Genetic analysis of *Vibrio parahaemolyticus* intestinal colonization

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Edited by Margaret J. McFall-Ngai, University of Hawaii at Manoa, Honolulu, HI, and approved April 18, 2016 (received for review January 31, 2016)

Vibrio parahaemolyticus **is the most common cause of seafood-borne gastroenteritis worldwide and a blight on global aquaculture. This organism requires a horizontally acquired type III secretion system (T3SS2) to infect the small intestine, but knowledge of additional factors that underlie *V. parahaemolyticus* pathogenicity is limited. We used transposon-insertion sequencing to screen for genes that contribute to viability of *V. parahaemolyticus* in vitro and in the mammalian intestine. Our analysis enumerated and controlled for the host infection bottleneck, enabling robust assessment of genetic contributions to in vivo fitness. We identified genes that contribute to *V. parahaemolyticus* colonization of the intestine independent of known virulence mechanisms in addition to uncharacterized components of T3SS2. Our study revealed that *toxR*, an ancestral locus in *Vibrio* species, is required for *V. parahaemolyticus* fitness in vivo and for induction of T3SS2 gene expression. The regulatory mechanism by which *V. parahaemolyticus* ToxR activates expression of T3SS2 resembles *Vibrio cholerae* ToxR regulation of distinct virulence elements acquired via lateral gene transfer. Thus, disparate horizontally acquired virulence systems have been placed under the control of this ancestral transcription factor across independently evolved human pathogens.**

Vibrio parahaemolyticus | transposon-insertion sequencing | type III secretion | bacterial pathogenesis | pathogen evolution

The gram-negative γ-proteobacterium *Vibrio parahaemolyticus* thrives in either pathogenic or symbiotic association with marine organisms and as a planktonic bacterium (1). This facultative human pathogen, abundant in aquatic environments, was first isolated following a food poisoning outbreak in 1952 and has emerged as the leading cause of seafood-associated gastroenteritis worldwide and a blight on global aquaculture (2, 3). Sequencing of the *V. parahaemolyticus* genome and the development of animal models of infection have demonstrated a critical role for type III secretion in *V. parahaemolyticus* virulence (4, 5).

Pathogenic isolates of *V. parahaemolyticus* encode two type III secretion systems (T3SSs), which are multiprotein structures that mediate the translocation of bacterial effector proteins directly into eukaryotic cells (4, 6). All *V. parahaemolyticus* strains encode a T3SS on the large chromosome (T3SS1), and the vast majority of clinical isolates, but few environmental isolates possess a horizontally acquired pathogenicity island (VPaI-7) encoding a second T3SS (T3SS2) and one or more pore-forming toxins (TDH) (7). Studies using the infant rabbit model of *V. parahaemolyticus* infection, which recapitulates manifestations of human gastrointestinal disease (e.g., profuse diarrhea, enteritis, epithelial disruption), revealed that although T3SS1 and TDH are dispensable for intestinal colonization and pathogenesis, colonization and pathology are dependent on T3SS2, consistent with the epidemiological association between T3SS2 and pathogenicity (5). Furthermore, T3SS2 gene expression is induced during intestinal colonization, likely in response to bile, which promotes production of *V. parahaemolyticus* T3SS2 regulator B (VtrB) (8, 9).

V. parahaemolyticus is presumed to use many bacterial factors, in addition to T3SS2, to survive and proliferate within the human gastrointestinal tract. Putative colonization factors have been reported using a murine orogastric model of *V. parahaemolyticus* infection; however, the relevance of these findings to human disease is unclear because T3SS2 is not required for colonization of the mouse intestine (10). Genome-wide analysis of *V. parahaemolyticus* colonization factors has not been performed in any host; however, for other pathogens, passage of transposon-insertion libraries through infection models has enabled highly effective, largely unbiased, and genome-wide identification of factors required for fitness in vivo (11).

We used transposon-insertion sequencing (TIS) to categorize genetic loci based on contributions to *V. parahaemolyticus* viability in vitro and in vivo (11). We discovered genes and regulatory networks that contribute to intestinal colonization independent of the few known *V. parahaemolyticus* virulence factors. We identified uncharacterized components of T3SS2 and found that ToxR, an ancestral *Vibrio* transcription factor previously thought to be dispensable for regulation of T3SS2 gene expression (9), is, in fact, critical for *V. parahaemolyticus* intestinal colonization and T3SS2 activity. ToxR is also an important regulator of horizontally acquired virulence genes in *Vibrio cholerae*, where it controls an unrelated set of virulence elements acquired via lateral gene transfer (12). Thus, pathogenic vibrios have independently linked ToxR, which is responsive to signals encountered in the intestine, to control of critical virulence factors.

Significance

We conducted a genome-wide screen to identify bacterial factors required for *Vibrio parahaemolyticus*, an important cause of seafood-borne gastroenteritis, to survive in vitro and colonize the mammalian intestine. Our analysis revealed uncharacterized components of a horizontally acquired type III secretion system linked to virulence (T3SS2) and hundreds of genes that likely contribute to colonization independent of T3SS2. Our work revealed that *toxR*, a conserved gene in vibrios that governs expression of horizontally acquired virulence factors in *Vibrio cholerae*, was critical for expression of T3SS2. Thus, expression of disparate virulence-linked elements, acquired via lateral gene transfer in independently evolved pathogenic vibrios, is controlled by a common ancestral transcription factor.

Author contributions: T.P.H., M.C.C., S.A., and M.K.W. designed research; T.P.H., C.J.B., and X.Z. performed research; T.P.H., S.A., and P.A.z.W. contributed new reagents/analytic tools; T.P.H., M.C.C., S.A., P.A.z.W., B.M.D., and M.K.W. analyzed data; and T.P.H., B.M.D., and M.K.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1601718113/-/DCSupplemental.

