Competitive Growth Advantage of Nontoxicogenic Mutants in the Stationary Phase in Archival Cultures of Pathogenic *Vibrio cholerae* Strains

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Spontaneous nontoxicogenic mutants of highly pathogenic *Vibrio cholerae* O1 strains accumulate in large numbers during long-term storage of the cultures in agar stabs. In these mutants, production of the transcriptional regulator ToxR was reduced due to the presence of a mutation in the ribosome-binding site immediately upstream of the *toxR* open reading frame. Consequently, the ToxR-dependent virulence regulon was turned off, with concomitant reduction in the expression of cholera toxin and toxin-coregulated pilus. An intriguing feature of these mutants is that they have a competitive fitness advantage when grown in competition with the parent strains in stationary-phase cocultures which is independent of RpoS, the only locus known to be primarily associated with acquisition of a growth advantage phenotype in bacteria.

The gram-negative, enteric bacterium *Vibrio cholerae* normally resides in coastal and estuarine water, from where it may be transmitted to the human body by ingestion of contaminated food and water. Within its human host, *V. cholerae* colonizes the small intestine and causes the diarrheal disease cholera (reviewed in reference 9). *V. cholerae* is a highly adapted organism capable of survival under the diverse environmental conditions found in the host intestine and in natural water reservoirs. Pathogenicity of *V. cholerae* is largely due to the production of cholera toxin (CT) and a toxin-coregulated pilus (TCP) coordinately expressed with CT that greatly enhances colonization of the intestinal epithelium by the bacterium (8, 19). Expression of CT, TCP, and several other virulence factors of *V. cholerae* is coordinately controlled by the expression of a regulatory cascade known, mainly for historical reasons, as the ToxR regulon (10, 11). At the top of the hierarchy are the transmembrane DNA binding proteins ToxR and TcpP, which act synergistically to activate transcription of *toxT* (7, 14). ToxT is the second transcriptional activator in the cascade, which directly activates expression of several virulence genes, including those encoding CT and TcpA, the major subunit of TCP (4).

In their natural environment, microbial populations are routinely exposed to nutrient insufficiency with only short and sporadic periods of nutrient availability. Starvation evokes a complex response, which in nonsporulating bacteria is mainly dependent on a stationary-phase-specific sigma factor, RpoS. RpoS controls the expression of several stationary-phase-inducible genes that confer resistance to multiple stresses (13). In addition to defensive measures to ensure survival under energy-depleted conditions, certain bacteria have evolved in-

teresting offensive strategies for competitive exclusion of related species or even subpopulations of the same species to facilitate their own survival (5, 22). For *Escherichia coli*, starvation induces a hypermutable state in a subpopulation, so that a starved culture is essentially genetically heterogeneous (20). Specific mutations in the *rpoS* gene and an RpoS-dependent enhanced ability to catabolize amino acids have been shown to confer a growth advantage phenotype (21, 23). We report here that spontaneous nontoxicogenic mutants arise in archival cultures of highly pathogenic *V. cholerae* strains, which have an RpoS-independent competitive growth advantage over their parent strains in the stationary phase.

Spontaneous nontoxicogenic mutants in aged stab cultures of *V. cholerae*. A collection of agar stab cultures of the *V. cholerae* O1 strains 569B and O395 stored in the laboratory for between 10 and 25 years was examined for CT production. Although the cultures were originally highly toxigenic, when retrieved from the agar stabs after long-term storage, a significant decrease in toxigenicity was observed. To examine if individual cells in these stabs uniformly produced low levels of CT or whether the population was heterogeneous with respect to CT production, the stab cultures were plated for single colonies and CT production was assayed with a large number of individual colonies. The results obtained indicated that the individual cells in a stab

TABLE 1. CT production and autoagglutination phenotypes of *V. cholerae* wild-type and mutant strains

<i>V. cholerae</i> strain	Amt of CT ^a	Autoagglutination ^b
O395	1.5 ± 0.95	+
O395-1C	0.02 ± 0.02	–
569B	1.85 ± 1.1	+
569B-2A	0.01 ± 0.009	–
JJM 43	ND	–

^a CT values are means ± standard deviations for three separate experiments. Results are expressed in micrograms per milliliter of culture supernatant at an optical density at 600 nm of 1. ND, not detected.

