)[5].

III. *Preparation of Oyster Media*

Fresh shucked oysters were purchased from a local seafood distributor and washed with distilled water. Two hundred grams of the oyster was homogenized with an equal volume of sterile PBS, supplemented with 1% NaCl, blended by a Waring blender at high speed for 1 min. The oyster homogenate was filtered through cheesecloth and used as the nonsterilized oyster medium. Indigenous vibrios in the nonsterilized oyster medium were monitored by plating on a selective medium, thiosulfate-citrate-bile salt-sucrose (TCBS) agar (Difco)[5].

IV. *Growth Competition*

New O3:K6 strains, 1137 (PFGE pattern I1) and 1121 (PFGE pattern I5), and environmental strain 1025 of *V. parahaemolyticus*, and *P. fluorescens* CCRC10304 were cultured in TSB-NaCl medium at 25˚C and shaken at 110 rpm for 5 hr to reach exponential phase. Bacterial cells were harvested by centrifugation at 8,000xg for 10 min, and were diluted in fresh culture medium to approximately 10^9 CFU/mL. *V. parahaemolyticus* and *P. fluorescens* cultures were inoculated into 100 mL of 1/10 TSB-1.0% NaCl in a 250 mL-Erlenmeyer flasks to a final inoculation level of 107 CFU/mL for both bacteria. These mixed cultures were incubated at 4˚C or 25˚C and shaken at 110 rpm for 7 (1/10 TSB-1.0% NaCl) or 24 hr (oyster). Viable cells were counted using TSA-NaCl after serial dilution in PBS-1% NaCl and were incubated at 25˚C for 16 hr. Colonies of *Vibrio* and *P. fluorescens* were counted. The tiny colonies of *P. fluorescens* could be easily distinguished from the large *Vibrio* colonies on this agar medium.

In the nonsterilzed oyster medium, *Vibrio* was counted on TCBS agar[5], while all bacteria were counted on the TSA-NaCl medium. The difference between these two bacterial counts was the number of indigenous bacteria.

V. *Statistical Analysis*

All experiments were repeated twice and the data were obtained from at least triplicate determinations. The data were analyzed using SPSS for Windows 6.0, (SPSS Inc., Chicago, Ill.) employing a t-test and analysis of variance[5].

RESULTS

I. *Acid Susceptibility of V. parahaemolyticus O3:K6 and Environmental Strains*

The susceptibility of these *V. parahaemolyticus* strains to acid inactivation at pH 4.0 or 4.2 has been performed and results showed high variations among different strains. The strains tested were highly resistant to the inactivation at pH 4.2 and 4.0, and the average D values for pH 4.0 inactivations were about 64 min for new O3:K6 strains and about 25 min for the environmental strains (data not shown). Also, the new O3:K6 group was significantly more tolerant to acid than the environmental group at pH 3.0 (Table 1).

Table 1. Information and acid susceptibility of *Vibrio parahaemolyticus* strains used in this study.

Strain no[a]	PFGE pattern[b]	Specimen	Location of isolation	D value at pH 3.0
New O3:K6				
1092	I1	Clinical	Taichung	1.41
1121	I5	Clinical	Miaoli	3.26
1132	I5	Clinical	Yunlin	2.87
1134	I5	Clinical	Taichung	1.44
1137	I1	Clinical	Yunlin	1.40
			mean ± se	2.08 ± 0.41*
Environmental				
1025	ND	seafood	Kaohsiung	1.38
1036	ND	seafood	Kaohsiung	0.69
1051	ND	seafood	Kaohsiung	0.68
1062	ND	seafood	Kaohsiung	0.69
1074	ND	seafood	Kaohsiung	0.68
			mean ± se	0.82 ± 0.19

I1 and I5 represent the major pattern of new O3:K6 strains[5]; ND, not determined; se, standard error of means; *, statistically significant at $p < 0.05$.