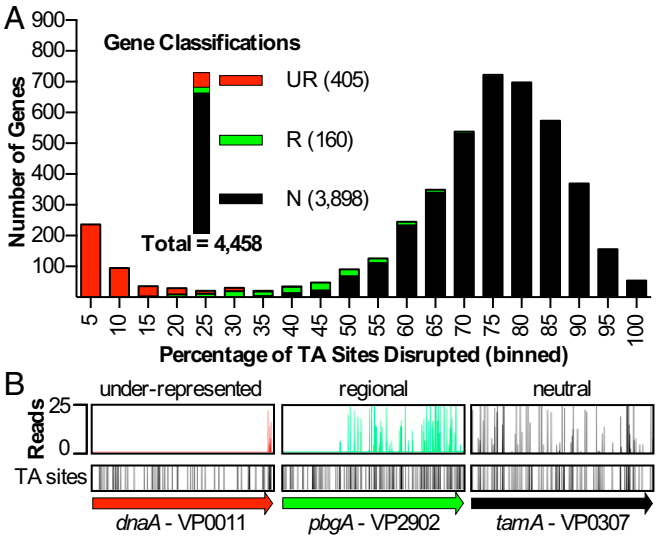

Fig. 1. EL-ARTIST gene classifications. (*A*) Distribution of percentage disruption for genes with 10+ TA sites. Genes classified as underrepresented (UR), regional (R), and neutral (N) are represented within each bin and in aggregate. (*B*) Transposon-insertion profiles of representative underrepresented, regional, and neutral genes. The vertical axis indicates the number of reads mapped to a TA site along the horizontal axis.

Results

TIS Identifies Genes Required for Viability in Vitro. We generated a high-density transposon-insertion library in a spontaneous streptomycin-resistant mutant (Sm^R) of *V. parahaemolyticus* RIMD 2210633, a sequenced clinical isolate of the pandemic O3:K6 serotype, using a Mariner-based transposon, which inserts at TA dinucleotides without additional sequence constraints (4, 13). The relative abundance of individual insertion mutants within such a library reflects each mutant's fitness (11). Sequencing-based characterization of the *V. parahaemolyticus* library identified at least 179,373 distinct mutants with an average of ~20 reads per insertion site (Fig. S1). Plotting the frequency of genes relative to the percentage of TA sites disrupted per gene revealed two distinct populations (Fig. 1*A*). The major peak, representing genes tolerant of transposon insertion, is centered at ~75% of TA sites disrupted, whereas the minor peak consists of genes with a lower frequency of disruption, many of which are likely required for bacterial viability (14).

We used EL-ARTIST, a TIS analysis pipeline, to categorize genes as "underrepresented" or "neutral" (Fig. 1*B*) based on the insertion profile across each locus relative to genomic context (14). Neutral loci are presumed to be dispensable for *V. parahaemolyticus* growth in vitro, in contrast to underrepresented loci, which lack corresponding insertion mutants. The analysis also identified "regional" loci (Fig. 1*B*), which lack transposon insertions across part, but not all, of a locus. All 4,831 annotated protein-coding genes were analyzed (Dataset S1); however, we excluded 372 genes with fewer than 10 TA sites to promote statistical confidence. Most *V. parahaemolyticus* genes (3,898 genes) were classified as neutral, whereas far fewer genes (565 genes) were classified as regional or underrepresented (Fig. 1*A*).

Gene set enrichment analysis using the cluster of orthologous group (COG) database indicated that the 565 nonneutral genes were disproportionately associated with a subset of biological processes (Fig. 2*A* and Dataset S2). The distribution of COGs among nonneutral genes closely paralleled the distribution of COGs associated with essential and domain-essential genes identified in a TIS assessment of genes required for *V. cholerae* viability in vitro (14) (Fig. S2 and Dataset S2*B*). Interspecies similarities in classification were also observed in gene-by-gene comparisons of the 565 *V. parahaemolyticus* nonneutral genes and homologs in *Escherichia coli* and *V. cholerae*, organisms in which the genes required for viability have been extensively characterized (Fig. 2*B* and Dataset S2*C*). Many nonneutral *V. parahaemolyticus* genes had homologs among the 287 protein-coding genes absent from the Keio collection (a proxy for the *E. coli* essential gene set), and most are reportedly essential or domain-essential in *V. cholerae* (15). Still, a notable fraction of the 565 genes had homologs that were individually dispensable for *E. coli* or *V. cholerae* viability in vitro or lacked homologs in these organisms. A small subset of *E. coli* essential genes (34 genes) was neutral in *V. parahaemolyticus* (Dataset S2*D*) and contained genes similar to those from a comparable analysis of *V. cholerae*, which may be indicative of *Vibrio*-specific adaptations in the associated processes (14). The 565 nonneutral genes likely encompassed all or almost all genes required for *V. parahaemolyticus* growth in vitro. Notably, this set of genes contained loci that have been deleted in previous studies, suggesting that the distribution of insertion mutants within a transposon-insertion library can reflect multiple factors, not solely the viability of each insertion mutant, and that our analysis likely overestimates the number of nonneutral loci in vitro.

