***Vibrio parahaemolyticus* Growth under Low-Iron Conditions and Survival under High-Magnesium Conditions**

CHIA-HSIN JU, P. S. MARIE YEUNG,[†] JESSICA OESTERLING,[‡] DANIEL A. SEIGERMAN,[§] AND KATHRYN J. BOOR*

Department of Food Science, Cornell University, Ithaca, New York 14853, USA

MS 05-449: Received 31 August 2005/Accepted 13 December 2005

ABSTRACT

Since 1996, *Vibrio parahaemolyticus* serotype O3:K6 and closely related strains have been associated with an increased incidence of *V. parahaemolyticus* gastroenteritis worldwide, suggesting the emergence of strains with enhanced abilities to cause disease. One hypothesis for the recent emergence of *V. parahaemolyticus* O3:K6 and related strains is an enhanced capacity for environmental survival relative to other strains, which might result in increased human exposure to these organisms. Therefore, the objective of this study was to test the hypothesis that survival or growth characteristics of clinical *V. parahaemolyticus* isolates differ from those of nonclinical isolates under different environmental conditions. Twenty-six *V. parahaemolyticus* isolates selected to represent either clinical or food sources were monitored for either survival following exposure to high magnesium (300 mM) or growth under iron-limited conditions. Isolates in each category (clinical or food) differed widely in survival capabilities following 24 h of exposure to 300 mM Mg^{2+} . Although 4 of 15 clinical isolates grew better at approximately 0.96 μM Fe^{2+} (iron-limited conditions) than at 50 μM Fe^{2+} (iron-rich conditions), as an entire group clinical isolates in this study were not more effective at growing under iron-limited conditions than were strains not associated with disease. Within the diverse collection of strains examined in these experiments, neither growth characteristics in low-iron environments nor survival capabilities following exposure to high magnesium concentrations were uniformly different between clinical and nonclinical *V. parahaemolyticus* isolates. Therefore, neither phenotypic characteristic can be used to reliably differentiate potentially pathogenic *V. parahaemolyticus* strains.

As a halophilic gram-negative inhabitant of warm coastal waters, *Vibrio parahaemolyticus* is commonly found in shellfish. Although not all strains of *V. parahaemolyticus* are pathogenic to humans, consumption of raw or improperly cooked seafood and shellfish contaminated with virulent strains of *V. parahaemolyticus* can cause acute gastroenteritis (6). Before 1996, *V. parahaemolyticus* outbreaks were generally sporadic and caused by diverse serotypes. However, since 1996, serotype O3:K6 and closely related strains, including some with serotypes O1:K25, O4:K68, and O1:KUT (untypeable), have been associated with an increased incidence of *V. parahaemolyticus* gastroenteritis worldwide (1, 5, 11, 27, 29). As a consequence, identification of factors contributing to seafood contamination by *V. parahaemolyticus*, and especially with O3:K6 and related strains, has become an important public health concern.

Iron and magnesium are important cofactors for bacterial growth and survival (7). In previous studies, greater adherence, increased hemolytic activity, and higher proliferation rates have been found for *V. parahaemolyticus*

grown under iron-limited conditions than under iron-adequate conditions (36). In the environment, free iron is not readily available because of its low solubility in the presence of oxygen and at neutral pH (7, 16). Within mammalian host cells, concentrations of free iron (approximately 10^{-18} M) are below that needed for optimal bacterial growth (16, 33) because of chelation by high-affinity iron-binding proteins in the cells (7, 8, 16, 31, 36). Because successful establishment of an infection within a host typically requires bacterial multiplication, the inability to obtain the iron needed for growth may limit bacterial virulence. Therefore, bacterial iron uptake mechanisms or other strategies for overcoming iron limitations can be important contributors to virulence (23). We hypothesized that *V. parahaemolyticus* strains associated with human illness might have enhanced growth capabilities at low iron concentrations relative to those of nonclinical *V. parahaemolyticus* strains; therefore, we examined growth characteristics of a diverse collection of *V. parahaemolyticus* isolates under conditions of limited iron availability.

Magnesium contributes to the structural stability of cells and is an essential cofactor for activation of a variety of enzymes (14). *V. parahaemolyticus* isolates from highly polluted coastal waters in India were found to be resistant to 300 mM Mg^{2+} , a concentration that is toxic to many other microorganisms (4). Thermally treated *V. parahaemolyticus* exhibited an enhanced capability to take up magnesium, indicating a possible increased requirement for Mg^{2+} for membrane and/or ribosome stability and repair

* Author for correspondence. Tel: 607-255-3111; Fax: 607-254-4868; E-mail: kjb4@cornell.edu.

[†] Present address: Department of Dairy Science, South Dakota State University, Brookings, SD 57007, USA.

[‡] Present address: Food Science Department, Purdue University, West Lafayette, IN 47907, USA.

[§] Present address: State University of New York, Upstate Medical University, Syracuse, NY 13210, USA.