II. *Growth Competition of V. parahaemolyticus New O3:K6 and Environmental Strains, Against P. fluorescens and Indigenous Bacteria*

The survival of *P. fluorescens* and different *V. parahaemolyticus* strains co-cultured in different media at room (25°C) or refrigerating temperature (4°C) was further examined. Competitive survival of bacteria was affected by various parameters, such as the strain, incubation medium and the incubation temperature (Fig. 1-4). When the co-

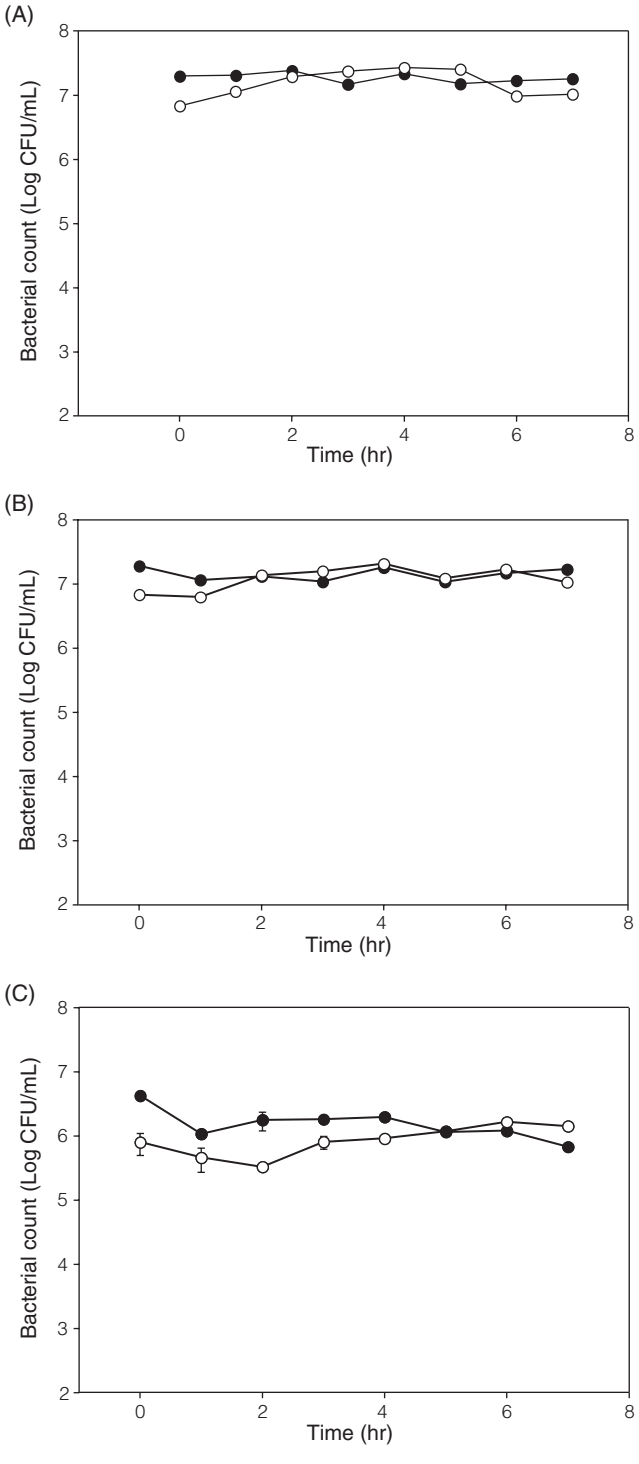

Figure 1. Growth of *Vibrio parahaemolyticus and Pseudomonas fluorescens* co-cultured in one-tenth strength of tryptic soy broth-NaCl medium at 25°C. Panel A, new O3:K6 strain 1137; B, new O3:K6 strain 1121; C, environmental strain 1025. ○, *P. fluorescens* CCRC10304; ●, *Vibrio parahaemolyticus* strain. Vertical bars represent the standard errors.

