**Vibrio cholerae** Persisted in Microcosm for 700 Days Inhibits Motility but Promotes Biofilm Formation in Nutrient-Poor Lake Water Microcosms

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Abstract

Toxigenic *Vibrio cholerae*, ubiquitous in aquatic environments, is responsible for cholera; humans can become infected after consuming food and/or water contaminated with the bacterium. The underlying basis of persistence of *V. cholerae* in the aquatic environment remains poorly understood despite decades of research. We recently described a “persister” phenotype of *V. cholerae* that survived in nutrient-poor “filter sterilized” lake water (FSLW) in excess of 700-days. Previous reports suggest that microorganisms can assume a growth advantage in stationary phase (GASP) phenotype in response to long-term survival during stationary phase of growth. Here we report a *V. cholerae* GASP phenotype (GASP-700D) that appeared to result from 700 day-old persister cells stored in glycerol broth at −80 °C. The GASP-700D, compared to its wild-type N16961, was defective in motility, produced increased biofilm that was independent of oxidative stress when grown specifically in FSLW (p<0.005). We propose that *V. cholerae* GASP-700D represents cell populations that may better fit and adapt to stressful survival conditions while serving as a critical link in the cycle of cholera transmission.

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Introduction

Cholera is a major public health threat worldwide, particularly in countries where safe drinking water, adequate sanitation and hygiene are suboptimal [1]. Cholera toxin (CT)-producing *V. cholerae* strains, generally in serogroups O1 and O139, are the cause of epidemic cholera. *V. cholerae* has two life styles, including transient passage through the human intestine where it causes profuse diarrhea (i.e. cholera), and a second existence in aquatic environments, including fresh, estuarine and marine environments [1,2,3]. In aquatic reservoirs, the microorganisms can survive either in planktonic (free-living) form or in biofilms [2,3]. Available data suggest that the bacteria survive between epidemics in these environments, particularly during inter-epidemic period, is poorly understood.

In this context, it has been suggested that *V. cholerae* can enter into a viable but non-culturable state (VBNC) in response to nutrient starvation and/or cold temperature [4,5]; however, the resuscitation of VBNC, under laboratory conditions, is inconsistent, raising questions about the role of the VBNC state in cholera epidemiology [6,7]. *V. cholerae* can also switch from a smooth colony type to a “rugose” (wrinkled) variant characterized by copious production of exopolysaccharide conferring resistance to chlorine, osmotic and oxidative stresses [8,9,10]. However, the role(s) of rugose variant of *V. cholerae* in epidemic cholera is limited because not all *V. cholerae* strains are capable of switching to rugose variant even in a medium promoting high-frequency rugose production [9].

Amid this conundrum, we recently reported that a subset of culturable *V. cholerae* assume what we have termed a “persister” phenotype in a “filter sterilized” lake water (FSLW) microcosm model [11]. In that study we found that only 13% of the microcosms yielded cells that persisted in excess of 700 days while 87% of the microcosms resulted in the death of cells by 120 days. Furthermore, we observed that persisting cells in 700-day old microcosms expressed a small colony phenotype associated with very small rod shaped cells with peritrichous flagella and a high degree of cell aggregation. In contrast, cells persisting in microcosms for 24 h exhibited normal colony phenotype with heterogeneous mixtures of cells with predominantly long helical cells with bipolar flagella [11]. A “growth advantage in stationary phase” (GASP) phenotype describes microorganisms that survive...
long-term in a stationary growth phase under stressful conditions [12,13,14]. For further analysis of 700 day-old cells, we subcultured the cells from microcosms onto L-agar and subsequently stored them in glycerol broth at −80°C. As we were not certain if 700 day-old persister cells of microcosm origin will retain their genetic and phenotypic traits unchanged upon storage in glycerol broth, for our convenience, we refer this glycerol-stored cells to GASP-700D phenotype; in contrast, wild-type *V. cholerae* N16961S strain grown overnight statically in FSLW at room temperature will be henceforth termed as N16961S-24 (Table 1).

Persister cells in other human pathogens exhibited biofilm formation conferring resistance to environmental stresses [15,16,17,18]. In *V. cholerae* the positive association of polar flagellum to biofilm formation has been demonstrated [19]. To better understand the GASP-700D phenotype of *V. cholerae* and to compare the differences, if any, between N16961S-24 and GASP-700D, we investigated the role(s) of novel flagella elicited by N16961S-24 and GASP-700D, respectively [11], in motility and biofilm formation. Here, we provide evidence that GASP-700D showed no motility in soft agar; produced biofilm only in nutrient-poor FSLW; and conferred resistance to oxidative stress when compared to N16961S-24.