TABLE 1. *Vibrio parahaemolyticus* isolates used in this study

Laboratory identification	FDA identification	Serotype	Source	Place of isolation	Year of isolation	Presence of <i>tdh</i>
FSL-Y1-003	T3980 ^a	O4:K13	Clinical	Japan	Unknown	+
FSL-Y1-010	T-3979 ^a	O5:K15	Clinical	Japan	Unknown	+
FSL-Y1-012	48432 ^a	O4:K12	Clinical	Washington	1991	—
FSL-Y1-013	47978 ^a	O6:K18	Clinical	Washington	1991	—
FSL-Y1-014	AN-5034 ^b	O4:K68 ^c	Clinical	Bangladesh	1996	+
FSL-Y1-015	AN-16000 ^b	O1:KUT ^{c,d}	Clinical	Bangladesh	1998	+
FSL-Y1-016	TX-2103 ^b	O3:K6	Clinical	Texas	1998	+
FSL-Y1-017	NY-3064 ^b	O3:K6	Clinical	New York	1998	+
FSL-Y1-021	U-5474 ^b	Old O3:K6 ^e	Clinical	Bangladesh	1980	+
FSL-Y1-022	AQ4037 ^b	Old O3:K6	Clinical	Traveler arriving in Japan from Maldives	1985	—
FSL-Y1-023	VP86 ^b	O3:K6	Clinical	Calcutta	1996	+
FSL-Y1-024	VP199 ^b	O3:K6	Clinical	Calcutta	1997	+
FSL-Y1-025	VP208 ^b	O3:K6	Clinical	Calcutta	1997	+
FSL-Y1-026	VP155 ^b	O3:K6	Clinical	Calcutta	1996	+
FSL-Y1-046	BAC-98-03255 ^b	O3:K6	Clinical	New York	Unknown	+
FSL-Y1-001	92000713 (1) ^a	O8	Food (clam)	Unknown	1992	—
FSL-Y1-002	NY477 ^a	O4:K8	Food (oyster)	New York	1997	+
FSL-Y1-004	CRAB ^a	Unknown	Food	Washington	1972	—
FSL-Y1-005	5C-1C ^a	O1	Food (oyster)	Washington	1988	—
FSL-Y1-006	M350A ^a	O5	Food (oyster)	Washington	1994	—
FSL-Y1-011	8332924 ^a	O1:K56	Food (oyster)	Gulf	1983	—
FSL-Y1-059	98-792-807 (27) ^b	O8:K74	Food (oyster)	Galveston Bay	1998	—
FSL-Y1-068	DI-A6-031699 ^b	O4:K9	Food (oyster)	Alabama	Unknown	+
FSL-Y1-069	DI-B11-031699 ^b	O4:K22	Food (oyster)	Alabama	Unknown	—
FSL-Y1-073	DI-F8-031699 ^b	O11:KUT	Food (oyster)	Alabama	Unknown	—
FSL-Y1-081	DI-A6-020800 ^b	O11:K61	Food (oyster)	Alabama	Unknown	—

^a Isolates provided by Charles Kaysner (formerly with the U.S. Food and Drug Administration, Bothell, Wash.).

^b Isolates provided by Angelo Depaola (U.S. Food and Drug Administration, Dauphin Island, Ala.).

^c Strains deemed genetically similar to O3:K6 strains by arbitrarily primed PCR, ribotype, and pulsed-field gel electrophoresis pattern analyses (12).

^d UT, untypeable.

^e Old O3:K6, O3:K6 strains isolated before 1996 that genetically differ from the O3:K6 strains isolated after 1996.

under these conditions (17). Because the ability to tolerate or possibly utilize high concentrations of magnesium may provide a competitive advantage for *V. parahaemolyticus* under some environmental conditions (e.g., in the presence of industrial pollutants), we hypothesized that clinical and nonclinical isolates may respond differently to high magnesium concentrations.

Previous observations of enhanced virulence-associated characteristics under low-iron conditions (36) and tolerance to high magnesium concentrations in *V. parahaemolyticus* isolated from a disease-pandemic region (4) led us to hypothesize that clinical and nonclinical *V. parahaemolyticus* isolates respond differentially and predictably to these conditions. The objective of this study was to test the hypothesis that phenotypes of clinical *V. parahaemolyticus* isolates, including those bearing the pandemic O3:K6 serotype, differ from those of nonclinical isolates under low-iron or high-magnesium conditions. Twenty-six *V. parahaemolyticus* isolates that had been selected from a large collection to represent either clinical or food sources were monitored for survival following exposure to high magnesium (300 mM) or for growth under iron-limited conditions.

