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Host-induced epidemic spread of the cholera bacterium

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Abstract

The factors that enhance the transmission of pathogens during epidemic spread are ill defined. Water-borne spread of the diarrhoeal disease cholera occurs rapidly in nature, whereas infection of human volunteers with bacteria grown *in vitro* is difficult in the absence of stomach acid buffering1. It is unclear, however, whether stomach acidity is a principal factor contributing to epidemic spread2. Here we report that characterization of *Vibrio cholerae* from human stools supports a model whereby human colonization creates a hyperinfectious bacterial state that is maintained after dissemination and that may contribute to epidemic spread of cholera. Transcriptional profiling of *V. cholerae* from stool samples revealed a unique physiological and behavioural state characterized by high expression levels of genes required for nutrient acquisition and motility, and low expression levels of genes required for bacterial chemotaxis.

Frequent cholera outbreaks occur in Dhaka, Bangladesh, providing the potential to study the bacterium within its natural setting. We collected stool samples from patients at the International Centre for Diarrhoeal Disease Research (ICDDR), Bangladesh, and performed serological analysis to identify stools that were positive for *V. cholerae* O1 Inaba El Tor. These were immediately used in murine infection studies. A strain grown *in vitro* (DSM-V984) was mixed with stool *V. cholerae* and the mixture was used to inoculate 3–5-day-old Swiss Webster mice by gavage. DSM-V984 is an O1 Inaba El Tor strain that was isolated the previous year at the ICDDR, and is marked by deletion of the *lacZ* gene to allow for enumeration of stool ($LacZ^+$) and *in vitro*-grown (DSM-V984; $LacZ^-$) bacteria. Mice were

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euthanized after 20–24 h, and bacteria were recovered by homogenization of the small intestine and plating on medium supplemented with a chromogenic substrate for LacZ. The output ratios of stool-sample *V. cholerae* to DSM-V984 were corrected for variations in the input ratios and represent the competitive indices (CI) of the stool *V. cholerae*. A CI above 1 indicates enhanced infectivity, whereas an index below 1 indicates decreased infectivity. As shown in Fig. 1a, *V. cholerae* shed from the human gastrointestinal tract (human-shed) showed greatly enhanced infectivity, out-competing the *in vitro*-grown strain by as much as 700-fold. A competitive advantage was consistently observed with samples collected from numerous patients on different days. The competitive advantage was entirely lost, however, when *V. cholerae* was colony purified from a stool sample and cultured *in vitro* in broth for 18 h before infection (CI geometric means of 1.1, 1.1 and 1.5 for three independent stool isolates and 8–10 mice per competition), indicating that passage through the human host transiently increased the infection potential of the stool-derived bacteria.

To have a significant role in the epidemic spread of cholera, the competitive advantage of stool-derived bacteria would have to persist after dissemination back into the environmental reservoir. We therefore tested whether human-shed *V. cholerae* maintained the hyperinfectious phenotype after incubation in local pond water. Freshly shed *V. cholerae* positive stool samples were diluted into pond water that was free of *V. cholerae* contamination. After incubation at room temperature (29 °C) for 5 h, the diluted samples were mixed with the *in vitro*-grown competitor strain, and this mixture was used to infect mice as described above. As shown in Fig. 1b, the hyperinfectious state of the human-shed bacteria remained intact. We propose that human passage of *V. cholerae* enhances subsequent water-borne spread of the cholera bacterium by effectively lowering the infectious dose in secondary individuals.

As the increased infectivity of stool-derived *V. cholerae* was lost after growth *in vitro*, the phenotype is transiently expressed. Therefore, to characterize the hypervirulent state we conducted transcriptional profiling of human-shed *V. cholerae* using a spotted DNA microarray3 containing representations of approximately 87% of the identified open reading frames (ORFs) of the Bangladeshi O1 El Tor reference strain N16961 (ref. 4). O1 Inaba El Tor positive stool samples were obtained from three patients in the ICDDR, filtered to remove particulate matter, then immediately frozen for later preparation of total RNA. These samples showed the canonical ‘rice water’ appearance that characterizes cholera stools, contained approximately 100 million *V. cholerae* per millilitre, and showed relatively few contaminating bacteria as assessed by dark-field microscopy and plating5. Stool RNA preparations were analysed by agarose gel electrophoresis to ensure RNA integrity and the absence of contaminating eukaryotic RNA. DSM-V999 was isolated from one of the three stool samples, and total RNA was isolated after *in vitro* growth to stationary phase (growth conditions identical to those used for the growth of DSM-V984 in the competition assays described above). One microgram of RNA from each of the samples was used for complementary DNA synthesis, labelled with Cy5 and hybridized to the microarray in the presence of a Cy3-labelled common reference (exponentially growing O1 Inaba El Tor strain 92A1552). Each sample was hybridized in quadruplicate and the relative fluorescent intensities determined by scanning with an Axon scanner. Data were quantified, normalized