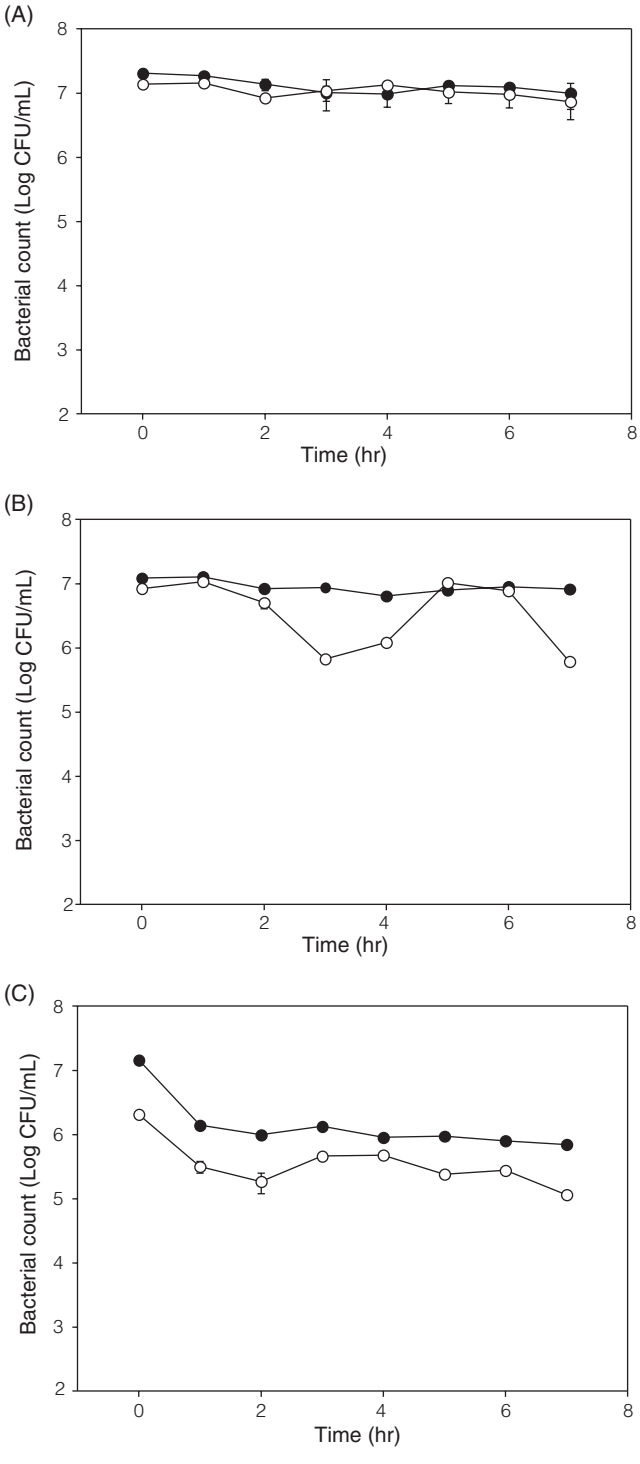

Figure 2. Growth of *Vibrio parahaemolyticus* and *Pseudomonas fluorescens* co-cultured in one-tenth strength of tryptic soy broth-NaCl medium at 4°C. Panel A, new O3:K6 strain 1137; B, new O3:K6 strain 1121; C, environmental strain 1025. ○, *P. fluorescens* CCRC10304; ●, *Vibrio parahaemolyticus* strain. Vertical bars represent the standard errors.

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cultures were incubated in 1/10 TSB-1.0% NaCl medium at 4 or 25˚C, *P. fluorescens* and all the *V. parahaemolyticus* strains showed similar change in bacterial counts (Fig. 1-4). Comparatively, population of both new O3:K6 strains remained nearly unchanged at 4 or 25˚C when co-culture with *P. fluorescens* in the diluted TSB medium (Fig. 1 A, 1B, 2A, 2B), whereas the environmental strain 1025 decreased by about one log CFU/mL at 4 or 25˚C (Fig. 1C, 2C). At 4˚C, *P. fluorescens* strain also decreased by about one log CFU/mL (Fig. 2C).

In the nonsterilized oyster medium, *V. parahaemolyticus* strains competed with indigenous bacteria which contained no *V. parahaemolyticus* as examined before the experiment. The population of indigenous vibrios in these nonsterilized oyster medium was below 100 CFU/mL and was negligible when compared to the inoculated *Vibrio* population (10^7-10^8 CFU/mL). When co-cultured with *V. parahaemolyticus* strains in this oyster medium, the indigenous bacteria proliferated slowly and reached maximum population in about 12 hr at 25˚C (Fig. 3) or in 12~16 hr at 4˚C (Fig. 4). Both of the new O3:K6 strains declined rapidly when cultured in this nonsterilized oyster medium, with population dropped to about 10^3-10^4 CFU/mL in 4 hr at 25˚C (Fig. 3A, 3B) and in 8 hr at 4˚C (Fig. 4A, 4B). On the other hand, the environmental strain 1025 proliferated in the first 4 hr and then decreased slowly to about 10^5-10^6 CFU/mL in 24 hr at both temperatures (Fig. 3C, 4C).

