

# The *Vibrio cholerae* virulence regulatory cascade controls glucose uptake through activation of TarA, a small regulatory RNA

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## Summary

***Vibrio cholerae* causes the severe diarrhoeal disease cholera. A cascade of regulators controls expression of virulence determinants in *V. cholerae* at both transcriptional and post-transcriptional levels. ToxT is the direct transcription activator of the major virulence genes in *V. cholerae*. Here we describe TarA, a highly conserved, small regulatory RNA, whose transcription is activated by ToxT from toxboxes present upstream of the ToxT-activated gene *tcpI*. TarA regulates *ptsG*, encoding a major glucose transporter in *V. cholerae*. Cells overexpressing TarA exhibit decreased steady-state levels of *ptsG* mRNA and grow poorly in glucose-minimal media. A mutant lacking the ubiquitous regulatory protein Hfq expresses diminished TarA levels, indicating that TarA likely interacts with Hfq to regulate gene expression. RNA hybrid analysis of TarA and the putative *ptsG* mRNA leader suggests potential productive base-pairing between these two RNA molecules. A *V. cholerae* mutant lacking TarA is compromised for infant mouse colonization in competition with wild type, suggesting a role in the *in vivo* fitness of *V. cholerae*. Although somewhat functionally analogous to SgrS of *Escherichia coli*, TarA does not encode a regulatory peptide, and its expression is activated by the virulence gene pathway in *V. cholerae* and not by glycolytic intermediates.**

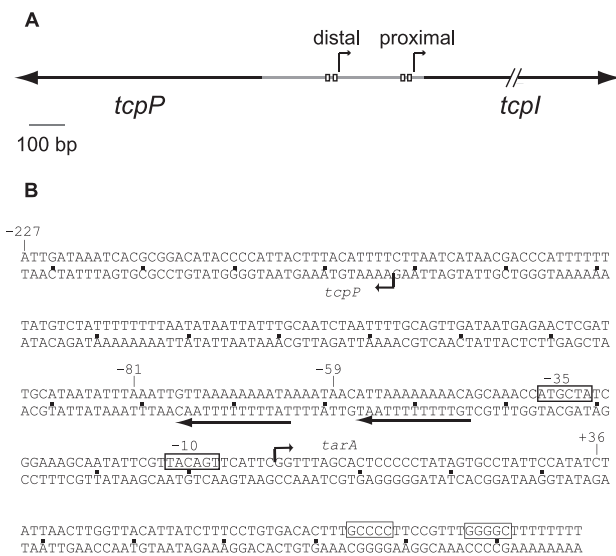
## Introduction

Asiatic cholera, a severe diarrhoeal disease, remains a significant cause of morbidity and mortality in many regions of the globe (Sack *et al.*, 2006). Cholera is caused by ingestion of the Gram-negative, highly motile curved bacillus *Vibrio cholerae* in contaminated food or water. *V. cholerae* are present in many coastal areas worldwide; however, of the hundreds of known *V. cholerae* serogroups, only the O1 and O139 serogroups can cause cholera (Reidl and Klose, 2002; Sack *et al.*, 2004).

The two major *V. cholerae* virulence factors are the cholera toxin (CT) and the toxin-coregulated pilus (TCP). Secretion of CT into the intestinal lumen by *V. cholerae* directly causes the secretory diarrhoea characteristic of cholera (Lonnroth and Holmgren, 1973; Gill, 1976). The genes encoding the two subunits of CT, *ctxAB*, are located within the genome of a filamentous bacteriophage, CTX $\Phi$  (Waldor and Mekalanos, 1996). The TCP, a type IV bundle-forming pilus, is required for intestinal colonization by *V. cholerae* and acts to initiate micro-colony formation (Taylor *et al.*, 1987; Herrington *et al.*, 1988; Attridge *et al.*, 1996; Thelin and Taylor, 1996). The genes encoding TCP are located within the *Vibrio* pathogenicity island (VPI), which may also be a mobile element but is not a bacteriophage (Manning, 1997; Karaolis *et al.*, 1998; Faruque and Mekalanos, 2003; Rajanna *et al.*, 2003). Other putative virulence genes located within the VPI are coordinately regulated with *ctxAB* and *tcp*, but their roles in pathogenesis are unclear.