### Materials and Methods

#### Bacterial Strains and Growth Conditions

Bacterial strains, including *V. cholerae* wild-type strain N16961S and its isogenic mutants (obtained either natural selection and/or

| Strain or Plasmid | Description | Reference |
|-------------------|-------------|-----------|
| **V. cholerae** strains | | |
| N16961S | A wild-type, smooth, O1 El Tor strain isolated in Bangladesh in 1971 | [9] |
| N16961S-24 | A growth of N16961S in nutrient-poor “filter sterilized” lake water incubated overnight statically at room temperature | This study |
| N16961R | A rugose variant of N16961S strain | [24] |
| N16961R-24 | A growth of N16961R in nutrient-poor “filter sterilized” lake water incubated overnight statically at room temperature | This study |
| GASP-700D | 700 days-old N16961S culture persisting in nutrient-poor FSLW was grown on L-agar and subsequently stored in FSLW supplemented with 30% glycerol at −80°C | [11] |
| AA212 | A ΔflaA null mutation in the background of N16961S strain | This study |
| AA215 | A ΔvpsR (420 bp internal in-frame deletion) created in the N16961S strain | This study |
| AA216 | A ΔvpsR (420 bp internal in-frame deletion) created in the N16961R | This study |
| AA217 | A GASP-700DΔvpsR (420 bp internal in-frame deletion) created in the background of GASP-700D strain | This study |
| AA218 | A ΔvpsA (VC_0917) in-frame null mutation was created in N16961S strain | This study |
| AA219 | A ΔvpsA (VC_0917) in-frame null mutation was created in N16961R strain | This study |
| AA220 | A GASP-700DΔvpsA (VC_0917) in-frame null mutation was created in the background of GASP-700D strain | This study |
| **E. coli** strains | | |
| DH5α | recA ΔlacZ169 prophage lacZΔM15 | Gibco, BRL |
| S17-1 λ pir | Pro hsdR hsdM’ Tmp’ Str’ | [41] |
| **Plasmids** | | |
| pWSK29 | Low-copy-number vector, Amp’, ori pSC101 | [23] |
| pCVD442 | Suicide vector, ori R6K, Amp’, sacB | [42] |
| pKEK93 | ΔfluA-Cm in pCVD442 | [20] |
| pAA69 | A 560-bp PCR fragment (SacII/XbaI) containing the 5’-end of vpsR gene of N16961 cloned into similarly digested pWSK29, Amp’ | This study |
| pAA72 | A 540-bp PCR fragment (SacII/SpeI) fragment upstream of vpsA gene of N16961 cloned into similarly digested pWSK29, Amp’ | This study |
| pAA73 | A 520-bp PCR fragment (XbaI-BamH1) was cloned into similarly digested pAA69, resulting in a plasmid (pAA73) containing 420-bp internal in-frame deletion. Amp’ | This study |
| pAA74 | A 360-bp PCR fragment (SpeI-EcoR1) downstream of vpsA was cloned into similarly digested pAA72, resulting in a plasmid (pAA74), Amp’ | This study |
| pAA77 | A 900-bp PCR fragment (SacII-SalI) from pAA74 was cloned into similarly digested pCVD442, Amp’ | This study |
| pAA78 | A 1080-bp PCR fragment (SacII-SalI) from pAA73 was cloned into similarly digested pCVD442, Amp’ | This study |
created by defined genetic mutations) used in this study are listed in Table 1. As reported earlier, we generated *V. cholerae* N16961 persisters (in excess of 700 days) in “filter sterilized” lake water microcosm model. Briefly, aliquots (500 ml) of lake water were sterilized using Nalgene 0.22 μm membrane filter units (Nalgene), and the microcosms were prepared as follows: 50 ml of “filter sterilized” lake water (FSLW) was transferred into a sterile 250 ml Erlenmeyer flask; for inoculum preparation a single colony of *V. cholerae* N16961 strain, obtained from L-agar grown overnight at 37°C, was inoculated into 3 ml L-broth. The culture was incubated overnight at 37°C with a shaking speed of 250 rpm, spun down and the resulting pellet was washed 2X in saline (0.85% NaCl), reconstituted in 3 ml saline, appropriately diluted, and 50 μl of diluted culture was inoculated into the microcosm flasks containing 50 ml FSLW. As confirmed by plate counts, the microcosms were prepared as follows: 50 ml of FSLW was transferred into a sterile 250 ml Erlenmeyer flask; for inoculum preparation a single colony of *V. cholerae* N16961 strain, obtained from L-agar grown overnight at 37°C, was inoculated into 3 ml of L-broth. The culture was incubated overnight at 37°C with a shaking speed of 250 rpm, spun down and the resulting pellet was washed 2X in saline (0.85% NaCl), reconstituted in 3 ml saline, appropriately diluted, and 50 μl of diluted culture was inoculated into the microcosm flasks containing 50 ml FSLW. As confirmed by plate counts, initial *V. cholerae* concentrations in the microcosms ranged from 10^3 to 10^6 cfu/ml. The culturable cells from microcosm were determined at intervals using standard plate counts. The 700 day-old cells were subcultured on L-agar and stored in glycerol broth at −80°C. While we cannot be certain that this is true for all GASp-700D cells, we observed that GASp-700D exhibited small colony phenotypes on L-agar for at least four consecutive days of subculture both at room temperature and at 37°C. However, when the cells were inoculated into L-broth and incubated overnight at 37°C with a shaking speed of 250 rpm, a mixture of small and large colonies were observed on L-agar upon plating. All the strains used in this study were subcultured from glycerol stock at −80°C onto L-agar and incubated overnight at 37°C before being used for specific experiments. As needed, antibiotic was added to the bacterial cultures as follows: ampicillin (100 μg/ml) and polymyxin B (50 U/ml).