Identification of Genes Required for Intestinal Colonization. We used the *V. parahaemolyticus* Sm^R library to identify genes required for colonization of the mammalian gastrointestinal tract. Infant rabbits were orogastrically inoculated with 10^9 cfu of the library and euthanized at the onset of disease (5). Transposon-insertion sites were sequenced for in vivo passaged libraries recovered from the distal small intestine (Fig. S1), the primary site of intestinal pathology (5). We compared the passaged library with the inoculum using a modified version of the Con-ARTIST pipeline, which identifies changes in transposon-insertion profiles following growth

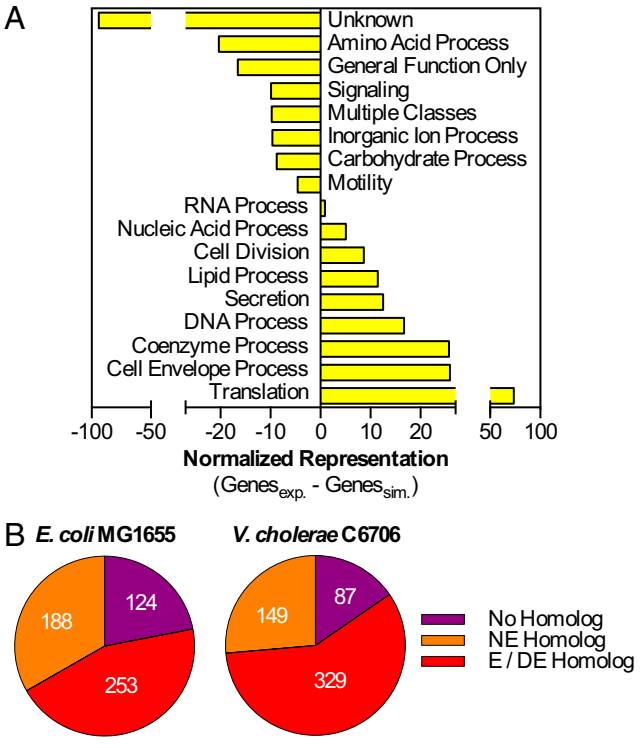

Fig. 2. Bioinformatic analysis of regional and underrepresented genes. (*A*) Abbreviated COG terms with statistically significant, differential representation in the regional and underrepresented datasets (exp.) relative to random sampling of the *V. parahaemolyticus* genome (sim.). (*B*) Status of *E. coli* and *V. cholerae* homologs of the *V. parahaemolyticus* genes classified as regional and underrepresented. E/DE, essential or domain-essential; NE, nonessential.

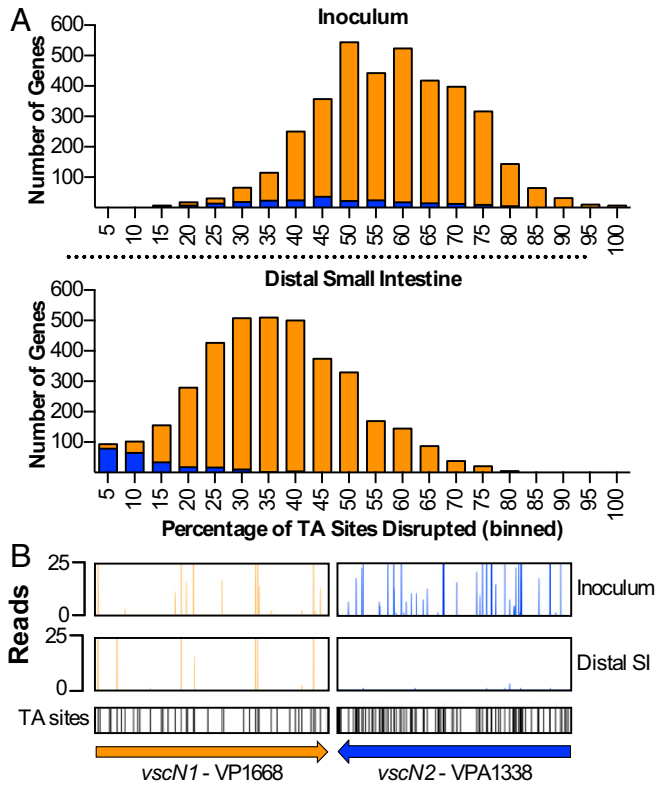

Fig. 3. Identification of conditionally depleted genes. (*A*) Distribution of percentage disruption for genes subjected to statistical analysis (*Materials and Methods*) in the bacterial inoculum (Inoculum) and a representative passaged library (Distal Small Intestine). Genes classified subsequently as conditionally depleted (blue) and all other genes queried (orange) are represented within each bin. (*B*) Transposon-insertion profiles of the neutral locus of *vscN1* and of *vscN2*, a representative conditionally depleted gene. The vertical axis indicates the number of reads mapped to a TA site along the horizontal axis for the simulated inoculum and a representative passaged library (Distal SI).

under a selective condition while compensating for stochastic changes in mutant representation due to experimental bottlenecks (16). Plots of the percentage of TA sites disrupted per gene revealed a leftward shift in a representative passaged library relative to the inoculum (Fig. 3*A*), indicative of an infection bottleneck. The number of unique mutants recovered from individual animals (80,000–100,000 mutants) reflects the minimum size of this bottleneck (Fig. S1), which is comparable to the size of the bottleneck observed for *V. cholerae* infection of the infant rabbit intestine (16–19). Consequently, our analyses were restricted to the 3,744 genes (Dataset S3*A*) found to have sufficient representation within the inoculum to enable discrimination between stochastic loss attributable to the infection bottleneck and loss due to negative selection (*Materials and Methods*). Due to this requirement, loci previously classified as underrepresented were not assayed in vivo.

Two hundred thirty *V. parahaemolyticus* genes were classified as "conditionally depleted" following colonization of the infant rabbit distal small intestine. Conditionally depleted loci displayed a robust, statistically significant, and reproducible reduction in the relative abundance of corresponding mutants in vivo (Fig. 3), suggesting that they are required for, or make a significant contribution to, intestinal colonization. Interestingly, only 34 of the 230 conditionally depleted genes were found to be up-regulated in previous in vivo transcriptional profiling, and the majority encode T3SS2 genes (8) (Fig. S3*A* and Dataset S4*A*). The 230 conditionally depleted genes include structural components of T3SS2, but no corresponding T3SS1 genes, consistent with evidence that T3SS2, but not T3SS1, is required for colonization (5) (Fig. 3*B*). Of 29 T3SS2 genes sufficiently represented in the inoculum to permit analysis, 22 were conditionally depleted, including 10 structural components; *vopV* (VPA1357), an effector protein required for colonization; *vtrB* (VPA1348), a transcriptional regulator; and 10 uncharacterized genes encoded in the T3SS2 gene cluster (6, 20–24) (Fig. 4*A* and Fig. S3*B*). The remaining seven genes were primarily T3SS2 effector proteins previously shown to be dispensable for infection.