MATERIALS AND METHODS

Bacterial isolates and preparation. The 26 isolates used in this study (Table 1) were provided by the U.S. Food and Drug Administration and included 15 clinical and 11 food isolates. The clinical isolates included several strains representing the recently emerged O3:K6 serotype and other closely related strains. Serotypes were determined previously by the Centers for Disease Control and Prevention, and the presence or absence of the virulence-associated *tdh* gene was determined previously (40). Additional descriptive information on the isolates used in this study can be accessed on line (www.pathogentracker.net). All isolates were streaked for isolation of single colonies on tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, Md.) supplemented with 2% NaCl (TSAS). Isolates were stored in tryptic soy broth supplemented with 2% NaCl (TSBS) and 20% glycerol at -80°C . Prior to each experiment, isolates from frozen stock were streaked on TSAS, incubated for 12 to 16 h, and then subcultured at least once in TSBS (37°C for 12 h at 250 rpm).

Growth media. For the iron study, M9 medium supplemented with casamino acids (M9GC) was prepared as described previously (2) to contain 11.28 g/liter M9 salt (which consists of 6.78 g/liter Na_2HPO_4 , 3 g/liter KH_2PO_4 , 0.5 g/liter NaCl, and 1

g/liter NH_4Cl (Difco, Becton Dickinson), 20 g/liter NaCl , 5 g/liter casamino acids, 2.5 g/liter glucose, 0.247 g/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.47 mg/liter CaCl_2 , and 1 mg/liter thiamine-HCl. All solutions were filter sterilized with 0.45- μm -pore-size Millipore membranes (Corning Incorporated, Corning, N.Y.). To remove residual iron, the M9GC medium was passed through Chelex-100 (Bio-Rad Laboratories, Hercules, CA) ion exchange resin (20, 21). Because Chelex-100 binds cations in addition to iron, 1 ml of 0.1 M MgSO_4 and 1 ml of 0.01 M CaCl_2 stock solutions were added (per liter of the medium) after the Chelex-100 treatment. The Chelex-treated medium, with an iron concentration of approximately 0.96 μM , as determined with an inductively coupled plasma atomic emission spectrometer (ICAP 61E thermal trace analyzer, Jarrel Ash Co., Franklin, Mass.) (22), was used for the iron-limited condition. Iron-rich medium was prepared by adding appropriate volumes of 0.1 M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, which had been prepared immediately prior to each experiment to minimize oxidation and precipitation, to the Chelex-treated M9GC medium. To determine optimal iron supplementation for the study, growth characteristics of seven *V. parahaemolyticus* isolates (FSL-Y1-004, FSL-Y1-005, FSL-Y1-011, FSL-Y1-012, FSL-Y1-014, FSL-Y1-016, and FSL-Y1-023), selected to represent the different *V. parahaemolyticus* subtypes and isolation sources (Table 1), were measured in the presence of six different iron concentrations (1, 50, 100, 150, 200, and 250 μM FeSO_4) in the Chelex-treated M9GC medium. FeSO_4 at 50 μM provided maximal growth yields as determined by optical density measurements at 600 nm (OD_{600}) for the test isolates and thus was selected as the optimal concentration for the iron-rich growth conditions. To prepare inocula for the growth assays, an overnight culture was prepared for each isolate (5 ml in TSBS at 37°C for 12 h at 250 rpm). A 1-ml sample was removed from each tube, and cells were harvested by centrifugation (3 min at $10,000 \times g$). Cell pellets were resuspended in 1 ml of phosphate-buffered saline (PBS), and the resulting suspensions were used to inoculate the iron-limited or iron-rich media (1% inoculum, 1.5 μl per 150 μl) in microtiter plate wells (Corning). Growth kinetics for each isolate were measured by monitoring the OD_{600} in the Fusion Universal microplate analyzer (Packard Instrument Co., Meriden, Conn.) at 37°C. Readings were taken every 15 min for approximately 8 h. Microtiter plates were subjected to orbital shaking during incubation. At least three independent experiments were conducted on each isolate. Plots of OD_{600} versus time were generated for each isolate, and the data were exported to Microsoft Excel (Microsoft Corporation, Redmond, Wash.) for further analyses.

Magnesium resistance assays. For the magnesium studies, procedures were similar to those described for the iron studies except that the M9 medium was prepared without MgSO_4 , and various concentrations of MgCl_2 were added prior to use. Conditions for the magnesium resistance trials were determined by testing the same seven isolates used for the iron trials for growth with various magnesium concentrations (0, 1, 50, 100, and 300 mM). Of the concentrations tested, 1 mM MgCl_2 yielded maximal growth and both 0 mM and 300 mM inhibited growth (data not shown). Therefore, to determine the effects of high Mg^{2+} concentrations on bacterial survival, 300 mM Mg^{2+} was selected as the magnesium challenge condition for all 26 isolates. Medium with 1 mM Mg^{2+} was used as a control condition that would enable growth. Prior to the magnesium challenge, *V. parahaemolyticus* cultures were grown in TSBS at 37°C with shaking at 250 rpm for 12 h, 10 ml of each culture was centrifuged, and cell pellets were resuspended in 10 ml of M9 supplemented with 300 mM Mg^{2+} . Control cultures for all isolates were prepared in parallel