and corrected to yield the relative transcript abundance of each gene as an intensity ratio with respect to that of the reference signal. These intensity ratios were then used to identify statistically significant differences in gene expression using the Statistical Analysis for Microarrays (SAM) program6. A two-class SAM analysis was conducted using the strain grown *in vitro* as class I, and each individual stool sample as class II. Genes with statistically significant changes in the level of expression—at least a twofold change—in each patient sample were chosen, and the derived data from individual stool samples were collapsed to identify genes that were differentially regulated in all three samples. According to these criteria, 237 genes were differentially regulated: 44 genes were induced and 193 genes were repressed in human-shed *V. cholerae* (Fig. 2 and Supplementary Information).

The transcriptomes (the complete collection of transcribed RNAs) of the stool-derived *V. cholerae* samples were similar to that of strain DSM-V999 cultured in rich medium, as 3,120 out of 3,357 ORFs examined were expressed at roughly equivalent levels. Notable differences included increased expression of an assortment of genes required for biosynthesis of amino acids, iron uptake systems, ribosomal proteins, and formation of a periplasmic nitrate reductase complex that may allow for respiration under low oxygen tension. This transcriptome is consistent with bacterial growth in an oxygen- and iron-limited host compartment—the conditions encountered in rice-water stools7. Combining these observations, we propose that *V. cholerae* departs from a rich growth environment within the small intestine and moves into a nutrient poor lumenal fluid that is rapidly purged. The physiological growth state of the stool bacteria and the capability for rapid growth could contribute to the observed hyperinfectious phenotype, as rapidly growing *V. cholerae* out-compete stationary phase cells (A.C., unpublished data, and J. J. Mekalanos, personal communication). However, hierarchical clustering of stationary phase, exponential phase and human-shed *V. cholorae* arrays revealed that *V. cholorae* isolated from patients cluster no closer to the exponential phase than to the stationary phase arrays, suggesting that on a genome-wide scale, the transcriptome bears traits of both of the growth phases (data not shown).

None of the genes that showed differential regulation were members of the ToxR/TcpP/ToxT virulence gene regulon8–11. Genes within this regulon include those for cholera toxin and factors coded for on the *Vibrio* pathogenicity island, such as the toxin co-regulated pilus. These factors are necessary for successful infection of humans and mice12. These results suggest that, before being shed, *V. cholerae* turns off expression of these particular virulence genes as part of a programme for dissemination to the environment and possible transmission to new hosts. These findings also indicate that the increased expression of this virulence regulon is not required for the increased infectivity exhibited by stool bacteria.

Among the genes differentially expressed in the stool samples are VC0028, VC0941, VC0869, VC0051, VC0647, VC0468, VC2350 and VCA0583, all of which were recently identified in a genetic screen for factors required for infection of infant mice13. VC0647 (*pnp*) and VC0468 (*gshB*) were additionally shown to have a role in the ability of *V. cholerae* to mount an acid tolerance response (ATR). *In vitro* induction of the ATR enhances the infectivity of *V. cholerae*14; however, our microarray analysis revealed that other genes known to be induced during the ATR were not differentially regulated in stool

samples, suggesting that if ATR has an involvement in the increased infectivity of human-shed *V. cholerae*, it is through pathways yet to be elucidated.

The most highly induced gene that we identified is one that was not annotated on completion of the *V. cholerae* genome sequence. As our *V. cholerae* microarray was constructed before annotation of the published sequence15, it contains sequences of some ORFs that did not meet cut-offs imposed during the course of annotation. 'VCA0934.5' (residues 885680–885886) lies in the region between VCA0933 and VCA0935, is transcribed in the same direction as the upstream VCA0933, and partially overlaps the divergently transcribed VCA0934. VCA0934.5 has a predicted strong Shine–Dalgarno sequence and would code for a protein comprising 69 amino acids that shows no significant similarity to any proteins contained in public databases and bears no known protein motifs. Elucidation of the function of this factor will be of great interest and its discovery emphasizes the importance of analysis of DNA sequences that lie outside of the cut-offs commonly used for genomic annotation in bacteria.