Relative to the 3,744 genes analyzed in vivo, conditionally depleted genes were disproportionately associated with a subset of biological processes (Fig. 4*B*), suggesting functions that may be particularly important for *V. parahaemolyticus* fitness in vivo. However, depletion of these mutants may also reflect general fitness defects that become evident during expansion of the library in vivo. To explore the latter possibility, we assessed relative fitness during competitive growth of the library in vitro and found that some in vivo conditionally depleted genes exhibited reduced fitness in vitro as well (Dataset S3*C*), including a subset with homologs that are required for the optimal fitness of *V. cholerae* in vitro and in vivo (16–18) (Dataset S4*B*). Our analysis also identified many in vivo conditionally depleted genes that appeared dispensable for optimal fitness in vitro. Because such genes can offer insight into processes required for in vivo survival, we performed further study of a subset of the genes that appear to contribute specifically to fitness in vivo.

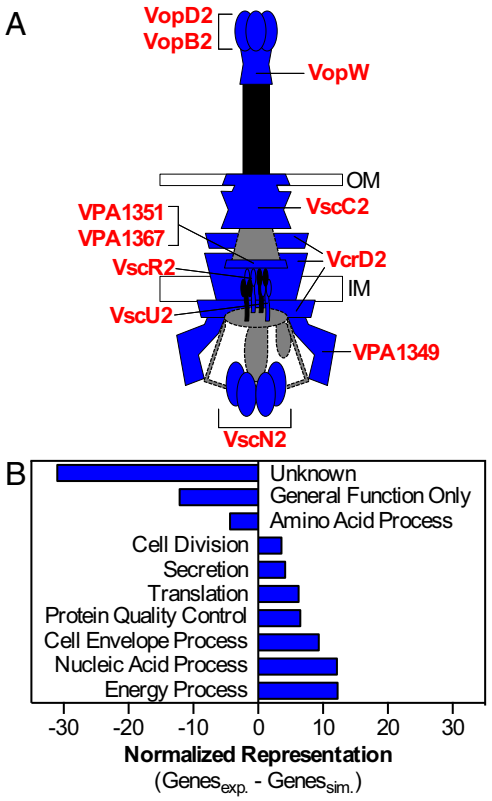

Fig. 4. Bioinformatic analysis of conditionally depleted genes. (*A*) Predicted structural schematic of T3SS2 indicating components that are conditionally depleted (blue), not queried in our analysis (black), or lacking known *V. parahaemolyticus* homologs (gray with dashed lines). (*B*) COG terms with statistically significant, differential representation in the conditionally depleted dataset (exp.) relative to random sampling of the 3,744 genes queried (sim.).

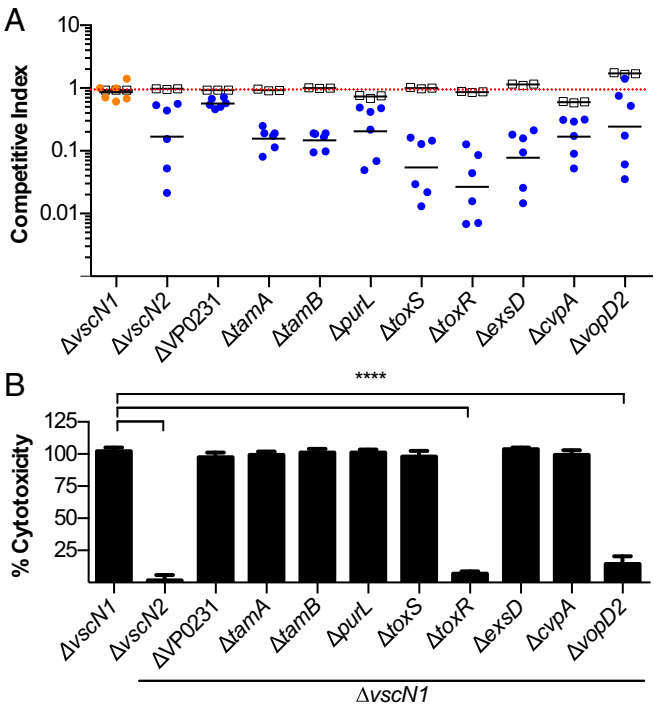

Fig. 5. Targeted validation of conditionally depleted genes. (*A*) Competitive indices of deletion mutants following coinfection of the distal small intestine (blue circles) or in vitro outgrowth (white squares). Deletion strains display in vivo competitive indices significantly lower (adjusted-$P < 0.05$) than the negative control (orange circles). Horizontal lines indicate the geometric mean of independent measurements. (*B*) T3SS2 activity of deletion strains (****$P < 0.0001$). *vscN1* was deleted from all strains to eliminate T3SS1-mediated cytotoxicity.

Validation of Selected Genes Required for Fitness in Vivo. We generated deletion mutants for 10 genes, including eight not previously implicated in *V. parahaemolyticus* infection of the infant rabbit gastrointestinal tract and two T3SS2 structural components [*vscN2* (VPA1338) and *vopD2* (VPA1361)] required for *V. parahaemolyticus* infection (5, 22). As a negative control, we deleted the T3SS1 gene *vscN1* (VP1668) (5) (Fig. 3*B*). We barcoded mutants with unique sequence tags stably integrated into a neutral locus, enabling enumeration of individual mutant frequencies within a complex population. A mixture of all mutants was competed against an excess of differentially tagged *V. parahaemolyticus* Sm^R in vitro and in vivo (Fig. 5*A*), and the competitive index of each deletion strain (a measure of relative fitness) was calculated from changes in individual tag frequencies. Most deletion strains exhibited in vitro fitness indistinguishable from the negative control (Fig. 5*A*), but the competitive indices of *purL* and *cvpA* were slightly, although significantly, lower. Importantly, all candidate deletion strains displayed in vivo competitive indices significantly lower than the in vivo competitive index of Δ*vscN1*, indicating in vivo attenuation relative to the negative control. Because our analysis controlled for the duration of population expansion without regard for potentially different in vitro and in vivo growth rates, these data do not preclude the possibility that the *purL* and *cvpA* mutants are attenuated in vivo solely due to the growth defects observed in vitro, provided that there was a substantially greater number of bacterial generations during in vivo growth. Collectively, these data validate the in vivo screen and suggest that our approach yielded a high confidence dataset that constitutes the most thorough dissection to date of the genetic requirements for *V. parahaemolyticus* intestinal colonization.