by resuspending cell pellets in M9 supplemented with 1 mM Mg^{2+} . The isolates were incubated at 37°C with constant shaking at 250 rpm for 0, 24, 48, 72, and 96 h. At each time point, 100- μl samples were obtained from each tube and serially diluted in PBS, and 100 μl of the appropriate dilutions were spread plated onto duplicate TSAS plates. The plates were incubated at 37°C for 16 to 18 h, and the bacterial concentrations (CFU per milliliter) were determined. Only data from plates bearing 30 to 300 CFU were used in the calculations. Percent survival for each sample was based on the following formula: survival (%) = $100 \times (\text{CFU/ml after } x \text{ h in } 300 \text{ mM } \text{Mg}^{2+} \text{ challenge} / \text{CFU/ml at } 0 \text{ h in } 300 \text{ mM } \text{Mg}^{2+})$.

Neither the low-iron nor high-magnesium concentrations evaluated in this study reflect conditions likely to be encountered within human cells or in the ocean, respectively. The concentration of iron in the human gastrointestinal tract varies with diet and interval since the last meal. The magnesium concentration in seawater varies with location, but at 35 ppt salinity (full strength seawater) it is typically 53 mM at 25°C. The preliminary studies described in this section were specifically designed to identify iron and magnesium concentrations that affect *V. parahaemolyticus* growth and survival, respectively, and might allow phenotypic differentiation of clinical and nonclinical *V. parahaemolyticus* isolates.

Statistical analyses. Growth kinetics in the iron-limited medium were compared with those in the iron-rich medium for all isolates with the Proc Genmod model (Statistical Analysis Systems software version 8.2, SAS Institute, Inc., Cary, N.C.). Survival results among the isolates from the magnesium resistance assay were compared with the two-sample *t* test to evaluate the significance of differences between groups of *V. parahaemolyticus* strains (Minitab version 14.0, Minitab, Inc., State College, Pa.). For all analyses, differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The objective of this study was to determine the growth and survival responses of multiple *V. parahaemolyticus* isolates when confronted with low-iron or high-magnesium conditions, respectively, and specifically to compare the responses between clinical and nonclinical isolates.

Effects of limited iron on growth. Bacteria require micromolar concentrations of bioavailable iron for optimal growth (16, 33). To enable growth in the human body, bacteria must compete for iron with mammalian iron-binding proteins such as transferrin and lactoferrin (7, 8, 16, 31, 36). We hypothesized that *V. parahaemolyticus* strains associated with human infections (clinical isolates) would be better able to grow under low-iron conditions than would isolates obtained from nonclinical sources (i.e., food isolates).

The OD_{600} values for *V. parahaemolyticus* cultures grown to maximal populations (early stationary phase, 4 h postinoculation) in iron-limited medium were compared with those generated in iron-rich medium, with OD_{600} at 4 h postinoculation as the dependent variable, iron as the independent variable, and strain as the repeated measure. The mean OD_{600} values in iron-rich and iron-limited media were 0.66 and 0.45, respectively. The OD_{600} at 4 h postinoculation for the 26 strains ranged from 0.26 to 0.92 in

TABLE 2. OD_{600} values for *V. parahaemolyticus* at 4 h postinoculation into iron-limited or iron-rich media

Isolate identification	OD_{600}^a		
	Iron limited	Iron rich	Difference
Clinical isolates			
FSL-Y1-003	0.39 ± 0.04	0.66 ± 0.07	0.27 ± 0.04
FSL-Y1-010	0.20 ± 0.10	0.37 ± 0.06	0.17 ± 0.10
FSL-Y1-012	0.34 ± 0.06	0.69 ± 0.08	0.35 ± 0.13
FSL-Y1-013	0.47 ± 0.17	0.49 ± 0.31	0.02 ± 0.39
FSL-Y1-014	0.37 ± 0.12	0.90 ± 0.12	0.54 ± 0.08
FSL-Y1-015	0.50 ± 0.08	0.35 ± 0.17	-0.15 ± 0.10
FSL-Y1-016	0.39 ± 0.11	0.85 ± 0.18	0.46 ± 0.11
FSL-Y1-017	0.43 ± 0.06	0.85 ± 0.10	0.42 ± 0.05
FSL-Y1-021	0.48 ± 0.03	0.34 ± 0.17	-0.14 ± 0.16
FSL-Y1-022	0.38 ± 0.16	0.46 ± 0.28	0.08 ± 0.27
FSL-Y1-023	0.31 ± 0.09	0.86 ± 0.06	0.54 ± 0.11
FSL-Y1-024	0.51 ± 0.05	0.26 ± 0.08	-0.25 ± 0.06
FSL-Y1-025	0.43 ± 0.06	0.73 ± 0.01	0.30 ± 0.16
FSL-Y1-026	0.66 ± 0.09	0.57 ± 0.13	-0.09 ± 0.09
FSL-Y1-046	0.49 ± 0.18	0.66 ± 0.22	0.17 ± 0.28
Food isolates			
FSL-Y1-001	0.43 ± 0.15	0.71 ± 0.15	0.28 ± 0.25
FSL-Y1-002	0.59 ± 0.09	0.56 ± 0.25	-0.03 ± 0.33
FSL-Y1-004	0.31 ± 0.07	0.72 ± 0.06	0.40 ± 0.07
FSL-Y1-005	0.38 ± 0.14	0.78 ± 0.24	0.40 ± 0.36
FSL-Y1-006	0.47 ± 0.09	0.71 ± 0.19	0.24 ± 0.21
FSL-Y1-011	0.37 ± 0.07	0.92 ± 0.21	0.55 ± 0.27
FSL-Y1-059	0.51 ± 0.17	0.74 ± 0.14	0.22 ± 0.31
FSL-Y1-068	0.52 ± 0.15	0.83 ± 0.14	0.31 ± 0.27
FSL-Y1-069	0.59 ± 0.06	0.58 ± 0.02	-0.01 ± 0.04
FSL-Y1-073	0.53 ± 0.15	0.84 ± 0.10	0.31 ± 0.22
FSL-Y1-081	0.59 ± 0.04	0.64 ± 0.05	0.05 ± 0.09

