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Genes Responsible for Size Reduction of Marine Vibrios during Starvation Are Located on the Chromosome

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In a survey of 21 marine *Vibrio* spp., all responded to nutrient deprivation by undergoing a reduction in size (dwarfing). However, only 43% of these strains possessed one or more plasmids, suggesting that the genes responsible for dwarfing were located on the chromosome rather than on the plasmids. This conclusion was confirmed by the observation that fragmentation and subsequent size reduction occurred in three strains from which the plasmids had been removed by curing. The cured strains lost certain characteristics, such as resistance to some heavy metals and antibiotics, that were restored when the plasmids were reintroduced by either transformation or electroporation.

A wide variety of microorganisms, most of which can be classified by metabolic and growth parameters as either oligotrophic or copiotrophic, exists within the marine habitat. Oligotrophic bacteria are capable of growing and multiplying in conditions of very low nutrient flux (13) because they accumulate nutrients that are widely dispersed in time (11) and utilize them for growth and proliferation. In contrast to oligotrophs, copiotrophic bacteria require high nutrient concentrations for growth (27). Copiotrophs appear to have developed specialized adaptations for long-term survival in oligotrophic habitats. Thus, some marine bacteria undergo size reduction, or "dwarfing," in response to nutrient deprivation (for examples, see references 2, 7, 10, 23–25, 30), a process suggested as a strategy for long-term survival during periods of nutrient stress (7, 24). Dwarf bacteria possess an ability to respond quickly by growing when more ideal conditions arise (18).

One of the most significant differences between dwarf and growing bacteria lies in their protein compositions. Although the total protein content per cell decreased considerably after only a few days of nutrient deprivation (2, 14, 16), the concentration of individual proteins was affected in different ways. Thus, a number of proteins that were present in growing cells disappeared during starvation, and new proteins, not present in actively growing cells, were synthesized (1, 16, 26). These findings suggest that some marine bacteria respond to starvation by expressing genes whose products are required for differentiation to a dwarf state. The so-called starvation proteins may be likened to the heat shock, SOS, and oxidation stress proteins in other systems, such as those of *Escherichia coli* and *Bacillus subtilis* (3, 9, 29, 32). It has been proposed that starvation proteins provide protection to the cell during long periods of nutrient deprivation. It has been reported elsewhere that the survival of a mutant strain of *Vibrio fluvialis*, unable to respond to starvation by size reduction, was decreased when compared with that of the parent strain (29a). One of the starvation-specific genes was presumably mutated, thus blocking the normal response. The question arose as to the location of the genes responsible for the starvation response. In the present investigation, we studied the possibility that genes responsible for dwarfing of marine bacteria are located on plasmids.

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MATERIALS AND METHODS

Bacterial strains. *V. fluvialis* NCTC 11328 was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London. *Vibrio* spp. AS1 through AS20 were isolated from Coogee Bay, New South Wales, Australia. Ten liters of seawater was collected at Coogee Bay in the late summer (water temperature, 21°C). Glass slides treated with 1% (wt/vol) water-soluble silicon (Siliclad, Clay Adams) were suspended in seawater for 30 min and then rinsed three times in modified Morita salts solution (MMS) (7) to remove any nonadherent or weakly adhering organisms. The slides were then smeared across the surface of MMS plates containing 2% (wt/vol) agar and 2 mM glucose. To identify those bacteria that were *Vibrio* spp., the 500 organisms isolated were tested for oxidase reaction, growth in medium A (6) containing 0.4 M sodium chloride and 20 mM mannitol, and sensitivity to the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropylpteridine phosphate; Sigma Chemical Co.) by placing disks containing 10 and 150 μg of this substance dissolved in dioxane on a lawn plate of each organism (4, 8). Isolates were presumed to be *Vibrio* spp. if they grew on mannitol as the sole source of carbon, were oxidase positive, and were sensitive to the agent 0/129.

Culture conditions. For most experiments, bacteria were grown in nutrient Morita (NM) broth (15) in shake flasks at 30°C. For the determination of bacterial growth rates and resistance to either antibiotics or heavy metals, bacteria were grown in medium A (6) supplemented with 0.4 M NaCl, 20 mM glucose, and 0.1% (wt/vol) Casamino acids (Difco Laboratories) (ANaGC).

Preparation of starved cells, measurement of bacterial size, and viable counts. Bacteria were grown to mid-exponential phase in NM broth and divided into 2 portions. Cells from one portion were used immediately for size measurements by using a Coulter Counter (Coulter Electronics, Inc.) (30). Cells from the other portion were starved of nutrients for 24 h, as described previously (30), prior to size determination. Viable counts were determined by plating serially diluted cultures onto nutrient agar containing 3% (wt/vol) sodium chloride.