Given the importance of T3SS2 for *V. parahaemolyticus* infection of the infant rabbit intestine, we investigated whether the in vivo attenuation of the validated mutants reflected a defect in T3SS2 activity. We measured each mutant's T3SS2-dependent cytotoxicity against cultured HT-29 colonic epithelial cells, an established readout of T3SS2 activity (25) (Fig. 5*B*). As expected, deletion of *vscN2* or *vopD2* abolished T3SS2-mediated cytotoxicity, but seven of the deletion strains displayed cytotoxicity levels equivalent to the Δ*vscN1*-positive control. Consequently, the in vivo attenuation of these seven mutants appears attributable to T3SS2-independent processes.

The seven genes are associated with processes not previously linked to *V. parahaemolyticus* infection of the intestinal tract. VP0231, a predicted UDP-galactose phosphate transferase involved in lipopolysaccharide biosynthesis, and *purL* (VP0666), an enzyme required for de novo synthesis of purine nucleotides, are thought to contribute to cell envelope biogenesis and nucleic acid metabolism, respectively (Dataset S4*A*); these processes are important for intestinal colonization by other enteric pathogens (16–18). *cvpA* (VP2186) is required for colicin V production and biofilm formation in *E. coli* and for *V. cholerae* survival in vivo, but it is unclear how this gene contributes to *V. parahaemolyticus* colonization (16–18, 26). The genes *tamA* (VP0307) and *tamB* (VP0308) encode components of a translocation and assembly module (Tam) found to mediate the assembly of autotransporters and the virulence of multiple pathogens (27). Finally, disruption of *exsD* (VP1698), which encodes a negative regulator of T3SS1 gene transcription, results in constitutive T3SS1 expression, suggesting that overexpression of T3SS1 genes in vivo impairs *V. parahaemolyticus* colonization (28).

In contrast to the seven mutants with unimpaired cytotoxicity, a *toxR* (VP0820) mutant lacked T3SS2-mediated cytotoxicity in vitro. ToxR is a transmembrane transcription factor present in all *V. parahaemolyticus* strains and in diverse *Vibrio* species. *V. cholerae* ToxR is this pathogen's master regulator of virulence factor expression; it modulates production of cholera toxin and the toxin coregulated pilus (TCP) that is required for intestinal colonization (12). A cotranscribed factor, ToxS, may augment the activity of *V. cholerae* ToxR through a direct interaction that protects ToxR from proteolytic degradation (29). In a mouse model of *V. parahaemolyticus* infection, ToxRS has been proposed to contribute to disease through regulation of the outer membrane protein OmpU; however, our data indicate that OmpU is dispensable for *V. parahaemolyticus* colonization (10) (Dataset S3*A*). Disruption of *V. parahaemolyticus toxS* (VP0819) impaired colonization of the intestine but had no effect on T3SS2 activity in vitro (Fig. 5), a phenotypic divergence that suggests *toxR* may contribute to in vivo fitness both in conjunction with, and independent of, *toxS*. Notably, *V. parahaemolyticus* ToxR has not previously been implicated in T3SS2 gene expression.

ToxR Is Required for Induction of *vtrB*. T3SS2 gene transcription depends on VtrA (VPA1332) and VtrB, two ToxR-like transcription factors encoded within the T3SS2 gene cluster in VPaI-7 (24). In vitro, VtrA- and VtrB-dependent T3SS2 expression is induced by crude bile, which promotes VtrA induction of the *vtrB* promoter through an unknown mechanism (9). VtrB then induces transcription of the full complement of T3SS2 genes (9, 24). We hypothesized that *toxR* might be required for the expression of these regulatory factors in *V. parahaemolyticus*. Consistent with this hypothesis, we found that deletion of *toxR* abolished bile-dependent induction of *vtrB* transcription (Fig. 6*A*); however, *toxR* was not required for production of VtrA (Fig. 6*B*). Interestingly, overexpression of VtrA restored *vtrB* induction and T3SS2 protein expression in the *toxR* mutant (Fig. 6*A* and Fig. S3*B*). Thus, our data suggest that *toxR* augments the activity of VtrA, specifically its induction of *vtrB* transcription. Finally, we observed that plasmid-based expression of VtrB restored the *toxR* mutant's production of VopD2 and T3SS2-mediated cytotoxicity to the level of the positive control (Fig.

7). Collectively, these data are consistent with a model in which ToxR augments the ability of VtrA to induce *vtrB* transcription.

Discussion

We harnessed the power of TIS to identify genes and regulatory networks that enable *V. parahaemolyticus* survival in vitro and in the intestine. We defined 565 genes likely required for the viability of this pandemic cause of seafood-borne enteritis, including loci homologous to essential genes in related bacteria and potential *V. parahaemolyticus*-specific essential genes. The latter could enable the design of targeted antimicrobials, reducing the use of broad-spectrum antibiotics in commercial aquaculture, which is currently plagued by this pathogen. The 230 stringently defined conditionally depleted genes, which are required for *V. parahaemolyticus* fitness in vivo, encompassed components of the pathogen's principal virulence factor (T3SS2) and numerous mediators of biological processes not previously linked to *V. parahaemolyticus* colonization in any model of infection. We also discovered that ToxR, an ancestral transmembrane transcription factor necessary for the expression of horizontally acquired virulence genes in *V. cholerae*, is critical for *V. parahaemolyticus* infection. Unexpectedly, ToxR proved necessary for induction of T3SS2 gene expression in *V. parahaemolyticus*; thus, as in *V. cholerae*, it governs expression of the pathogen's key virulence factor.