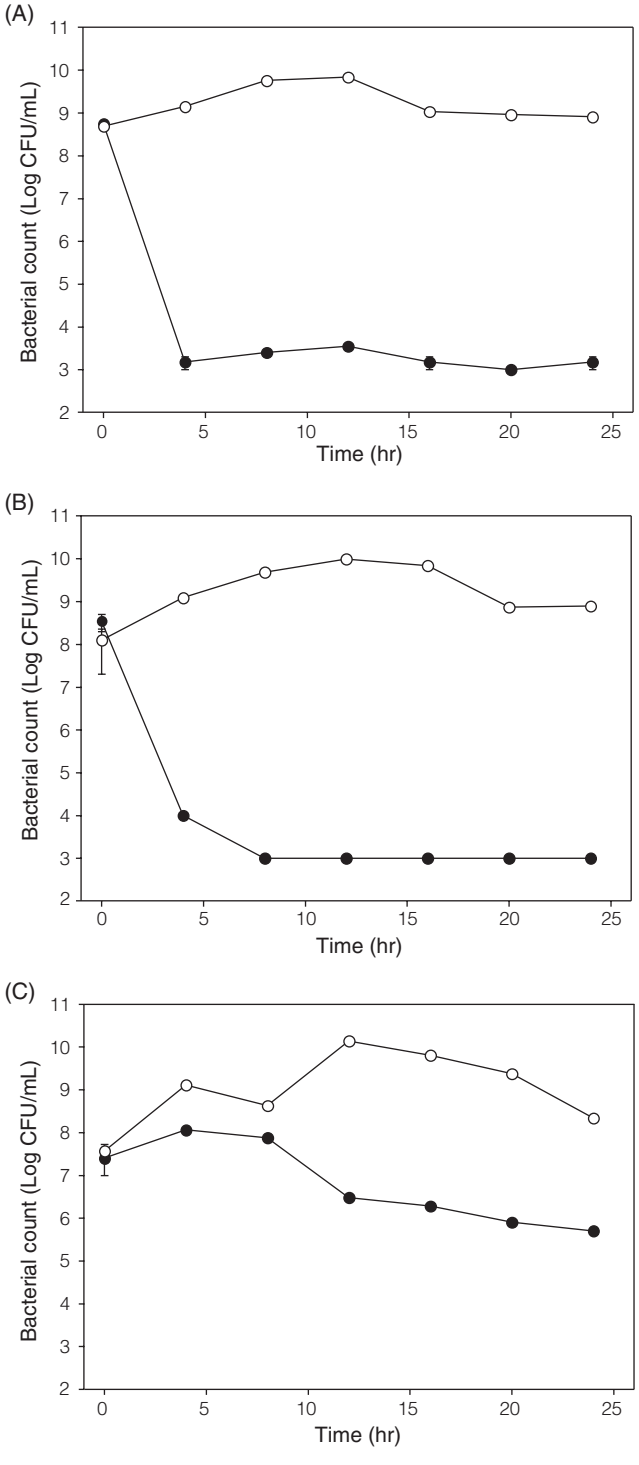

Figure 3. Growth of *Vibrio parahaemolyticus* and indigenous bacteria co-cultured in oyster medium at 25˚C. Panel A, new O3:K6 strain 1137; B, new O3:K6 strain 1121; C, environmental strain 1025. ○, indigenous bacteria; ●, *Vibrio parahaemolyticus* strain. Vertical bars represent the standard errors.

DISCUSSION

Some enteric pathogens tolerate acid, such as *Salmonella typhimurium*[13] and *Escherichia coli*[14]. Acid shock treatment normally enhances the tolerance of these pathogens to acid challenge, cross-protected against other stresses and also associated with the expression of some virulence factors[15,16]. *V. parahaemolyticus* is more vulnerable to acid stress than *Salmonella* and *E. coli*; however, sublethal mild acid treatment of the former induces acid tolerance and enhances pathogenicity[12]. Previously, the authors examined the acid susceptibility of these new O3:K6 strains in the stationary phase and found no significant difference from other environmental strains[5]. Normally, bacteria in the stationary phase will develop tolerance of a variety of stresses. This phenomenon differs from tolerance induced in the exponential phase[7,8]. This work demonstrated that the new O3:K6 group was significantly more tolerant of acid than the environmental group (Table 1). Such high acid tolerance enhances the survival of these new O3:K6 strains in a food-processing environment and also enables them to successfully pass the gastric acid challenge, enhancing initial viable population in the adherence and colonization of these strains in the intestine.