^a Values represent mean ± standard error from at least three independent trials in which triplicate samples were simultaneously tested.

the iron-rich medium and from 0.20 to 0.66 in the iron-limited medium. The significant difference ($P < 0.0001$) in OD_{600} between the iron-rich (50 μM Fe^{2+}) and the iron-limited (approximately 0.96 μM Fe^{2+}) media indicates that the Chelex-100 treatment of M9GC generated a physiologically relevant difference in the iron concentration in this medium. Differences in OD_{600} between cultures grown in iron-rich and those grown in iron-limited media are summarized in Table 2. Nineteen *V. parahaemolyticus* isolates had higher OD_{600} readings in the iron-rich medium than in the iron-limited medium. Four clinical isolates, i.e., two O3:K6 isolates (FSL-Y1-024 and FSL-Y1-026), one "old" O3:K6 isolate (FSL-Y1-021, an O3:K6 strain isolated before 1996 that differs genetically from O3:K6 strains isolated after 1996 (29)), and O1:KUT (FSL-Y1-015, which is genetically similar to post-1996 O3:K6 strains (12)) consistently had significantly higher OD_{600} readings in the iron-limited than in the iron-rich medium. These results indicate that the optimal iron concentration for growth of these four isolates is lower than that required by the remaining strains, which supports the hypothesis that some clinical isolates

can grow effectively under iron-limited conditions. Two food isolates (FSL-Y1-002 and FSL-Y1-069) and one clinical strain (FSL-Y1-013) grew similarly under both iron-rich and iron-limited conditions. Overall, however, our analyses indicate that isolates obtained from clinical sources are not universally more effective at growing under iron-limited conditions than are nonclinical isolates.

During investigations of *V. parahaemolyticus* outbreaks, relevant isolates are typically serotyped and screened for the presence of hemolysin-producing genes, such as the *tdh* (thermostable direct hemolysin) and the *trh* (thermostable direct hemolysin-related hemolysin) gene, which are considered to be important virulence factors in *V. parahaemolyticus*-induced gastroenteritis (18, 19). Therefore, we also conducted statistical analyses on isolate OD_{600} values after categorizing isolates by serotype (i.e., O3:K6 versus non-O3:K6) or by the presence of the *tdh* gene. No significant differences in OD_{600} values were observed between these groups under iron-rich or iron-limiting conditions.

Some microorganisms, including *Vibrio cholerae* (35) and *V. parahaemolyticus* (38, 39), have evolved specialized systems for overcoming iron restrictions in host organisms that enable multiplication to sufficient numbers to cause infections (8, 16), whereas others have evolved to require minimal iron. For example, the bacterial pathogen *Borrelia burgdorferi* is believed to require little iron for growth because very few of its proteins appear to require iron as a cofactor (32). *Blastomyces dermatitidis* can proliferate for short periods in the absence of ferric iron (15). At least two mechanisms for iron acquisition have been identified in *V. parahaemolyticus*: (i) acquisition of iron directly from host iron sources, including hemin and hemoglobin (30, 37), and (ii) synthesis of siderophores, which strongly bind Fe^{3+} , enabling delivery of iron to the microbe via high-affinity siderophore uptake systems (28). Some bacterial siderophores also contribute to virulence. For example, Yamamoto et al. (38, 39) reported that vibrioferrin, a siderophore synthesized by *V. parahaemolyticus*, was present at significantly higher concentrations in clinical isolates than in environmental isolates, suggesting that the production of more vibrioferrin by clinical isolates may confer a selective advantage for microbial survival and proliferation under conditions of iron limitation, such as those encountered in the human host. In the present study, although the majority of *V. parahaemolyticus* isolates grew optimally in the presence of approximately 50 μM Fe^{2+} , four clinical isolates had higher OD_{600} values under our iron-limited conditions (approximately 0.96 μM) and three isolates had similar growth regardless of iron concentration. These observations suggest that iron requirements for individual *V. parahaemolyticus* strains vary considerably and that some strains may need relatively little iron for optimal growth. Further studies of specific mechanisms used by the four clinical isolates to overcome low iron availability could contribute to our understanding of *V. parahaemolyticus* pathogenesis.