A marked number of genes involved in chemotaxis showed altered expression levels: expression of these genes was repressed in the stool-derived *V. cholerae*. The role of chemotaxis in virulence of *V. cholerae* is unclear16,17, although several genes required for chemotaxis are known to be crucial for expression of cholera toxin during infection18. Notably, these latter genes, including *cheA-2*, *cheY-3* and *cheZ*, did not show altered expression in the stool samples. Instead, most of the remaining chemotaxis genes were repressed, including genes for all three CheW linker proteins, three out of four CheV linker proteins, all three CheR methyltransferases, and 17 methyl-accepting chemotaxis proteins. Although the stool-derived *V. cholerae* were highly motile as assessed by dark-field and phase-contrast microscopy, many of the gene families that are essential for chemotactic signalling and adaptation to chemoattractant and chemorepellent gradients were repressed. These results suggest that these motile bacteria are non-chemotactic on exiting the human host, and imply that downregulation of the chemotactic response is linked to dissemination from the host and re-entry to the aquatic environment. One possibility is that repression of chemotaxis increases shedding from the gastrointestinal tract. Finally, loss of the chemotactic response in otherwise motile *V. cholerae* was recently shown to markedly increase infectivity in an infant mouse model of cholera18. It is possible that the motile but non-chemotactic state combined with the unique physiological state of human-shed *V. cholerae* mediates the observed increase in infectivity. Because this transcriptional profiling provides only a 'snap-shot' of gene expression at the moment of dissemination, an important next step will be the elucidation of the proteome of human-shed *V. cholerae*.

Our findings identify the process by which cholera epidemics may be propagated by the human host, and provide insight into gene expression patterns of the disseminating microorganisms. Passage through the human gastrointestinal tract induces a hyperinfectious state, which is perpetuated even after purging into natural aquatic reservoirs. The fact that the human host not only provides a suitable niche for growth but also prepares *V. cholerae* for infection of additional humans has interesting implications for the study of human-to-human spread of other virulent microorganisms. Perhaps other epidemic pathogens are spread in a manner whereby the agent is made more infectious before or during exit from its

primary host. Elucidation of the gene expression profiles of disseminating microorganisms should provide new targets for antimicrobial therapy to disable transmission and for vaccine development to prevent infection.

Methods

Strains, sample collection and competition assays

Strain DSM-V984 was differentially marked at the *lacZ* locus by mobilization of the suicide plasmid pGP704::*lacZ* and selection on media supplemented with ampicillin and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) as described previously19. Patients admitted to the ICDDR, Bangladesh, showing clinical manifestations of cholera had rice-water stools pre-screened by dark-field microscopy to verify the presence of *V. cholerae*, and by inhibition of dark-field motility using monoclonal antibodies to determine serogroup and serotype. Freshly shed stool samples were collected in sterile beakers, filtered through cheesecloth, and a portion was frozen at –80 °C for subsequent RNA preparation. The remainder of the stool sample was used in competition assays. Human studies protocols were reviewed and approved by the Research Review Committee and Ethical Review Committee at the ICDDR, Bangladesh, and by the Institutional Review Board at the Massachusetts General Hospital.

Competition assays were conducted by mixing DSM-V984 grown on LB medium overnight with stool bacteria in a ratio of 1:10 (v/v). A total of 50μl of a 1,000-fold dilution of this mixture, representing 10^4–10^5 colony-forming units (c.f.u.), was administered to 3–5-day-old Swiss Webster mice by gavage as described14. After 20–24 h, animals were euthanized, the small intestines removed, homogenized and plated on LB medium supplemented with X-gal to allow for subsequent enumeration of $LacZ^+$ and $LacZ^-$ colonies. Outputs ranged from 10^5 to 10^7 c.f.u. per small intestine. Ratios of output bacteria were corrected for deviations in input ratios from 1:1 and represent the competitive index of the strains in question. Competitions with water-incubated stool-derived bacteria were conducted as above with the exception that stool-derived *V. cholerae* were diluted into pond water and were incubated at room temperature for 5 h before mixing with DSM-V984 and inoculation into mice. The pH of the two pond water samples was 7.0–7.5.

Microarray analysis

The construction of the *V. cholerae* DNA microarray will be described in greater detail elsewhere. Briefly, after the completion of the genomic sequence of N16961 by the Institute for Genomic Research, but before annotation of the genome, predicted unique ORFs were found using two separate ORF-finding programs, and a portion of each ORF was amplified by polymerase chain reaction (PCR) and spotted onto poly-L-lysine-coated glass slides15. The array consists of 5,222 spots representing 3,357 ORFs with an average size of 600 base pairs per PCR product.