^b +, clumping of cells; –, no clumping.

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TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>V. cholerae</i>		
569B	Sm ^s , wild type, CT producing	Laboratory collection
569B-Sm ^R	Sm ^r derivative of 569B, CT producing	This study
569B-2A	Sm ^s , CT ⁻ , isolated from aged stabs	This study
569B-2A-Sm ^R	Sm ^r derivative of 569B-2A, CT ⁻	This study
O395	Wild type, CT producing	Laboratory collection
O395-1C	CT ⁻ , isolated from aged stabs	This study
JJM43	O395-N1 (Δ <i>toxR</i>)	19
Plasmids		
pVM7	pBR 327 containing <i>toxR</i> gene	14
pKEK156	pMAL carrying <i>toxT</i> gene	18
pAG120	pBS containing <i>V. cholerae</i> <i>tcp PH</i> genes	This study

culture either produced normal levels of CT (1.5 to 2 μ g of CT per ml per A_{600} unit) or produced only about 10 to 20 ng of CT (Table 1). Under identical conditions, no CT was detected (<2 ng) for the *V. cholerae* *toxR* mutant strain JJM44 (Table 1). The proportions of toxigenic and nontoxigenic variants in individual vials of stabs differed appreciably, but in general the older the stab, the greater the proportion of cells that did not produce CT. The CT⁻ phenotype was stably inherited upon repeated subculturing and single-colony isolation, suggesting that it was due to a mutation. A large number of such mutant colonies were isolated from aged Luria-Bertani (LB) agar stabs, and two mutants, 569B-2A and O395-1C (Table 2), were studied in detail. Southern blot and PCR analysis indicated that the *ctxAB* gene was present in both the mutant strains (data not shown).

Transcriptional repression of *ctxAB* and *tcpA* genes in the strains 569B-2A and O395-1C. To examine if the inhibition of CT production in the *V. cholerae* strains 569B-2A and O395-1C was at the level of transcription, reverse transcription (RT)-PCR and Northern blot analyses were performed with RNA isolated from the parent and mutant strains grown under permissive conditions (LB, pH 6.6, 30°C) (15). *V. cholerae* strain JJM43 (Δ *toxR*) (Table 2), in which the entire ToxR virulence regulon is shut off (16, 19), was used as a negative control. RT-PCR analysis indicated that *ctxAB*-specific mRNA was practically not produced in the strains 569B-2A and JJM43, and in strain O395-1C, *ctxAB* mRNA was drastically reduced by more than 15-fold from levels for the parent strain, O395 (Fig. 1A). In these experiments, 16S rRNA production was

used as an internal control (Fig. 1C), and RNA that had not been reverse transcribed was used as a negative control (Fig. 1D). Similar results were obtained by Northern blot analysis (data not shown).

Expression of the *tcpA* gene, known to be coordinately regulated with *ctxAB* gene expression (19), was examined in the strains 569B-2A and O395-1C. RT-PCR analysis indicated a statistically significant reduction in *tcpA* expression for both the mutants compared to the corresponding parent strains ($P = 0.001$). After normalization according to the amounts of 16S rRNA present in each RNA population (Fig. 1C), the amount of *tcpA*-specific mRNA in strains O395-1C and 569B-2A was 10- to 12-fold lower than that in the corresponding parent strains (Fig. 1B). Practically no *tcpA*-specific transcript was detected in strain JJM43 (Fig. 1B, lane e).

Expression of the regulatory genes *toxT*, *toxR*, and *tcpP*. Since *ctxAB* and *tcpA* expression was reduced for strains 569B-2A and O395-1C, expression of *toxT* encoding the transcriptional activator of both the *ctxAB* and *tcpA* genes (4) was examined for these strains. For strains 569B-2A and O395-1C, *toxT* expression was about 20-fold lower than for the corresponding parent strains (Fig. 2A and B), while no *toxT* mRNA was detected in strain JJM43 (Fig. 2A, panel I, lane h). CT production was restored to wild-type levels for both strains 569B-2A and O395-1C carrying the cloned *toxT* gene in plasmid pKEK156 (Table 2), although the plasmid had almost no effect on CT production in the wild-type strain (Fig. 2C).