**Genetic Manipulations**

A ΔflaA mutant (AA212; Table 1) was created in the background of N16961S strain (Table 1) using a ΔflaA gene targeting vector described previously [20]. For creating in-frame mutation in *hydrD*/*vpsR* [8,21] and in a rugosity-associated gene, *vpsA* (VC0917, encoding UDP-N-acetylgalactosamine 2-epimerase [sevC]) [22] in the back ground of N16961S, N16961R and GASp-700D (Table 1), a two-step PCR cloning strategy was used. Briefly, two PCR products flanking an internal deletion (420-bp) in *vpsR* were engineered. Each PCR product carries a restriction endonuclease site at its 5’ end; however, 3’ ends of the forward and reverse PCR products carried a common restriction site to facilitate deletion mutation. For *vpsR*, *SacII* and *XbaI* sites were introduced at 5’ and 3’ ends, respectively, of the forward PCR product amplicon (560-bp) while 5’ and 3’ ends of reverse PCR product (520-bp) were introduced with *XbaI* and *BamHI* sites, respectively. Primers aa212 and aa213 (Table S1) were used to amplify forward PCR fragment using N16961S chromosomal DNA as a template with standard PCR conditions. The PCR product was purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA). The purified PCR product was digested with *SacII* and *XbaI*, the digested product was purified, and the PCR product was ligated with a similarly digested vector, pWSK29, [23] resulting in a plasmid (pAA69). The plasmid was transformed into *Escherichia coli DH5α* as described previously [24]. Next, two convergent PCR primers, including aa214 and aa215 (Table S1) were used to amplify the reverse PCR product; the amplicon was purified and digested with *XbaI* and *BamHI*. The digested products were purified and ligated into a similarly digested plasmid (pAA69), resulting in a plasmid pAA73 containing a 420-bp internal deletion of *vpsR*. The plasmid was transformed into DH5α. Subsequently, plasmid pAA73 was digested with *SacII* and *SalI* to retrieve a 1080-bp fragment and the fragment was gel purified. The purified fragment was ligated into a similarly digested suicide vector, pCVD442, [23] and transformed into an *E. coli* S17 λ pir resulting in a plasmid pAA78 (Table 1). *E. coli* S17 λ pir carrying pAA78 was conjugated to *V. cholerae* N16961S, N16961R and GASp-700D. Selection of transconjugants, counter selection, and chromosomal mutation using homologous recombination of *vpsR* was performed as described previously [21,24]. Mutants sustained an internal in-frame deletion in *vpsR* (SΔvpsR, mutation in smooth background [AA215, Table 1], RΔvpsR, mutation in rugose background [AA216] and GASp-700DΔvpsR, mutation in GASp-700D background [AA217]) were verified by PCR and DNA sequencing as described previously [24]. A similar approach was also used for creating a null mutation in the *vpsA* gene in the background of N16961S, N16961R and GASp-700D, resulting in the mutants AA218, AA219 and AA220, respectively. Primers (aa264 and aa265, aa266 and 267) used to create null mutation in *vpsA* are listed in Table S1.