Survival of *V. parahaemolyticus* under magnesium challenge. In our preliminary experiments, we initially as-

FIGURE 1. Mean percent survival of food (a) and clinical (b) *V. parahaemolyticus* isolates after 24 h of magnesium challenge (300 mM Mg^{2+}) from two independent experiments performed in duplicate. Percent survival for each sample was based on the following formula: survival (%) = $100 \times (\text{CFU/ml after 24 h of } 300 \text{ mM } Mg^{2+} \text{ challenge} / \text{CFU/ml after 0 h in } 300 \text{ mM } Mg^{2+})$.

essed the growth of *V. parahaemolyticus* in media with various magnesium concentrations. The tested *V. parahaemolyticus* strains did not reproduce at 300 mM Mg^{2+} , but 1 mM allowed optimal growth. OD_{600} values were lower in media with 50 and 100 mM Mg^{2+} than in media with 1 mM Mg^{2+} . Mean percent survival of *V. parahaemolyticus* isolates challenged for 24 h with 300 mM Mg^{2+} are summarized and illustrated in Figure 1. Three nonclinical isolates (FSL-Y1-002, FSL-Y1-059, and FSL-Y1-081) and five clinical isolates (FSL-Y1-014, FSL-Y1-016, FSL-Y1-017, FSL-Y1-026, and FSL-Y1-046) maintained >50% survival after 24 h of exposure to 300 mM Mg^{2+} . Data analyses revealed that the group of clinical isolates did not differ from the group of nonclinical (food) isolates in resistance to a high magnesium concentrations after 24 h of exposure ($P = 0.52$). No viable cells of food isolates or clinical isolates were detected after 72 or 96 h, respectively, in medium with 300 mM Mg^{2+} .

Magnesium is an essential divalent cation that functions as a cofactor for numerous proteins and as a regulatory and stabilizing factor for membranes, ribosomes, cellular structures, and pH balance (13, 34). Although magnesium is essential for the growth of many marine bacteria (24, 25), high concentrations of magnesium can be toxic to many microorganisms (9, 14). Bhattacharya et al. (4) cultured *V. parahaemolyticus* from highly polluted seawater in India and found that the isolates were unusually resistant to high magnesium ion concentrations. In these isolates,

resistance to Mg^{2+} (300 mM) was associated with the presence of a 5.5-kb plasmid (4). In another study, metal resistance characteristics were compared for environmental and clinical *V. parahaemolyticus* isolates (10). *V. parahaemolyticus* strains isolated from marine and freshwater fish from the coastal area near Calcutta, India, were resistant to arsenate, but most clinical isolates were susceptible to this compound, suggesting that a resistant phenotype might be a possible marker for differentiating environmental and clinical isolates (10). *V. parahaemolyticus* appears able to acquire resistance to potentially harmful compounds in the presence of high concentrations of heavy metals in natural water ecosystems (3). Therefore, *V. parahaemolyticus* isolates that are exposed to highly polluted seawater may develop tolerance to various pollutants, including magnesium.

Although a subset of four clinical isolates had higher OD_{600} values under the iron-limited conditions than under the iron-rich conditions used in this study, in general *V. parahaemolyticus* clinical isolates are not universally better able to grow under low-iron conditions than are nonclinical isolates. Eight *V. parahaemolyticus* strains, including three food isolates and five clinical isolates, had higher survival rates following 24 h of exposure to 300 mM Mg^{2+} than did the remaining 18 strains. Wide and overlapping ranges in survival capabilities under high-magnesium conditions and in OD_{600} values under iron-limited conditions were observed among strains grouped by isolation source (clinical or food). Therefore, specific responses to iron limitation or to the presence of 300 mM Mg^{2+} did not produce unique phenotypic characteristics that would allow differentiation between clinical and nonclinical isolates. The recent availability of the complete genome sequences for *V. parahaemolyticus* (26) will facilitate application of techniques such as microarrays to provide more insight into the complex phenomena of bacterial responses to exposure to low-iron or high-magnesium conditions.