Expression of *toxT* requires the transmembrane DNA binding proteins, ToxR and TcpP, which act synergistically to activate transcription from the *toxT* promoter (3, 7). No statistically significant difference was observed in *tcpPH* expression between strain 569B-2A or O395-1C and the corresponding parent strain ($P \geq 0.25$) (Fig. 2A [panel II] and B). However, expression of *toxR* in strains O395-1C and 569B-2A was lower than in the corresponding parent strains (Fig. 2A [panel III] and B).

ToxR can restore CT production in strains 569B-2A and O395-1C. It has previously been reported that nontoxigenic mutants carrying a frame shift mutation in the coding sequence of *tcpPH* arise in large numbers during growth of *V. cholerae* under permissive condition (2). However, plasmid pAG120 carrying the full-length *tcpPH* genes (Table 2) could not complement the nontoxigenic phenotype of the mutant strains 569B-2A and O395-1C. Also, the nucleotide sequence of the

FIG. 1. RT-PCR analysis of *ctxAB* and *tcpA* expression. RT-PCR was performed with RNA isolated from strains 569B (lanes a), O395 (lanes b), 569B-2A (lanes c), O395-1C (lanes d), and JJM43 (lanes e) for estimation of *ctxAB* mRNA (A), *tcpA* mRNA (B), and 16S rRNA (C). DNase-treated RNA samples that had not been reverse transcribed were used as negative controls (D).

FIG. 2. (A) *toxT*, *toxR*, and *tcpP* gene expression. RT-PCR was performed for 25 (lanes a and b) or 35 (lanes c to h) cycles with RNA isolated from *V. cholerae* strains 569B (lanes a and c), 569B-2A (lanes b and d), O395 (lane f), O395-1C (lane g), and JJM43 (lane h) for estimation of *toxT* (I), *tcpP* (II), and *toxR* (III) mRNA. Panel I, lane e, 35 cycles RT-PCR with RNA from *V. cholerae* strain 569B-2A carrying plasmid pVM7 for estimation of *toxT* expression. (B) Densitometric analysis of the *toxT*-, *toxR*-, and *tcpP*-specific RT-PCR products from strains O395, 569B, O395-1C, and 569B-2A after normalization according to the amount of RT-PCR product corresponding to 16S rRNA detected in the same cDNA sample. (C) Restoration of CT production by ToxT and ToxR. Strains O395, 569B, O395-1C, and 569B-2A with or without plasmid pKEK156 (ToxT) or plasmid pVM7 (ToxR) were grown to the stationary phase, and CT was measured in culture supernatants corresponding to 10^9 CFU and expressed as percentages of the amounts obtained in culture supernatants of strain O395. Data represent the average for three independent experiments. Error bars indicate standard errors of the means.

tcpPH genes in the mutants was identical to that in the corresponding parent strains.

Plasmid pVM7 carrying the cloned wild-type *toxR* gene (Table 2) could restore CT production to wild-type levels in strains 569B-2A and O395-1C (Fig. 2C). Plasmid pVM7 could also restore *toxT* expression in these strains (Fig. 2A, panel I, lane e). These results suggested that the strains O395-1C and 569B-2A might carry a mutation in *toxR*. The nucleotide sequence of the *toxR* open reading frame and promoter region up to -103 bases was determined for strains 569B-2A and

O395-1C and compared with the sequence of the parent strains. The only difference between the mutant and parent strains was the deletion of an A residue 10 nucleotides upstream of the *toxR* open reading frame (Fig. 3) at the predicted

569B : CACAACCTCAAAAGAGATATCG ATGAGT CAT ATT
M S H I
569B-2A: CACAACCTCAAA^{*}GAGATATCG ATGAGT CAT ATT
M S H I

FIG. 3. Nucleotide and deduced amino acid sequence at the 5' end of *toxR*. The A residue at the RBS of *toxR* in strain 569B denoted in bold was deleted in strain 569B-2A. The position of the deleted A nucleotide in 569B-2A is marked by an arrowhead.

FIG. 4. Immunoblot analysis of ToxR. Total cellular proteins in *V. cholerae* strains O395 (lane a), O395-1A (lane b), 569B (lane c), 569B-2A (lane d), and JJM43 (lane e) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with anti-*V. cholerae* ToxR serum.