**Motility Assay**

Motility of *V. cholerae* strains was determined using motility agar plates as described previously [24] with minor modifications. The experiment was performed with cells grown both in L-broth and FSLW. Briefly, N16961S, N16961R, GASp-700D and ΔflaA mutant were grown in L-broth and incubated overnight statically at room temperature. As for FSLW, the strains were first subcultured onto L-agar; a single colony from L-agar was grown in 3 ml L-broth and incubated overnight statically at room temperature. Subsequently, the cultures were spun down at 7,000 rpm for 5 min in a table top centrifuge; the pellet was washed 2X with FSLW and resuspended into 3 ml FSLW and the culture was incubated overnight statically at room temperature. An inoculating wire was dipped into each culture and then stabbed into the motility agar plate. The plates were incubated for 8 h and overnight at 37°C. Zones of migration of bacterial strains around the inoculating sites were measured at 8 h and after overnight incubation of the plates. If no zone was detected, a block of agar was cut around the inoculating site, homogenized in saline (0.85% NaCl), appropriately diluted in saline, and then plated on L-agar to determine if any culturable cells were survived in the inoculation site.

**Quantitative Real-time Reverse Transcription PCR (qRT-PCR)**

For qRT-PCR, *V. cholerae* strains, including N16961S-24, N16961R-24 and GASp-700D (Table 1) were grown in FSLW overnight statically at room temperature. Total RNA was extracted and purified from each culture using the RNasy kit (Qiagen, Valencia, CA); the contaminating DNA in the preparation was eliminated on-column by DNase digestion. Total RNA (10 ng) was converted to cDNA, and the RT-PCR assay were performed using iScript one-step RT-PCR kit with SYBR green (Bio-Rad, Hercules, CA) and CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA) following manufacturer’s instructions. Primers used in this study are listed in Table S1. For each sample, the mean cycle threshold of the test transcript was normalized to that of *toxR* (*toxR* was equally expressed both in L-broth and in FSLW) and presented relative to *V. cholerae* N16961S-24 strain that has arbitrarily been taken as 1 (Figure S1). Values above 1 or less than 1of a selected gene indicate that the transcript was present in higher or lower numbers, respectively, than that of control strain. Data are based on three independent experiments. Previous report using qPCR demonstrated that *V. cholerae* expressed *phoB* and Pvt system genes while repressed *lp* genes when grown in “filter
sterilized pond water microcosms compared to its growth in nutrient-rich L-broth [7]. To validate our qRT-PCR data, we compared the differential gene expression by growing V. cholerae N16961S strain in nutrient-rich L-broth incubated overnight statically, and in nutrient-deficient FSLW under identical growth conditions. Expression of transcripts was determined as described above except that the threshold of transcript was presented relative to V. cholerae N16961S strain grown in L-broth.