Plasmid extraction and electrophoresis. For plasmid extraction and electrophoresis, the method of Kado and Liu (17)

was modified as follows. A 10-ml volume of a mid-exponential-phase culture was harvested, and the cell pellet was suspended in 0.7 ml of 10 mM Tris hydrochloride buffer (pH 8.0) with 1 mM EDTA (TE buffer). A 1.5-ml volume of lysing solution (100 mM Tris [pH 12.5] containing 3% [wt/vol] sodium dodecyl sulfate) was added, and the mixture was maintained at 65°C for between 20 and 60 min (depending on the strain used) and then emulsified after the addition of 0.5 ml of 1.0 M Tris hydrochloride (pH 7.0) and 5 ml of phenol-chloroform (1:1). Precipitated protein was removed by centrifugation at 6,800 × g for 20 min. The aqueous layer was removed, and sodium chloride was added to a final concentration of 3% (wt/vol). The DNA was precipitated with ethanol and collected by centrifugation at 6,800 × g for 20 min, and the pellet was dried in a vacuum and subsequently suspended in 14 μl of TE buffer. A 3-μl volume of RNase (1 mg/ml) was added, and the suspension was incubated at 37°C for 25 min after which 3 μl of a tracking dye (50 mM Tris-acetate [pH 7.8] with 15% [wt/vol] Ficoll 400, 0.25% [wt/vol] bromophenol blue, and 0.25% [wt/vol] xylene cyanole ZZ) was added. Electrophoresis was at 100 V (3.6 V/cm) for 4 h in a 0.6% (wt/vol) Seakem ME agarose gel (FMC Corp.) in TAE buffer (0.04 M Tris-acetate [pH 8.0] containing 1 mM EDTA). Plasmid sizes were determined by running *E. coli* V 517 plasmid standards (20) alongside *Vibrio* spp. plasmids. The gel was stained for 30 min in TE buffer containing ethidium bromide (1 μg/ml). Plasmid bands were visualized with a UV transilluminator (model UVP-TM36, Ultra-Violet Products, Inc.) and photographed by using Polaroid type 665 film.

Large-scale isolation of DNA was performed by using the method described above except that 60 times the volume of cell cultures and reagents was used for each step. The plasmid DNA (dissolved in TE buffer) was purified by CsCl-ethidium bromide density gradient centrifugation (21). After removal of the ethidium bromide, the purified plasmid DNA was dialyzed against TE buffer and stored frozen at −20°C. Isolation of plasmids from preparative gels (0.4% [wt/vol] SeaPlaque low-melting-point agarose [FMC]) and subsequent electroelution from the agarose matrix were performed as described by Maniatis et al. (21).

Plasmid curing. Plasmids were removed from several *Vibrio* spp. by incubating cell cultures in MMS supplemented with 20 mM glucose, 0.1% (wt/vol) Casamino acids, and novobiocin (100 to 250 μg/ml; Sigma) as described by McHugh and Swartz (22). After incubation for 12 h, the cultures were diluted in MMS and plated onto nutrient agar containing 3% (wt/vol) NaCl (NAS). Individual colonies were subsequently tested for plasmid loss.

Transformation of plasmids into marine vibrios. *Vibrio* strains were grown to mid-exponential phase in 20 ml of modified Luria broth (containing, per liter of distilled water, 10 g of tryptone, 5 g of yeast extract, and 30 g of NaCl [pH 7.5]). Cells were washed twice in 10 ml of 10 mM MOPSO [3-(*N*-morpholino)-2-hydroxypropanesulfonic acid] (pH 7.0), containing 10 mM rubidium chloride and 0.25 M sucrose. The cells were harvested and suspended in 10 ml of solution X (10 mM MOPS [pH 6.5] containing 50 mM calcium chloride, 10 mM rubidium chloride, and 0.25 M sucrose). The suspension was held at 4°C for 30 min, after which time the cells were harvested and suspended in 2 ml of solution X. A 3-μl volume of dimethyl sulfoxide was added to a portion (0.2 ml) of this cell suspension in a microcentrifuge tube and mixed thoroughly by gently inverting the tube several times. Purified plasmid DNA (2 μg) was added, and the mixture was incubated at 4°C for 30 min. A 1-ml volume of modified Luria broth was added, and the cells were incubated for 1 h at 30°C. Cells were pelleted and suspended in 1 ml of modified Luria broth, and serial dilutions were spread on NAS and incubated at 30°C for 24 to 48 h. Colonies chosen at random were screened for the presence of plasmids.