All 10 of the previously annotated T3SS2 structural components assayed in our study were conditionally depleted (Fig. S3*A*). Consistent with a previous report, a T3SS2 effector-encoding gene (*vopV*) was required for intestinal colonization (23). Additionally, 10 conditionally depleted genes encoded within the T3SS2 gene cluster lacked prior annotation; these genes may encode components of the secretion apparatus, given that many components of the general T3SS machinery have not yet been defined for T3SS2 (6). BLAST analysis did not identify sequence homology between

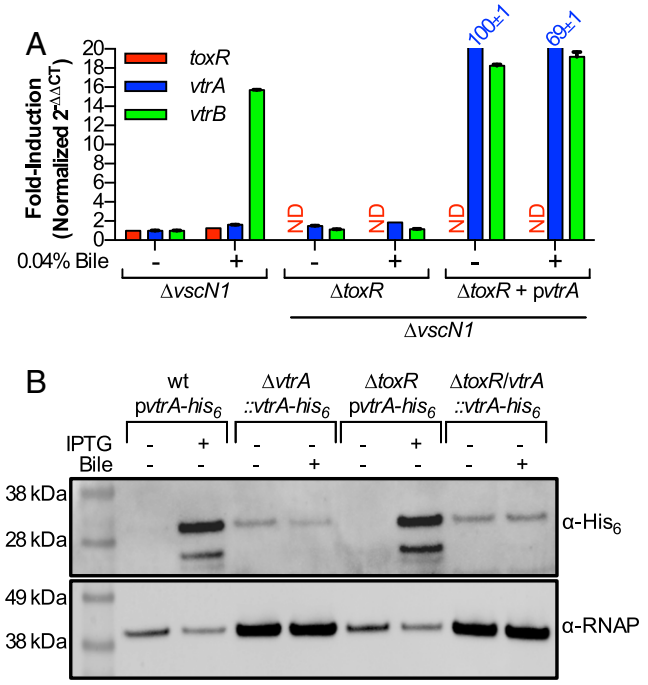

Fig. 6. *toxR* is required for induction of *vtrB* transcription. (*A*) Relative abundance of mRNA transcripts in the presence or absence of crude bile. ND, none detected. (*B*) VtrA-His_6 expression in the presence or absence of crude bile via immunoblotting (~29 kDa). Due to elevated VtrA-His_6 levels, less lysate was run for samples containing p*vtrA-his*$_6$. IPTG, isopropyl β-D-1-thio-galactopyranoside; α-RNAP, α-RNA polymerase; wt, wild type.

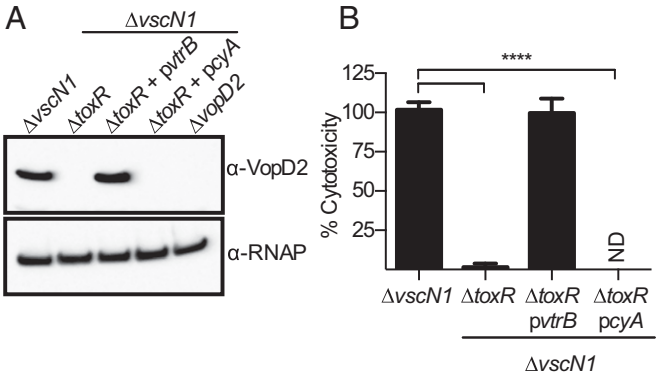

Fig. 7. Expression of *vtrB* bypasses the requirement for *toxR*. (*A*) Immunoblotting of the T3SS2 component VopD2 in Δ*toxR* strains grown in crude bile and exogenously expressing *vtrB* (p*vtrB*) or an unrelated gene (p*cyA*). (*B*) T3SS2 activity in Δ*toxR* strains grown in crude bile and exogenously expressing p*vtrB* or p*cyA* (****$P < 0.0001$). *vscN1* was deleted from all strains to eliminate T3SS1-mediated cytotoxicity. ND, none detected.

any of the 10 genes and known T3SS components; however, structure-based prediction (HHpred) revealed that VPA1351 bears structural homology to the inner membrane ring of the *Salmonella enterica* (serovar Typhimurium) SPI-1 T3SS, whereas VPA1352 and VPA1365 likely encode T3SS chaperones (6, 30) (Fig. S3*A*). The uncharacterized gene VPA1350 may correspond to a needle-associated component of T3SS2 (31). Notably, the other six genes are conserved in the T3SS found in *V. cholerae* AM-19226, and may correspond to unidentified components of the T3SS2 structural apparatus in this organism as well; however, we cannot rule out the possibility that these genes encode T3SS2 effector proteins or factors that contribute to in vivo fitness through T3SS2-independent processes (32).

Our genome-wide approach identified numerous *V. parahaemolyticus* genes outside of VPaI-7 that contribute to intraintestinal survival independent of T3SS2. Given their putative function in autotransporter assembly, the requirement for *tamA* and *tamB* in *V. parahaemolyticus* intestinal colonization is somewhat surprising, in that no autotransporter proteins have been reported in a *Vibrio* species. *V. parahaemolyticus* may produce noncanonical autotransporters that are individually or collectively required for colonization. Alternatively, the function of Tam in vibrios may not be restricted to insertion of autotransporters into the outer membrane, as previously postulated (33). Tam proteins contribute to the pathogenesis of several gram-negative bacteria, and TamB is required for colonization of the squid light organ by the commensal *Vibrio fischeri*, suggesting that these membrane proteins play a potentially broad role in diverse host–microbe interactions (33, 34). Interestingly, only *tamB* was required for *V. cholerae* colonization of the infant rabbit gastrointestinal tract (Dataset S4*B*), highlighting the variation in processes underlying disease linked to enteropathogenic vibrios.