Expression of genes encoding *V. cholerae* virulence factors is regulated by a cascade involving transcription activators, repressors and proteases (Matson *et al.*, 2007); however, the direct transcription activator of virulence genes is ToxT (Matson *et al.*, 2007). ToxT is a member of the AraC/XylS family of transcription factors (Higgins *et al.*, 1992; Ogierman and Manning, 1992). ToxT binds to a 13 bp degenerate DNA sequence, the toxbox, to activate transcription (Withey and DiRita, 2006), using toxboxes configured as direct repeats, inverted repeats, and single toxboxes to activate transcription of different operons (Withey and DiRita, 2005a; 2006).

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**Fig. 1.** Schematic and diagram of *tcpI* promoters and the surrounding region.

**A.** Schematic. Scale bar: 0.5 cm represents 200 bp of DNA. Bent arrows indicate putative transcriptional start sites, and long black arrows represent coding sequences of the known genes *tcpP* and *tcpI*. *tcpI* has been shortened from 1.6 kb for clarity. Toxboxes, or ToxT-recognition sequences, are indicated by paired boxes.

**B.** Diagram of *tcpI*-distal promoter, *tarA*, and surrounding sequences. Bent arrows indicate transcriptional start sites for *tcpP* and *tarA*. Numbers indicate position relative to *tarA* transcriptional start site. ToxT binding sites (toxboxes) for the *tcpI*-distal promoter are underlined with horizontal arrows. The -35 and -10 elements for *tarA* are indicated by thick boxes, and thin boxes represent potential base-pairing regions in putative hairpin structure.

Binding sites for ToxT have been characterized at most of the promoters it is known to activate (Withey and DiRita, 2005a,b; 2006). An exception is the *tcpI* promoter, located on the VPI and encoding a putative methyl-accepting chemotaxis protein with an unknown role in pathogenesis (Harkey *et al.*, 1994). Our sequence analysis identified two pairs of putative toxboxes upstream of *tcpI*; one pair, in an inverted configuration, is positioned proximal to the *tcpI* open reading frame (Withey and DiRita, 2006). The other pair, in a direct repeat configuration, is positioned at a site upstream of the proximal toxboxes (Fig. 1A), and thus we refer to them as the distal toxboxes. Primer extension analysis performed previously by others identified two different start sites of *tcpI* transcription that would potentially correspond to transcription activated by ToxT from each set of toxboxes, although when those studies were carried out, the toxboxes had not yet been identified (Thomas *et al.*, 1995; Murley *et al.*, 2000). We performed experiments to characterize the role of ToxT in transcription from both of these putative sites and have found that there are indeed two separate ToxT-dependent transcripts produced upstream of *tcpI*. However, the *tcpI*-proximal promoter alone is responsible

for transcription of *tcpI* itself (J.H. Withey and V.J. DiRita, unpublished).

Here we describe a small RNA (~91 nt), which we named TarA for ToxT-activated RNA, whose transcription from a gene located between *tcpI* and *tcpP* is activated by ToxT at the promoter distal to *tcpI*. The TarA nucleotide sequence is highly conserved among both epidemic and environmental *V. cholerae* strains that carry the VPI. We demonstrate that TarA influences glucose uptake through its effect on the transcript encoding the glucose transporter PtsG.

## Results

### *ToxT* activates transcription of a small non-coding RNA

To characterize ToxT-dependent activation of *tcpI*, we constructed *lacZ* fusions and assayed  $\beta$ -galactosidase activity in *V. cholerae* having either wild-type *toxT* or a *toxT* deletion. Our results indicated that the minimal ToxT-responsive DNA sequence for activating a *tcpI-lacZ* fusion at this *tcpI*-proximal promoter extended only to -94 relative to the proximal *tcpI* promoter. Because a second separate start site for *tcpI* transcription had been previously described at -243 relative to the AUG (Harkey *et al.*, 1994; Murley *et al.*, 2000), we also constructed *tcpI::lacZ* fusions extending to -431 relative to the start site of transcription from the *tcpI*-proximal promoter to include both putative promoters (Fig. 1A). However, additional upstream DNA, even if it contained the putative *tcpI*-distal promoter, did not affect the level of  $\beta$ -galactosidase produced. This result suggested that the distal promoter, if functional, does not read into *tcpI* under the conditions tested.