TABLE 1. Growth, size, reduction, and plasmid size of marine vibrios

Vibrio strain	Generation time[a] (min)	Dwarfing (%)[b]	Plasmid size (MDa)
AS1	59	38	NF[c]
AS2	66	42	NF
AS3	125	48	11.8
AS4	46	44	34.2
AS5	73	39	NF
AS6	34	40	NF
AS7	55	43	NF
AS8	111	52	56.0
AS9	36	49	NF
AS10	67	30	NF
AS11	64	49	7.5, 15.2[d]
AS12	64	56	13.4
AS13	97	42	8.2, 9.1, 18.3
AS14	146	47	NF
AS15	123	48	NF
AS16	42	54	NF
AS17	39	52	NF
AS18	58	58	3.2
AS19	61	40	NF
AS20	45	38	49.7
V. fluvialis	35	56	31.8

[a] Generation times were determined in ANaGC.
[b] Percentage of mid-exponential-phase cells reaching the size range 0.22 to 0.39 μm^3 after starvation for 24 h.
[c] NF, None found.
[d] Appears to be a dimer of the 7.5-MDa plasmid.

Electroporation of plasmids into marine vibrios. Cells derived from 20 ml of an exponential-phase culture (10^7 CFU/ml) were washed three times in 2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 6.8) containing 100 mM sucrose and suspended in 10 ml of 2 mM HEPES (pH 6.8) containing 100 mM sucrose and 5 mM calcium chloride. Cells from 1 ml of this suspension were washed three times in 1.5-ml portions of 10% (wt/vol) glycerol. The supernatant was removed, and the cell pellet was suspended in 100 μl of 10% (wt/vol) glycerol. A sample (50 μl) of this suspension was electroporated with purified plasmid DNA (1 μg) in a Gene Pulser (Bio-Rad Laboratories) with an electric pulse of 12,500 V/cm for 5 to 12.5 ms. The electroporated sample was incubated in 9 ml of NM broth for 4 h at 30°C. The cells were harvested (at 10,000 × g for 10 min) and suspended in 1 ml of NM broth, and serial dilutions were subsequently spread on NAS and incubated for 24 to 48 h at 30°C. Colonies chosen at random were screened for the presence of plasmids.

RESULTS AND DISCUSSION

Isolation and characteristics of marine vibrios. Since some dwarf cells have been found to be more adhesive than growing cells (for example, see reference 7), 500 adhesive bacteria were isolated from Coogee Bay. Of these, 20 were found to be *Vibrio* spp. (see Materials and Methods). When starved, all isolated vibrios as well as *V. fluvialis* were found to undergo size reduction to varying degrees (Table 1). To overcome the problem of loss of viability that occurred when *V. fluvialis* was starved for periods of more than 2 to 3 days

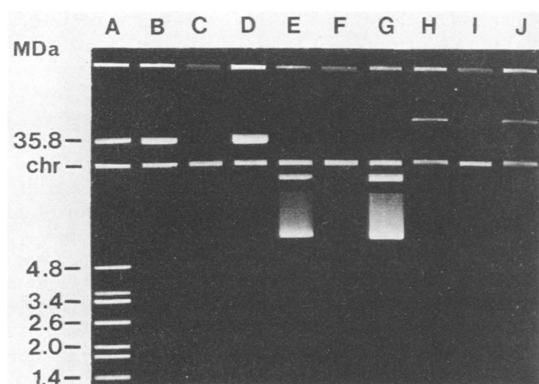

FIG. 1. Plasmid profiles of *Vibrio* strains AS4, AS8, and AS11 before and after curing and with their plasmid(s) reintroduced. Lanes: A, plasmid standards isolated from *E. coli* V157; B, AS4 showing a 34.2-MDa plasmid; C, AS4c (AS4 cured of its plasmid); D, AS4cr (AS4c with its plasmid reintroduced); E, AS11 showing 7.5- and 15.2-MDa plasmids; F, AS11c (AS11 cured of its plasmids) (7.5 and 15.2 MDa); G, AS11cr (AS11c with the 15.2-MDa plasmid reintroduced); H, AS8, showing a 56.0-MDa plasmid; I, AS8c (AS8 cured of its plasmid); J, AS8cr (AS8c with its plasmid reintroduced). chr, Chromosomal DNA.

(30), samples for size reduction measurements were taken early. The results were obtained on cultures that had been starved for 24 h (Table 1). At that sampling time, all strains were found to have retained full viability as determined by plate counts (data not shown). Differences in growth rate, plasmid profiles, and extent of dwarfing suggested that the isolates were different strains (Table 1). Of the 21 strains tested, 43% contained at least one plasmid. Strain AS11 appeared to contain two plasmids, but further tests (see below) suggested that the 15.2-megadalton (MDa) plasmid was a dimer of the 7.5-MDa plasmid. Strain AS13 appeared to contain three plasmids. It is possible that plasmids present in low copy numbers escaped detection. In addition, large plasmids (>100 MDa) may not have been detected by the method used. The proportion of strains containing plasmids (43%) may therefore be an underestimate. However, this result agrees with the findings of Baya et al. (5) and Hermansson et al. (12) that 45 and 39%, respectively, of marine isolates contained plasmids.