ToxR is another conditionally depleted locus encoded outside of VPaI-7 that contributes to intestinal colonization. Our data, which we are unable to reconcile with a contradictory report from Gotoh et al. (9), indicate that ToxR is required for induction of the key T3SS2 regulator, *vtrB*. Consequently, T3SS2 expression can be restored in a *toxR* mutant via either complementation with ToxR (Fig. S4) or exogenous expression of VtrB. Importantly, *toxR*-dependent T3SS2 expression is not an artifact of Δ*vscN1* deletion or the streptomycin-resistant parent strain because an identical *toxR* mutation constructed in the wild-type background yields the same phenotype (Fig. S4). The *toxR* mutant's inability to activate bile-dependent T3SS2 gene expression could by itself account for the mutant's in vivo attenuation.

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However, ToxR may also contribute to virulence via regulation of additional pathways, potentially in conjunction with the cotranscribed conditionally depleted gene *toxS*. This regulatory paradigm is similar to ToxR-dependent virulence gene regulation in *V. cholerae*, although a distinct set of virulence elements is involved. In *V. cholerae,* ToxR forms a disulfide-linked complex with a related protein, TcpP, that governs transcription of the virulence regulator *toxT*. ToxR's regulatory role can be bypassed by overexpression of either TcpP or ToxT (35). The *V. parahaemolyticus* regulator VtrA lacks periplasmic cysteine residues, precluding disulfide bond formation like that required for TcpP–ToxR interaction in *V. cholerae;* however, as with TcpP, exogenous expression of VtrA overcomes the requirement for *toxR*, restoring *vtrB* transcription and T3SS2 protein expression. Additional studies are needed to identify the precise means by which ToxR enables virulence gene expression. Collectively, our data suggest that *V. parahaemolyticus* ToxR augments VtrA induction of the *vtrB* promoter, a posttranslational regulatory mechanism that bears striking similarity to that observed in *V. cholerae* regulation of distinct virulence elements.

A critical step in the evolution of pathogenic *V. parahaemolyticus* and *V. cholerae* involved acquisition of foreign DNA by nonpathogenic precursor strains. For *V. parahaemolyticus*, acquisition of T3SS2 appears to have enabled colonization of the human intestine. For *V. cholerae*, sequential acquisition of the TCP biogenesis genes and the cholera toxin genes *ctxAB* allowed this organism to thrive in the small intestine and promote its own dissemination (12). Strikingly, *V. cholerae* and *V. parahaemolyticus* appear to have independently linked control of disparate horizontally transmitted elements critical for infection to the same ancestral transcription factor, ToxR. This strategy may have facilitated the evolution of these two highly successful pathogens because ToxR is responsive to signals encountered in the host small intestine (36). Furthermore, the common route of acquisition for these distinct virulence elements, lateral gene transfer, suggests a broader role for ToxR in the regulation of foreign DNA.

Materials and Methods

Transposon (16) and animal infection studies (5) were performed as previously described with modifications outlined in *SI Materials and Methods*. Detailed descriptions of all strains (Dataset S5), reagents, and methodologies used in this study are provided in *SI Materials and Methods*.

The animal protocols for these studies were reviewed and approved by the Harvard Medical Area Standing Committee on Animals (Institutional Animal Care and Use Committee protocol 04308, Animal Welfare Assurance of Compliance A3431-01). All animal studies were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the NIH (37) and the Animal Welfare Act of the United States Department of Agriculture.

ACKNOWLEDGMENTS. We thank Drs. Tetsuya Iida and Toshio Kodoma for providing reagents, Jennifer Pena for technical assistance, and members of the M.K.W. laboratory for helpful discussions. This work was supported by NIH Grants R37 AI-042347 (to M.K.W.), F31 AI-120665 (to T.P.H.), and F32 GM108355-02 (to M.C.C.); the Howard Hughes Medical Institute (M.K.W.); Northern Norway Regional Health Authority Grants SFP1293-16 (to S.A.) and A67600 (to P.A.z.W.); Research Council of Norway Grant 249979/RU (to S.A.); and Bill & Melinda Gates Foundation Grant OPP1111658 (to P.A.z.W.). C.J.B. is supported by a Pew Latin American Fellowship and a Becas Chile Postdoctoral Fellowship.