ACKNOWLEDGMENTS

We are indebted to Angelo DePaola and Charles Kaysner (U.S. Food and Drug Administration) for providing the *V. parahaemolyticus* isolates used in this study. We thank Vincent Yeung for analyzing the iron contents in the various media. This work was supported by the Cooperative State Research, Education, and Extension Service, National Research Initiative Competitive Grants Program (NRI proposal 2001-35201-10951) of the U.S. Department of Agriculture.

REFERENCES

- Abbott, S. L., C. Powers, C. A. Kaysner, Y. Takeda, M. Ishibashi, S. W. Joseph, and J. M. Janda. 1989. Emergence of a restricted bioserovar of *Vibrio parahaemolyticus* as the predominant cause of *Vibrio*-associated gastroenteritis on the west coast of the United States and Mexico. *J. Clin. Microbiol.* 27:2891–2893.
- Atlas, R. M., and L. C. Parks. 1993. Handbook of microbiological media, p. 529–530. CRC Press, Boca Raton, Fla.
- Bhattacharya, M., P. Choudhury, and R. Kumar. 2000. Antibiotic- and metal-resistant strains of *Vibrio parahaemolyticus* isolated from shrimps. *Microb. Drug Resist.* 6:171–172.
- Bhattacharya, M., S. S. Roy, D. Biswas, and R. Kumar. 2000. Effect of Mg^{2+} ion in protein secretion by magnesium-resistant strains of *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* isolated from the coastal water of Haldia port. *FEMS Microbiol. Lett.* 185: 151–156.