Location of genes essential for dwarfing. The results (Table 1) revealed that, although 43% of the *Vibrio* strains contained plasmids, all responded to starvation by dwarfing, suggesting that the genes responsible for size reduction were located on the chromosome. However, it was possible that diversity existed among the strains tested and that, whereas in some strains the size reduction genes were located on the chromosome, in others they may have been located on either a plasmid(s) or the chromosome or dispersed between the chromosome and plasmids. To test this possibility, five plasmid-containing strains (AS3, AS4, AS8, AS11, and *V. fluvialis*) were treated with novobiocin to remove the plasmids. Colonies from three strains (AS4, AS8, AS11) were found to have lost their plasmids (Fig. 1). The cured strains were tested for dwarfing, growth rate, and resistance to antibiotics and heavy metals to determine any possible loss of traits. Upon curing, strains AS8 and AS11, but not AS4, showed the loss of several traits associated with heavy metal and antibiotic resistance (Table 2). A slight increase in growth rate was found in all three cured strains, an observation which is consistent with the loss of plasmids which require cellular energy for replication. Dwarfing of the cured strains was not affected, indicating that in these three strains the genes essential for the early stages of size reduction, including fragmentation and the reductive division phase (19), were located on the chromosome. Because size reduction appears to be part of a starvation survival process in some marine bacteria, it is probable that genes encoding the pathway for the continuous phase of size reduction are located on the chromosome rather than on plasmids, which may be lost during periods when the functions encoded thereon are not required by the cell. This suggestion could be tested by examining the response of cured strains to periods of starvation lasting several days or weeks.

To confirm that the characteristics lost from the cured strains were plasmid encoded, the plasmids were reintroduced and the resulting strains were tested for phenotype. Transformation with $CaCl_2$ shock was found to restore the plasmid in strain AS4c (Fig. 1) at a frequency of 16×10^1 transformants per μg of plasmid DNA, but was not a suitable method for strains AS8c and AS11c. Electroporation proved to be a suitable alternative method to $CaCl_2$ shock for strains AS8c and AS11c (Fig. 1), with frequencies of 4.8×10^1 and 3.9×10^1 transformants per μg of plasmid DNA, respectively, being obtained. The low frequencies of plasmid transfer that were obtained by using electroporation in these

TABLE 2. Effect of plasmid removal from strains AS4, AS8, and AS11

Vibrio strain	Generation time[a] (min)	Dwarfing (%)[b]	Resistance to[c]:					
			Streptomycin	Tetracycline	$CdCl_2$	$HgCl_2$	$ZnCl_2$	K_2CrO_4
AS4	46	44	+	+	−	−	−	+
AS4c[d]	42	44	+	+	−	−	−	+
AS4cr[e]	47	43	+	+	−	−	−	+
AS8	111	52	+	−	−	+	+	+
AS8c[d]	108	53	+	−	−	+	−	+
AS8cr[e]	114	52	+	−	−	+	+	+
AS11	64	49	+	+	+	+	−	+
AS11c[d]	62	49	−	−	−	−	−	+
AS11cr[e]	64	49	+	+	−	+	−	+

[a] Determined in AnaGC broth. Average of three experiments.
[b] Percentage of mid-exponential-phase cells reaching the size range of 0.22 to 0.39 μm^3 after starvation for 24 h.
[c] Determined on AnaGC plates. Concentrations used (in μg/ml): streptomycin, 50; tetracycline, 10; $CdCl_2$, 60; $HgCl_2$, 80; $ZnCl_2$, 250; K_2CrO_4, 40. Strains that grew equally well in the presence or absence of antibiotics or heavy metals were classified as resistant (+).
[d] Plasmid-cured strain.
[e] Plasmid-cured strain with its plasmids reintroduced by either transformation or electroporation.

experiments were comparable to previously published data (28, 31), indicating that conditions were not optimum for the *Vibrio* strains used. Introduction of the plasmid into strain AS8c restored $ZnCl_2$ resistance (Table 2). The 15.2-mDa plasmid of AS11 was separated from the 7.5-MDa plasmid by using a preparative agarose gel (see Materials and Methods). Introduction of the 15.2-MDa plasmid into AS11c by electroporation restored all lost functions except for $CdCl_2$ resistance. Failure to restore $CdCl_2$ resistance might have occurred through a random mutational event in the plasmid DNA. Both the 15.2- and 7.5-MDa plasmids were present in clones transformed by using 15.2-MDa plasmid DNA, suggesting that the 15.2-MDa plasmid is a dimer of the 7.5-MDa plasmid (Fig. 1). Introduction of the 34.2-MDa plasmid of AS4 into AS4c did not alter the phenotype of the host (Table 2).

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