Biofilm Assays

Quantitative assessment of biofilm produced by V. cholerae strains grown both in L-broth and in FSLW was measured as described previously [19] with modifications. Twenty-four well polystyrene plastic plates (Corning Incorporated, Corning, NY) were used as the surface for bacterial attachment. For assessment of biofilm produced in L-broth, V. cholerae strains, including N16961S, ΔvpsR, ΔvpsA, N16961R, ΔvpsR, ΔvpsA, GASP-700D, GASP-700D ΔvpsR and GASP-700DΔvpsA (Table 1) were examined. Biofilm assay was performed as described previously [19]. For measurement of biofilm produced in FSLW, V. cholerae strains, including N16961S-24, ΔvpsR, ΔvpsA, N16961R-24, ΔvpsR, ΔvpsA, GASP-700D, GASP-700D ΔvpsR and GASP-700DΔvpsA (Table 1) were investigated. Briefly, a single colony of each strain grown overnight on L-agar was inoculated into 3 ml L-broth and the cultures were incubated overnight with shaking (250 rpm) at 37°C. The culture was spun down and the pellet was washed 2X with FSLW and subsequently reconstituted into 3 ml FSLW. Fifty µl culture was then mixed to 450 µl fresh FSLW (ca. 10^8 cfu/ml) in a well of plastic plate; the culture was incubated for measurement of biofilm produced in FSLW, V. cholerae vpsA and GASP-700D strains grown both in L-broth and in FSLW was measured as described previously [19] with modifications. Twenty-four well polystyrene plastic plates (Corning Incorporated, Corning, NY) were used as the surface for bacterial attachment. For assessment of biofilm produced in L-broth, V. cholerae strains, including N16961S, ΔvpsR, ΔvpsA, N16961R, ΔvpsR, ΔvpsA, GASP-700D, GASP-700D ΔvpsR and GASP-700DΔvpsA (Table 1) were examined. Biofilm assay was performed as described previously [19]. For measurement of biofilm produced in FSLW, V. cholerae strains, including N16961S-24, ΔvpsR, ΔvpsA, N16961R-24, ΔvpsR, ΔvpsA, GASP-700D, GASP-700D ΔvpsR and GASP-700DΔvpsA (Table 1) were investigated. Briefly, a single colony of each strain grown overnight on L-agar was inoculated into 3 ml L-broth and the cultures were incubated overnight with shaking (250 rpm) at 37°C. The culture was spun down and the pellet was washed 2X with FSLW and subsequently reconstituted into 3 ml FSLW. Fifty µl culture was then mixed to 450 µl fresh FSLW (ca. 10^8 cfu/ml) in a well of plastic plate; the culture was incubated for measurement of biofilm produced in FSLW, V. cholerae vpsA and GASP-700D strains grown both in L-broth and in FSLW was measured as described previously [19] with modifications. Twenty-four well polystyrene plastic plates (Corning Incorporated, Corning, NY) were used as the surface for bacterial attachment. For assessment of biofilm produced in L-broth, V. cholerae strains, including N16961S, ΔvpsR, ΔvpsA, N16961R, ΔvpsR, ΔvpsA, GASP-700D, GASP-700D ΔvpsR and GASP-700DΔvpsA (Table 1) were examined. Biofilm assay was performed as described previously [19].
to the culture before supplementing the culture with stress ingredient, and then multiplying the result by 100.

**Statistical Analysis**

One-way ANOVA was performed in STATA v 12 (StataCorp, College Station Texas, USA) to determine the significant differences in diverse traits assessed in the study. Equal variance within groups was assessed using Bartlett’s test, and a Bonferroni correction was implemented to control type I error for multiple comparisons between the wild-type and its isogenic mutants or variants. A p-value of <0.005 was considered as statistically significant.

**Results**

Comparison of Motility between N16961S-24 and GASP-700D of *V. cholerae*

*Vibrio cholerae* carries a single polar flagellum required for its motility. Since we are the first to describe that *V. cholerae* can switch, in response to nutrient-starvation in FSLW, from a canonical single polar flagellum to bipolar and peritrichous flagella in N16961S-24 and GASP-700D, respectively [11], we were interested to investigate the role(s) of bipolar and peritrichous flagella, if any, in motility using motility agar. We also included a ΔflaA mutant strain that is non-motile because it lacks the major flagellin subunit [20], and a (motile) rugose variant of *V. cholerae* (N16961R). When the bacterial strains were grown in L-broth before inoculating into motility agar, both N16961S (smooth variant) and N16961R (rugose variant) were motile (Figure 1, #1 and #2), with the rugose variant exhibiting approximately 2.5-fold reduced motility, which is consistent with previous reports described by our group and others [9,28]. To our surprise GASP-700D was non-motile (Figure 1, #3). As expected, the ΔflaA mutant was non-motile (Figure 1, #4). When grown in nutrient-poor FSLW, both N16961S-24 and N16961R-24 strains demonstrated motility, with N16961S-24 exhibiting increased motility compared to the rugose variant (Figure 1, #5 and #6) further corroborating that the rugose variant is less motile than its smooth counterpart. Interestingly, GASP-700D, in contrast to N16961S-24, did not move from the point of inoculation, even after 24 h of growth in motility agar (Figure 1, #7). As expected, the ΔflaA mutant was non-motile (Figure 1, #8). Our data suggest that unlike the bipolar flagella of N16961S-24, GASP-700D did not facilitate productive motility both in L-broth and FSLW. To ensure that GASP-700D was viable at the inoculation site, we examined a block of agar consisting of the entire inoculation site as described in methods section. We obtained ca. 1×10⁶ cfu, confirming that GASP-700D was surviving inside the agar but defective in motility.