FIG. 5. Viability of *V. cholerae* 569B and *V. cholerae* 569B-2A in LB. Individual cultures in LB medium were incubated at 37°C with aeration, and CFU were counted at regular intervals.

ribosome binding site (RBS) of *toxR* (12, 16). Such -1 deletions in small mononucleotide repeats are commonly present in stationary-phase mutants that arise in nondividing cells (17). Since the mutation at the *toxR* RBS might affect translation efficiency, ToxR levels in the mutant and wild-type strains were examined by immunoblot analysis, using anti-ToxR antibody. A very weakly immunoreactive band was detected in lysates of the mutant strains 569B-2A and O395-1C which comigrated with the stronger immunoreactive band observed in lysates of the wild-type strains, 569B and O395 (Fig. 4). A comigrating band was not detected in lysates of the strain JJM43 ($\Delta toxR$), although a weak band corresponding to a smaller protein was sometimes detected in these lysates (Fig. 4, lane e). These results suggest that the strains 569B-2A and O395-1C carry a mutation in the Shine-Dalgarno sequence upstream of *toxR* that resulted in reduced ToxR levels in these cells. The low levels of ToxR may be insufficient for activation of *toxT* expression, since supplementation of ToxR by introduction of the multicopy plasmid pVM7 could restore *toxT* expression in these strains (Fig. 2A, panel I, lane e).

Mutants 569B-2A and O395-1C have a competitive growth

advantage over parent strains. Since mutations that confer a growth advantage are known to arise in nondividing cells (1, 6, 20), growth of the strains 569B-2A and O395-1C, isolated from aged agar stabs, was examined both in individual cultures and in mixed cultures with the isogenic wild-type parent strain.

When grown separately, the growth rates of the mutant and the parent strains were similar in the logarithmic phase, and their survival in the stationary phase was also comparable (Fig. 5). Next, the mutant strains were cocultured with the corresponding wild-type parent strains, and each population in the mixed culture was monitored by viable count assay. The two populations in a culture could be successfully distinguished because one carried a neutral antibiotic resistance marker (Sm^r). The marker was switched between the parent and mutant strains, and cocultures were performed reciprocally to confirm that the marker did not affect fitness. When strain 569B was cocultured with the mutant strain 569B-2A, the relative proportions of each strain in the mixed culture remained unchanged in the logarithmic phase of growth (Fig. 6A). However, after 24 h, the proportion of 569B-2A was almost fourfold higher than that of 569B, and by 72 h, 569B cells were practically eliminated from the coculture (Fig. 6B). When strain O395 was cocultured with the mutant O395-1C, a growth advantage for the mutant was also observed in the stationary phase. However, in this case, even after 150 h, the parent strain O395 was not completely eliminated from the mixed culture (Fig. 6C).

RpoS is normal in strains 569B-2A and O395-1C. In *E. coli* a specific mutation in the *rpoS* gene, encoding the stationary-phase sigma factor, has been shown to confer a growth advantage in the stationary phase, and these mutants could finally take over the population in competition experiments with the wild-type cells (21). To determine if the growth advantage of strains 569B-2A and O395-1C was due to a similar mutation, the sequence of the *rpoS* gene recovered by PCR from strains O395-1C and 569B-2A was determined and compared with that of the corresponding wild-type strains. No difference in *rpoS* sequence was detected between the parent and mutant strains, indicating that RpoS has no role in the observed com-

FIG. 6. Selective advantage of *V. cholerae* strains 569B-2A and O395-1C in mixed cultures with wild-type strains. Fifty microliters of 18-h cultures of wild-type and mutant cells were added to 5 ml of fresh LB, and the mixed cultures were grown with aeration at 37°C. CFU of each population was counted at regular intervals by plating on LB agar with or without antibiotic. (A) Mixed cultures of 569B and 569B-2A in the logarithmic phase of growth. (B) Competition between 569B and 569B-2A in the stationary growth phase. (C) Competition between O395 and O395-1C in the stationary phase.

petitive fitness gain of the mutants. In view of the fact that starvation induces a state of hypermutability in a subpopulation of cells, which contain a high frequency of mutations (20), the possibility exists that unselected mutations are present in strains 569B-2A and O395-1C which contribute to their fitness gain.

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