5. Bhuiyan, N. A., M. Ansaruzzaman, M. Kamruzzaman, K. Alam, N. R. Chowdhury, M. Nishibuchi, S. M. Faruque, D. A. Sack, Y. Takeda, and G. B. Nair. 2002. Prevalence of the pandemic genotype of *Vibrio parahaemolyticus* in Dhaka, Bangladesh, and significance of its distribution across different serotypes. *J. Clin. Microbiol.* 40: 284–286.
6. Blake, P. A., R. E. Weaver, and D. G. Hollis. 1980. Diseases of humans (other than cholera) caused by vibrios. *Annu. Rev. Microbiol.* 34:341–367.
7. Braun, V., and H. Killmann. 1999. Bacterial solutions to the iron-supply problem. *Trends Biochem. Sci.* 24:104–109.
8. Brown, J. S., and D. W. Holden. 2002. Iron acquisition by gram-positive bacterial pathogens. *Microbes Infect.* 4:1149–1156.
9. Chamnongpol, S., and E. A. Groisman. 2002. Mg²⁺ homeostasis and avoidance of metal toxicity. *Mol. Microbiol.* 44:561–571.
10. Chauhan, B. S., N. M. Shaikh, S. P. De, S. Sil, P. K. Ganguly, and R. Kumar. 1997. Arsenate resistance as a possible marker in the differentiation of environmental and clinical isolates of *Vibrio parahaemolyticus*. *Zentralbl. Bakteriologie* 285:486–490.
11. Chiou, C. S., S. Y. Hsu, S. I. Chiu, T. K. Wang, and C. S. Chao. 2000. *Vibrio parahaemolyticus* serovar O3:K6 as cause of unusually high incidence of food-borne disease outbreaks in Taiwan from 1996 to 1999. *J. Clin. Microbiol.* 38:4621–4625.
12. Chowdhury, N. R., S. Chakraborty, T. Ramamurthy, M. Nishibuchi, S. Yamasaki, Y. Takeda, and G. B. Nair. 2000. Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. *Emerg. Infect. Dis.* 6:631–636.
13. Dulka, J. J., and T. H. Risby. 1976. Ultratrace metals in some environmental and biological systems. *Anal. Chem.* 48:640A–653A.
14. Flatman, P. W. 1991. Mechanisms of magnesium transport. *Annu. Rev. Physiol.* 53:259–271.
15. Giles, S. S., and C. J. Czuprynski. 2004. Extracellular calcium and magnesium, but not iron, are needed for optimal growth of *Blastomyces dermatitidis* yeast from cells in vitro. *Clin. Diagn. Lab. Immunol.* 11:426–429.
16. Guerinot, M. L. 1994. Microbial iron transport. *Annu. Rev. Microbiol.* 48:743–772.
17. Heinis, J. J., L. R. Beuchat, and F. C. Boswell. 1978. Antimetabolite sensitivity and magnesium uptake by thermally stressed *Vibrio parahaemolyticus*. *Appl. Environ. Microbiol.* 35:1035–1040.
18. Honda, T., M. A. Abad-Lapebla, Y. X. Ni, K. Yamamoto, and T. Miwatani. 1991. Characterization of a new thermostable direct haemolysin produced by a Kanagawa-phenomenon-negative clinical isolate of *Vibrio parahaemolyticus*. *J. Gen. Microbiol.* 137(2):253–259.
19. Honda, T., Y. X. Ni, and T. Miwatani. 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. *Infect. Immun.* 56:961–965.
20. Imbert, M., and R. Blondeau. 1998. On the iron requirement of lactobacilli grown in chemically defined medium. *Curr. Microbiol.* 37: 64–66.
21. Kadurugamuwa, J. L., H. Anwar, M. R. Brown, G. H. Shand, and K. H. Ward. 1987. Media for study of growth kinetics and envelope properties of iron-deprived bacteria. *J. Clin. Microbiol.* 25:849–855.
22. Kosse, J. S., A. C. Yeung, A. I. Gil, and D. D. Miller. 2000. A rapid method for iron determination in fortified foods. *Food Chem.* 75: 371–376.
23. Litwin, C. M., and S. B. Calderwood. 1993. Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev.* 6:137–149.
24. Macleod, R. A., and E. Onofrey. 1956. Nutrition and metabolism of marine bacteria. II. Observations on the relation of sea water to the growth of marine bacteria. *J. Bacteriol.* 71:661–667.
25. Macleod, R. A., E. Onofrey, and M. E. Norris. 1954. Nutrition and metabolism of marine bacteria. I. Survey of nutritional requirements. *J. Bacteriol.* 68:680–686.
26. Makino, K., K. Oshima, K. Kurokawa, K. Yokoyama, T. Uda, K. Tagomori, Y. Iijima, M. Najima, M. Nakano, A. Yamashita, Y. Kubota, S. Kimura, T. Yasunaga, T. Honda, H. Shinagawa, M. Hattori, and T. Iida. 2003. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. *Lancet* 361: 743–749.
27. Matsumoto, C., J. Okuda, M. Ishibashi, M. Iwanaga, P. Garg, T. Rammamurthy, H. C. Wong, A. Depaola, Y. B. Kim, M. J. Albert, and M. Nishibuchi. 2000. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and toxRS sequence analyses. *J. Clin. Microbiol.* 38:578–585.
28. Neilands, J. B. 1995. Siderophores: structure and function of microbial iron transport compounds. *J. Biol. Chem.* 270:26723–26726.
29. Okuda, J., M. Ishibashi, E. Hayakawa, T. Nishino, Y. Takeda, A. K. Mukhopadhyay, S. Garg, S. K. Bhattacharya, G. B. Nair, and M. Nishibuchi. 1997. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. *J. Clin. Microbiol.* 35:3150–3155.
30. O'Malley, S. M., S. L. Mouton, D. A. Occhino, M. T. Deanda, J. R. Rashidi, K. L. Fuson, C. E. Rashidi, M. Y. Mora, S. M. Payne, and D. P. Henderson. 1999. Comparison of the heme iron utilization systems of pathogenic vibrios. *J. Bacteriol.* 181:3594–3598.
31. Payne, S. M., and R. A. Finkelstein. 1978. The critical role of iron in host-bacterial interactions. *J. Clin. Invest.* 61:1428–1440.
32. Posey, J. E., and F. C. Gherardini. 2000. Lack of a role for iron in the Lyme disease pathogen. *Science* 288:1651–1653.
33. Ratledge, C., and L. G. Dover. 2000. Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* 54:881–941.
34. Scherrer, R., and P. Gerhardt. 1973. Influence of magnesium ions on porosity of the *Bacillus megaterium* cell wall and membrane. *J. Bacteriol.* 114:888–890.
35. Sigel, S. P., and S. M. Payne. 1982. Effect of iron limitation on growth, siderophore production, and expression of outer membrane proteins of *Vibrio cholerae*. *J. Bacteriol.* 150:148–155.
36. Wong, H. C., C. C. Liu, C. M. Yu, and Y. S. Lee. 1996. Utilization of iron sources and its possible roles in the pathogenesis of *Vibrio parahaemolyticus*. *Microbiol. Immunol.* 40:791–798.
37. Yamamoto, S., Y. Hara, K. Tomochika, and S. Shinoda. 1995. Utilization of heme and hemoglobin as iron sources by *Vibrio parahaemolyticus* and identification of an iron-repressible heme-binding protein. *FEMS Microbiol. Lett.* 128:195–200.
38. Yamamoto, S., N. Okujo, S. Matsuura, I. Fujiwara, Y. Fujita, and S. Shinoda. 1994. Siderophore-mediated utilization of iron bound to transferrin by *Vibrio parahaemolyticus*. *Microbiol. Immunol.* 38: 687–693.
39. Yamamoto, S., N. Okujo, T. Yoshida, S. Matsuura, and S. Shinoda. 1994. Structure and iron transport activity of vibrioferrin, a new siderophore of *Vibrio parahaemolyticus*. *J. Biochem. (Tokyo)* 115: 868–874.
40. Yeung, P. S., M. C. Hayes, A. DePaola, C. A. Kaysner, L. Kornstein, and K. J. Boor. 2002. Comparative phenotypic, molecular, and virulence characterization of *Vibrio parahaemolyticus* O3:K6 isolates. *Appl. Environ. Microbiol.* 68:2901–2909.