As GASP-700D exhibited no motility in soft agar, we further investigated using qRT-PCR to determine whether flagellar genes, including flaA (encodes critical flagellin), βCF (encodes regulator of Class III flagellar genes), motL and motB (encode flagellar motor) and flaC (encodes master regulator of all flagellar genes) were repressed in GASP-700D relative to N16961S-24. A previous study reported that phoB and Pst system genes of *V. cholerae* were expressed in nutrient-poor FSLW compared to nutrient-rich L-broth, whereas ctxA and tcp genes were repressed under the same conditions [7].

We first compared the relative expression of phoB, Pst-system genes, ctxA and tcp genes by N16961S-24 and GASP-700D grown in nutrient-poor FSLW to that of wild-type *V. cholerae* N16961S grown in nutrient-rich L-broth in otherwise identical growth conditions (Figure S1). The phoB and Pst-system genes were highly expressed, while tcp genes and ctxA were repressed, by N16961S-24 and GASP-700D grown in FSLW relative to N16961S grown in nutrient-rich L-broth, confirming the results of the previous study [7]. Additionally, expression of the flagellar genes, except βCF, was also down-regulated in GASP-700D, as well as in the rugose N16961R-24 variant, compared to their expression in N16961S-24 when grown in FSLW. Strikingly, βA, the master flagellar regulatory gene, was 1,000-fold down-regulated (p<0.005) in GASP-700D compared to N16961S-24, suggesting that flagellar synthesis is down-regulated in GASP-700D (Figure 2). Taken together, our results suggest that GASP-700D may have lost peritrichous flagella and/or some flagellar gene(s) might have sustained mutation(s) in GASP-700D resulting in the defect of productive motility. Indeed, microorganisms surviving for long-time in stressful stationary growth cultures can result in the selection of mutants that express GASP phenotype [12].

**Comparative Assessment of Biofilm Formation between N16961S-24 and GASP-700D of *V. cholerae***

We previously reported that 700 days-old persister cells showed a high degree of cell to cell aggregation compared to N16961S-24 [11]. Furthermore, the flagella of N16961S-24 allow motility, whereas GASP-700D does not facilitate productive motility. Because *V. cholerae* motility and the polar flagellum contribute to biofilm formation [19,29], we were interested in determining the role(s) of the novel bipolar and possible non-productive/deleted peritrichous flagella elicited by N16961S-24 and GASP-700D, respectively, in biofilm formation when grown in nutrient-poor FSLW. As *V. cholerae* biofilm is produced and positively regulated by vps genes and vpsR gene, respectively, we created vpsR and vpsA in-frame deletion mutations in the background of N16961S, N16961R and GASP-700D. As expected, vpsR and vpsA mutants inhibited rugose colony phenotype (Figure 3A) [8,24]. We initially
measured biofilm production by *V. cholerae* strains, including N16961S-24, N16961R-24 and GASP-700D, and in-frame deletion mutants of the *vpsR* and *vpsA* biofilm genes, in the background of N16961S (Δ*vpsR* and Δ*vpsA*), N16961R (Δ*vpsR* and Δ*vpsA*) and GASP-700D (GASP-700DΔ*vpsR* and GASP-700DΔ*vpsA*). Figure 4B shows the quantitative analysis of biofilm formation which indicates that all strains except SAΔ*vpsR* produced increased biofilm (p<0.005) compared to N16961S-24. We stained biofilms formed by N16961S-24, N16961R-24 and GASP-700D in FSLW with ruthenium red, and examined the biofilm matrix using transmission electron microscopy (TEM) (Figure 5). Copious amounts of exopolysaccharide matrix could be detected surrounding the N16961R-24 cells, whereas very little exopolysaccharide matrix could be seen in the biofilm of N16961S-24. Likewise, GASP-700D biofilms appeared to contain very little exopolysaccharide matrix, suggesting that GASP-700D forms VPS-independent biofilms. Taken together, our data support the idea that GASP-700D produced biofilm specific to FSLW and that this biofilm is independent of VPS-mediated biofilm.