Acid tolerance of some pathogenic bacteria is attributed to the presence of urease which neutralizes acid in local environment. In the presence of urease, *Helicobacter pylori* successfully colonizes in high acid gastric environment[17]. In *V. parahaemolyticus*, urease is found only with another TDH-related hemolysin in some of the pathogenic but Kanagawa-negative strains without TDH[18,19]. The new O3:K6 strains are known to be urease-negative[6]. All the strains used in this research were also urease-negative.

The means how these new O3:K6 strains acquire this acid tolerance is unknown.

While exhibiting acid tolerance, the new O3:K6 strains may also outgrow other bacteria in competition, and thus successfully inhabit the natural environment and survive in the food-processing chain. When meat, milk and seafood are stored at low temperatures, psychrotrophic bacteria become the dominant microflora. *P. fluorescens* is a common spoilage microorganism in these foods[20]. *V. parahaemolyticus* is prevalent in various seafood[11,21], and around half of the environmental *V. parahaemolyticus* strains and other vibrios isolated from refrigerated seafood are cold adapters. Accordingly, *V. parahaemolyticus* and *P. fluorescens* are likely to co-inhabit a single niche and compete with each other, especially in refrigerated seafood.

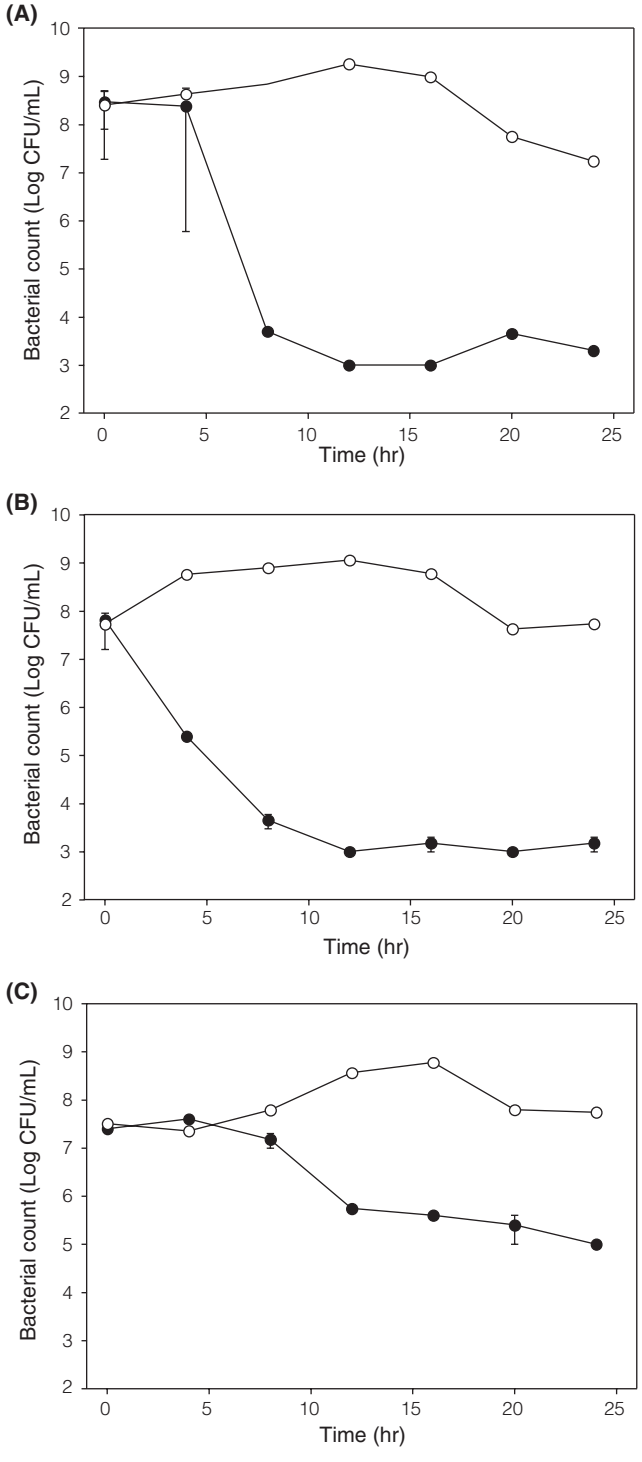

Figure 4. Growth of *Vibrio parahaemolyticus* and indigenous bacteria co-cultured in oyster medium at 4˚C. Panel A, new O3:K6 strain 1137; B, new O3:K6 strain 1121; C, environmental strain 1025. ○, indigenous bacteria; ●, *Vibrio parahaemolyticus* strain. Vertical bars represent the standard errors.