Total *V. cholerae* RNA was collected from stool samples and strain DSM-V999 grown overnight *in vitro* using TRIzol reagent (GIBCO-BRL) as described15, with the exception that RNA was collected on an RNeasy column (QIAGEN) immediately after isopropanol precipitation. DNase treatment to remove DNA contamination was conducted before elution

from the column. Quantities of RNA were determined by measuring absorbance at 260/280 nm (A_{260}/A_{280}) and integrity was verified by visualization on a 1% agarose gel. Equal concentrations of each test RNA and the common reference RNA (exponentially growing 92A1552) were used for reverse transcription reactions that directly incorporate Cy5- and Cy3-labelled nucleotides into the cDNA as described15. Labelling reactions were performed in duplicate on two separate days, resulting in quadruplicate arrays for each strain derived from stool samples or grown *in vitro*. The hybridizations and data analyses were performed as described in the main text. Control arrays were also hybridized to identify genes potentially affected by the stool-freezing process, and statistically significant changes that matched patient stool sample regulation were eliminated from subsequent analysis (all microarray data are provided at http://genome-www5.stanford.edu/microarray/SMD/).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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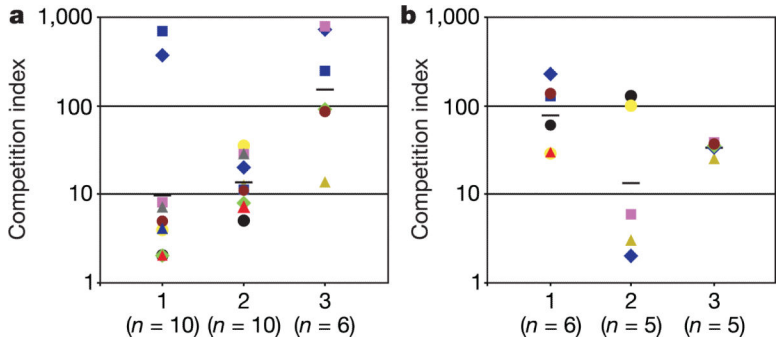

Figure 1.

Human-shed *V. cholerae* are hyperinfectious in competition assays in infant mice. **a**, **b**, Freshly isolated stool samples from six patients (representing experiments 1, 2 and 3 on the *x* axes in **a** and **b**) were either mixed directly with *in vitro*-grown DSM-V984 (**a**), or incubated in local pond water for 5 h before mixing (**b**), and then used for intragastric infection of animals. Data points represent the output ratio of stool-derived *V. cholerae* to *in vitro*-grown competitor strain after correction for deviations in input ratios. Each data point represents the output from a single animal and symbols and colours are used to facilitate visual discrimination of data values. The geometric mean is indicated by a horizontal bar. *n*, total number of animals per experiment.

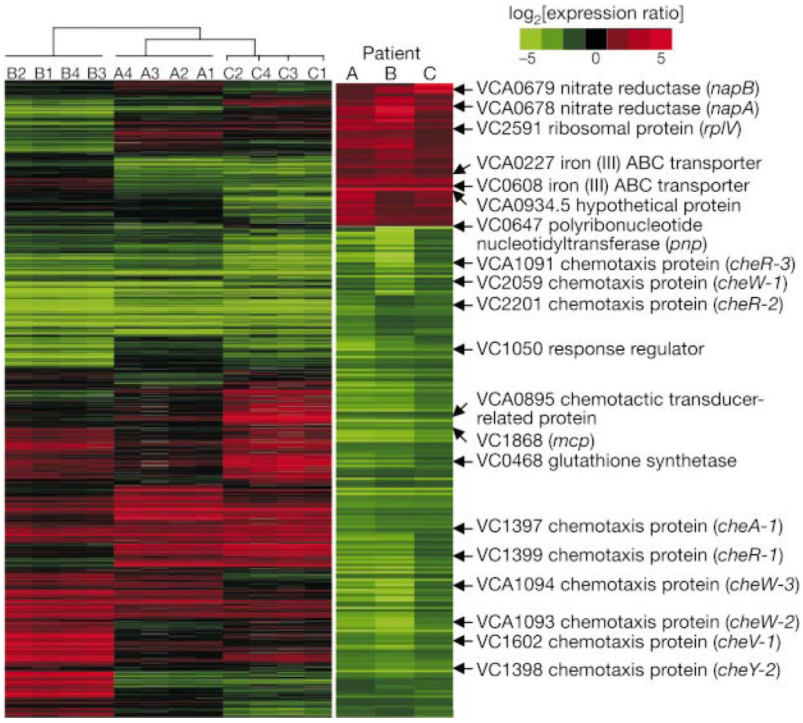

Figure 2.

Transcription profile of human-shed *V. cholerae*. Cluster diagram showing the whole-genome expression profile of *V. cholerae* recovered from the stools of three ICDDR patients (left panel). Patient samples are labelled A–C and replicate arrays are indicated by the numbers 1–4. The dendrogram shows tight clustering of technical replicates. The right panel is a cluster diagram of genes showing consistent differential regulation from patients A, B and C. Fold changes relative to *V. cholerae* grown *in vitro* were calculated using the average values from the quadruplicate arrays shown on the left, and are schematically represented here. Red indicates a minimum twofold increase in expression; green represents a minimum twofold reduction in expression. Representative genes are listed and their relative location indicated by an arrow. See Supplementary Information for a complete list with relative fold changes.