**Stress Resistance**

We and others have previously reported that *V. cholerae* rugose variants, that produce copious amounts of exopolysaccharide and biofilm, can resist chlorine, oxidative, and osmotic stresses [8,9,10]. As GASP-700D produced FSLW-specific biofilm, we investigated whether this phenotype, like rugose phenotype can resist diverse stresses [31,32]. To this context, we subjected GASP-700D to H₂O₂, chlorine, and NaCl stresses. We note that there were no obvious growth differences among *V. cholerae* strains grown in L-broth and examined in this study (data not shown). Interestingly, we observed that, like N16961R-24, GASP-700D was more resistant to H₂O₂ in FSLW (p<0.005) compared to N16961S-24 (Figure 6). However, unlike N16961R-24, GASP-700D was as susceptible as N16961S-24 when exposed to chlorine and osmotic stresses (data not shown).

**Discussion**

Recently, we reported a *V. cholerae* “persister” phenotype which is a key step in the understanding of the long-term survival of *V. cholerae* in the environment. However, substantial work still needs to be done to understand this phenotype, and to assess its role in cholera transmission. In the current study, we provide evidence...
that glycerol stored persister cells (700 days-old cells) have transitioned to what appeared to be a growth advantage in stationary phase (GASP) phenotype. Compared to its wild-type strains (N16961S-24 and N16961S), GASP-700D phenotype of *V. cholerae* exhibited: (i) non-motile phenotype, (ii) enhanced exopolysaccharide production and biofilm formation that are specific to

**Figure 3. Colony morphology and associated biofilms (measured quantitatively) produced by each *V. cholerae* strain.**

(A) Colony morphology: each *V. cholerae* strain was subcultured on L-agar and incubated overnight at 37°C before images were acquired; (B) Quantitative measurement of biofilm produced by each *V. cholerae* strain in nutrient-rich L-broth; and (C) Quantitative measurement of biofilm produced by each *V. cholerae* strain in nutrient-poor FSLW. All the values are expressed as means ± standard deviation (SD) from at least triplicate experiments. P-values are computed by comparing the biofilm formation of each strain with that of N1961S-24 using one-way ANOVA test. A p-value of <0.005 was considered statistically significant.

**Figure 4. Topography and architecture of *V. cholerae* biofilms.**

Each strain was grown in a 4-well cell culture plate containing 500 µl FSLW. A glass cover slip was dipped into each culture well and incubated overnight statically at room temperature. The glass cover slips were stained with SYTO 9 and the images were obtained using a laser scanning confocal microscopy with an excitation and emission wavelengths of 484 and 500 nm, respectively. (A) Images of x–y sections (top panels) and x–z projections of the same biofilms (bottom panels) were analyzed with DAIME software; magnification, x200. (B) Average biofilm heights (µm) for each strain measured across five random x–z sections. (C) Total volume of biofilm (µm³) for each strain calculated by x–y and x–z projections. A p-value of <0.005 was considered statistically significant.
FSLW, and independent of θφ, (iii) resistance to oxidative stress, and (iv) small colony phenotype. The storage and subculture of persister cells in glycerol broth at −80 °C may have influenced the observed phenotype seen with GASP-700D as described above.

We hypothesize that, during long-term survival (700 days) in stressful stationary culture, *V. cholerae* may have adopted two responses, including: (i) assume “persister” phenotype [11], and (ii) select GASP mutants that successfully adapt to stressful growth conditions [12]. Although we currently have no supporting evidence to conclude that GASP-700D genome has any mutation, we did observe that GASP-700D is defective in productive motility implying that GASP-700D may have possible mutation(s)/alteration in its genome. We propose that GASP-700D represents a GASP phenotype. Indeed, previous reports demonstrated that GASP phenotypes with genetic mutations are common in microorganisms surviving long-term in stressful and stationary growth phase.