1. McCarter L (1999) The multiple identities of Vibrio parahaemolyticus. *J Mol Microbiol Biotechnol* 1(1):51–57.
2. Letchumanan V, Chan K-G, Lee L-H (2014) Vibrio parahaemolyticus: A review on the pathogenesis, prevalence, and advance molecular identification techniques. *Front Microbiol* 5:705.
3. Tang KF, Lightner DV (2014) Homologues of insecticidal toxin complex genes within a genomic island in the marine bacterium Vibrio parahaemolyticus. *FEMS Microbiol Lett* 361(1):34–42.
4. Makino K, et al. (2003) Genome sequence of Vibrio parahaemolyticus: A pathogenic mechanism distinct from that of V cholerae. *Lancet* 361(9359):743–749.
5. Ritchie JM, et al. (2012) Inflammation and disintegration of intestinal villi in an experimental model for Vibrio parahaemolyticus-induced diarrhea. *PLoS Pathog* 8(3): e1002593.
6. Portaliou AG, Tsolis KC, Loos MS, Zorzini V, Economou A (2015) Type III Secretion: Building and Operating a Remarkable Nanomachine. *Trends Biochem Sci* 41(2):175–189.
7. Boyd EF, et al. (2008) Molecular analysis of the emergence of pandemic Vibrio parahaemolyticus. *BMC Microbiol* 8(1):110.
8. Livny J, et al. (2014) Comparative RNA-Seq based dissection of the regulatory networks and environmental stimuli underlying Vibrio parahaemolyticus gene expression during infection. *Nucleic Acids Res* 42(19):12212–12223.
9. Gotoh K, et al. (2010) Bile acid-induced virulence gene expression of Vibrio parahaemolyticus reveals a novel therapeutic potential for bile acid sequestrants. *PLoS One* 5(10):e13365.
10. Whitaker WB, Parent MA, Boyd A, Richards GP, Boyd EF (2012) The Vibrio parahaemolyticus ToxRS regulator is required for stress tolerance and colonization in a novel orogastric streptomycin-induced adult murine model. *Infect Immun* 80(5): 1834–1845.
11. Barquist L, Boinett CJ, Cain AK (2013) Approaches to querying bacterial genomes with transposon-insertion sequencing. *RNA Biol* 10(7):1161–1169.
12. Matson JS, Withey JH, DiRita VJ (2007) Regulatory networks controlling Vibrio cholerae virulence gene expression. *Infect Immun* 75(12):5542–5549.
13. Yamaichi Y, et al. (2015) High-resolution genetic analysis of the requirements for horizontal transmission of the ESBL plasmid from Escherichia coli O104:H4. *Nucleic Acids Res* 43(1):348–360.
14. Chao MC, et al. (2013) High-resolution definition of the Vibrio cholerae essential gene set with hidden Markov model-based analyses of transposon-insertion sequencing data. *Nucleic Acids Res* 41(19):9033–9048.
15. Kato J, Hashimoto M (2007) Construction of consecutive deletions of the Escherichia coli chromosome. *Mol Syst Biol* 3:132.
16. Pritchard JR, et al. (2014) ARTIST: high-resolution genome-wide assessment of fitness using transposon-insertion sequencing. *PLoS Genet* 10(11):e1004782.
17. Fu Y, Waldor MK, Mekalanos JJ (2013) Tn-Seq analysis of Vibrio cholerae intestinal colonization reveals a role for T6SS-mediated antibacterial activity in the host. *Cell Host Microbe* 14(6):652–663.
18. Kamp HD, et al. (2013) Gene fitness landscapes of Vibrio cholerae at important stages of its life cycle. *PLoS Pathog* 9(12):e1003800.
19. Abel S, et al. (2015) Sequence tag-based analysis of microbial population dynamics. *Nat Methods* 12(3):223–226.
20. Park K-SS, et al. (2004) Functional characterization of two type III secretion systems of Vibrio parahaemolyticus. *Infect Immun* 72(11):6659–6665.
21. Zhou X, et al. (2012) The hydrophilic translocator for Vibrio parahaemolyticus, T3SS2, is also translocated. *Infect Immun* 80(8):2940–2947.
22. Kodama T, et al. (2008) Identification of two translocon proteins of Vibrio parahaemolyticus type III secretion system 2. *Infect Immun* 76(9):4282–4289.
23. Hiyoshi H, et al. (2011) VopV, an F-actin-binding type III secretion effector, is required for Vibrio parahaemolyticus-induced enterotoxicity. *Cell Host Microbe* 10(4):401–409.
24. Kodama T, et al. (2010) Two regulators of Vibrio parahaemolyticus play important roles in enterotoxicity by controlling the expression of genes in the Vp-PAI region. *PLoS One* 5(1):e8678.
25. Kodama T, et al. (2007) Identification and characterization of VopT, a novel ADP-ribosyltransferase effector protein secreted via the Vibrio parahaemolyticus type III secretion system 2. *Cell Microbiol* 9(11):2598–2609.
26. Hadjifrangiskou M, et al. (2012) Transposon mutagenesis identifies uropathogenic Escherichia coli biofilm factors. *J Bacteriol* 194(22):6195–6205.
27. Selkrig J, et al. (2012) Discovery of an archetypal protein transport system in bacterial outer membranes. *Nat Struct Mol Biol* 19(5):506–510, S1.
28. Zhou X, Konkel ME, Call DR (2010) Regulation of type III secretion system 1 gene expression in Vibrio parahaemolyticus is dependent on interactions between ExsA, ExsC, and ExsD. *Virulence* 1(4):260–272.
29. Almagro-Moreno S, Root MZ, Taylor RK (2015) Role of ToxS in the proteolytic cascade of virulence regulator ToxR in Vibrio cholerae. *Mol Microbiol* 98(5):963–976.
30. Soding J, Biegert A, Lupas AN (2005) The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res* 33(Web Server issue): W244–W248.
31. Zhou X, et al. (2013) A Vibrio parahaemolyticus T3SS effector mediates pathogenesis by independently enabling intestinal colonization and inhibiting TAK1 activation. *Cell Reports* 3(5):1690–1702.
32. Dziejman M, et al. (2005) Genomic characterization of non-O1, non-O139 Vibrio cholerae reveals genes for a type III secretion system. *Proc Natl Acad Sci USA* 102(9): 3465–3470.
33. Brooks JF, 2nd, et al. (2014) Global discovery of colonization determinants in the squid symbiont Vibrio fischeri. *Proc Natl Acad Sci USA* 111(48):17284–17289.
34. Selkrig J, et al. (2015) Conserved features in TamA enable interaction with TamB to drive the activity of the translocation and assembly module. *Sci Rep* 5:12905.
35. Morgan SJ, et al. (2015) Formation of an Intramolecular Periplasmic Disulfide Bond in TcpP Protects TcpP and TcpH from Degradation in Vibrio cholerae. *J Bacteriol* 198(3): 498–509.
36. Herrington DA, et al. (1988) Toxin, toxin-coregulated pili, and the toxR regulon are essential for Vibrio cholerae pathogenesis in humans. *J Exp Med* 168(4):1487–1492.
37. Committee on Care and Use of Laboratory Animals (1996) *Guide for the Care and Use of Laboratory Animals* (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.