P. fluorescens and many other strains of *Pseudomonas* are known to inhibit the growth of several human and fish pathogens, including *Aeromonas sobria, Staphylococcus aureus* and *V. anguillarum*[22]. However, the competition among each bacterium in a co-culture system may alter when the growth condition changes. Pseudomonas spp. can inhibit, stimulate, or have no effect on *Listeria monocytogenes* as reported by different authors[23]. In brain heart infusion broth, *P. fluorescens* reduces the maximum population density reached by *L. monocytogenes* and is affected by salt and acidity in the medium[23]. Preincubation of *P. fluorescens* in milk at low temperature stimulates the growth of *L. monocytogenes* probably by hydrolyzing the milk proteins[24].

When cultured separately in TSB-NaCl at 25˚C, *P. fluorescens* CCRC10304 is a slow growing bacterium as compared to *V. parahaemolyticus*. *P. fluorescens* takes five more hours than *V. parahaemolyticus* to reach an absorbance of 1 at 600 nm. Also, *P. flurorescens* forms much smaller colonies than *V. parahaemolyticus* on TSA-NaCl after being incubated at 25˚C overnight (data not shown). However, *P. fluorescens* and *V. parahaemolyticus* strains showed similar survival ability in 1/10 TSB-NaCl medium incubated at 4 or 25˚C (Fig. 1, 2). Such low nutrient broth mimics normal marine environment for *V. parahaemolyticus*, thus the competition in this medium may represent their ability in the natural environment.

In nonsterilized oyster medium, the growth of *V. parahaemolyticus* was inhibited more sharply by indigenous bacteria except the environmental *V. parahaemolyticus* (Fig. 3, 4). Such inhibition is probably attributed to the presence of indigenous bacteria which exhaust the nutrition or growing space. Other inhibitory factors cannot be excluded. Since the oysters purchased from the market were fresh and contained a mixture of different bacterial species. The microflora of these oyster samples may change during the storage at 4˚C. Nevertheless, psychrotrophic *Pseudomonas* spp. may not have enough time to become the dominant species when the oyster is incubated at refrigerating temperature for only 24 hr in this study. The mixture of indigenous bacteria may also produce antagonistic metabolites against the vibrios[22]. The production of peroxides and other harmful free radicals by active metabolism of these mixture of indigenous bacteria may inhibit the proliferation of *V. parahaemolyticus*[25-27]. Protective agents, such as catalase and superoxide dismutase, are synthesized by *V. parahaemolyticus* and balance the inhibitory

effect of these harmful metabolites[28]. According to our unpublished study, a significant quantity of superoxide dismutase is present in the exponential phase cells of *V. parahaemolyticus*. Therefore, the growth of *V. parahaemolyticus* in raw oyster may also depend on the balance of protective agents and the harmful metabolites.

Strains of new O3:K6 isolated from different geographic regions or from different years show extremely high genetic homogeneity, as demonstrated by pulsed-field gel electrophoresis[5] or other molecular methods[4,6,29,30]. Survival ability was similar in both of the new O3:K6 strains 1121 and 1137 representing two major PFGE patterns I1 and I5 (Fig. 1-4).

The competitive ability of the new O3:K6 and the environmental strains in relation to the *P. fluorescens* or indigenous bacteria may be different depending on the culture conditions. The survival ability of environmental strain was slightly inferior as compared to the new O3:K6 strains in 1/10 TSB at 25°C (Fig. 1, 2), but much better than these new O3:K6 strains in the nonterilized oyster medium (Fig. 3, 4). Reason for this difference is unknown. These results demonstrated that in general the environmental strain of *V. parahaemolyticus* is probably a better survivor than the new O3:K6 strains in the natural environment. Therefore, survival competition in the natural environment is not likely to be the reason for the widespread of these pandemic strains.

In conclusion, the exponential phase cultures of the new O3:K6 strains of *V. parahaemolyticus* were more resistant to pH 3.0 than the environmental strains. The competition between the new O3:K6, environmental strains of *V. parahaemolyticus* and the *V. parahaemolyticus* and indigenous bacteria depends on bacteria strains, incubation medium and incubation temperature. The new O3:K6 strains declined faster than the environmental strain in the oyster medium in the presence of indigenous bacteria. These data provide some hints concerning the survival of the new O3:K6 strains and their ability to spread.

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Health of the Republic of China for financially supporting this research under Contract Nos. DOH89-TD-1053 and DOH90-TD-1025.

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