The nutrient-poor growth conditions in FSLW affect the motility of *V. cholerae* even before its transition to GASP-700D in a phase-dependent manner. The smooth variant exhibited reduced motility in soft agar after 24 h growth in FSLW in contrast, the rugose variant, which normally shows reduced motility in comparison with the smooth variant, was unaltered for motility after 24 h growth in FSLW. Once the bacteria have transitioned into GASP-700D, however, they appear non-motile in this assay (Figure 1). qRT-PCR revealed a dramatic down-regulation (1000-fold) of flaA expression in GASP-700D (Figure 2). FlrA is the “master regulator” of the flagellar transcription hierarchy [33]. It is the sole Class I flagellar factor that activates σ^54^-dependent transcription of Class II flagellar genes, thus initiating flagellar synthesis. It is not known how flaA transcription is itself controlled in *V. cholerae*, but expression of the FlrA homologue FleQ in *Pseudomonas aeruginosa* has been shown to be negatively regulated by the alternate sigma factor AlgP, which results in loss of motility that is simultaneous with increased polysaccharide expression and biofilm formation [34]. It is not clear whether the reduction in flaA transcription is responsible for the non-motile phenotype, because interestingly, transcription of other flagellar genes within the transcription hierarchy, including the Class III regulator flcC, the motor genes motB and motE, and the major core flagellin, flaA, were not dramatically reduced in GASP-700D. It has been shown previously that mutation of flhG leads to the expression of multiple polar flagella, and the flaA* V. cholerae* strain appears non-motile in soft agar, possibly due to an inability to effectively coordinate flagellar function [35].

Previous studies of *V. cholerae* biofilm formation have mostly focused on nutrient-rich growth conditions either in static and/or in flow-cell methods [9,36]. Under these conditions, the rugose variant produces robust biofilms with three dimensional pillars and columns [36]. Here, we studied biofilms formed in nutrient-poor FSLW conditions that more closely mimic the natural environment of *V. cholerae* [4,37]. We found that nutrient-poor conditions promote much less biofilm formation than the nutrient-rich conditions; even with the rugose variant (Figures 3B and 3C). Our previous study demonstrated that a number of sugars, including sucrose and glucose, inhibited *V. cholerae* exopolysaccharide expression [38]. In contrast, glucose promoted biofilm production by *Staphylococcus aureus* [39,40]. Our observations suggest that physical and chemical parameters, including nutrient composition, pH, and attachment surfaces, can influence the outcome of biofilm formation by *V. cholerae*.

GASP-700D produces a well-developed biofilm in FSLW that appears predominantly coalesced rather than scattered. In contrast, the rugose variant forms well-developed but scattered biofilms (Figure 4A). However, in the absence of the VPS genes...
Δψr or ΔψdA, the rugose variant forms biofilms with similar coalesced characteristics to GASP-700D in this medium, as does a ΔψdA, GASP-700DΔψr and GASP-700DΔψdA mutants (Figures 4A and 4B). This suggests that GASP-700D and the strains lacking ψr genes form biofilms that are independent of V. cholerae 14. Zinser ER, Kolter R (2004) Evolution of microbial diversity during prolonged V. cholerae 13. Finkel SE, Kolter R (1999) Oxidative stress resistance may be due to the alternative biofilms resistant to oxidative stress than either smooth or rugose variants. 

GASP-700D using qRT-PCR. N16961S was grown both 

in contrast to the abundant exopolysaccharide in the rugose variant biofilms, suggesting that the GASP-700D biofilms may contain yet to be defined biofilm matrix. Such a putative extracellular matrix could drive the development of the alternative, coalescing biofilms seen in the GASP-700D which is more resistant to oxidative stress than either smooth or rugose variants. Oxidative stress resistance may be due to the alternative biofilm matrix. Ruthenium red staining failed to detect exopolysaccharide in the GASP-700D biofilms in FSLW (Figure 6), in 

Supporting Information

Figure S1  Comparative analysis of the differential gene expression among V. cholerae strains N16961S and GASP-700D using qRT-PCR. N16961S was grown both in nutrient-rich L-broth and in nutrient-poor FSLW (N16961S-24) (ca. 10^8 cfu/ml), and the cultures were incubated overnight statically at room temperature. GASP-700D was grown (ca. 10^6 cfu/ml) in FSLW only. Expression of each gene was normalized to that of toxR, and subsequently compared to that of the wild-type N16961S grown in L-broth. Data represent the average results of three independent experiments and error bars indicate as means ± standard deviation (SD).

(TIF)

Table S1 Oligonucleotide primers used in this study. (DOCX)

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Author Contributions

Conceived and designed the experiments: AA. Performed the experiments: MJ KR AA. Analyzed the data: MJ KR AA. Contributed reagents/materials/analysis tools: KEK MY OA AA. Wrote the paper: JGM AA.

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