To determine whether the *tcpI*-distal promoter is functional, we created a set of *lacZ* fusions whose 3' endpoints were located upstream of the *tcpI*-proximal promoter (at -173 relative to its start site and +36 relative to the distal promoter start site) and with 5' endpoints that extended to -59, -81 or -223 relative to the start of transcription from the distal promoter (Fig. 1B). Results of  $\beta$ -galactosidase assays using these constructs indicated that the *tcpI*-distal promoter was indeed functional and highly activated by ToxT (Table 1). Sequence analysis identified two consensus toxboxes configured as a direct repeat upstream of the *tcpI*-distal promoter -35 box that had the same spacing as the toxboxes we observed at the *tcpA* promoter (Withey and DiRita, 2006) (Fig. 1B). Deletion of one of the two toxboxes resulted in a complete loss of ToxT-directed transcription from the *tcpI*-distal promoter [Table 1, Fig. 1B (-59 construct)].

Downstream of the start site of transcription is a G/C rich region that could potentially form a stem-loop structure (boxes in Fig. 1B), followed by a tract of T nucleotides on

**Table 1.** Nested *tarA::lacZ* fusion analysis.

5' Endpoint	3' Endpoint	Miller units (wild-type <i>toxT</i> )	Miller units ( $\Delta$ <i>toxT</i> )	Fold difference ( <i>toxT</i> / $\Delta$ <i>toxT</i> )
-223	+36	11 800 $\pm$ 500	804 $\pm$ 137	15 $\times$
-81	+36	18 000 $\pm$ 551	436 $\pm$ 44.8	41 $\times$
-59	+36	94.6 $\pm$ 9.35	93.1 $\pm$ 16.5	1 $\times$

5' and 3' endpoints indicate region of *V. cholerae* chromosome ligated into pTL61T plasmid. Fusion plasmids were electroporated into wild-type O395 and an isogenic  $\Delta$ *toxT* strain. Cultures were grown under inducing conditions for 3 h and then tested for  $\beta$ -galactosidase activity, with data reported in Miller units. Fold difference was determined by taking the ratio of wild-type activity to  $\Delta$ *toxT* activity.

**Table 2.** Conservation of TarA and upstream sequences.

<i>V. cholerae</i> strain	Biotype	O antigen	TarA conservation	Upstream DNA conservation
O395 <sup>a</sup>	Classical	O1	99/99 nt	99/99 bp
Z17561 <sup>a</sup>	Classical	O1	99/99 nt	99/99 bp
N16961 <sup>a</sup>	El Tor	O1	96/99 nt	62/99 bp
H1 <sup>a</sup>	El Tor	O1	96/99 nt	75/99 bp
SCE4	Environmental	O8	99/99 nt	75/99 bp
SCE200 <sup>a</sup>	Environmental	O44	99/99 nt	91/99 bp
SCE256	Environmental	O42	99/99 nt	68/99 bp
SCE226	Environmental	O35	98/99 nt	85/99 bp

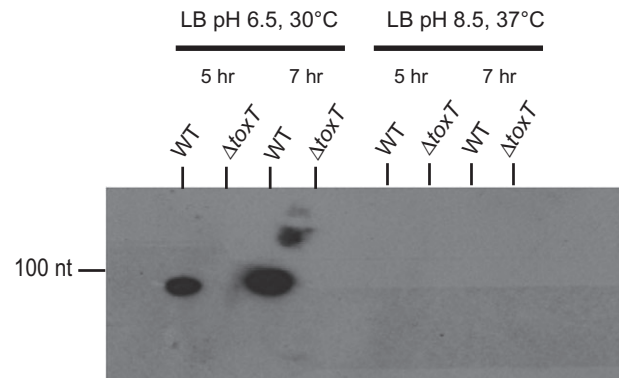
a. Toxigenic strain.

the non-template strand, suggesting the presence of a transcription terminator. To determine if a small RNA product initiating at the *tcpI*-distal promoter and ending at the putative terminator is produced, we performed Northern blots on whole-cell RNA purified from *V. cholerae* having a wild-type or a deleted *toxT* gene, probing with an oligonucleotide complementary to the putative sRNA sequence. A single RNA product of the expected size, < 100 nt, was detected in the Northern blotting experiments (Fig. 2). Production of the sRNA required functional ToxT, was detectable beginning at 3 h of growth (data not shown), continued through logarithmic phase, and peaked at 7 h of growth under ToxT-inducing conditions; no TarA was detected in non-inducing conditions (Fig. 2). These findings are consistent with previous observations of ToxT activity (Yu and DiRita, 1999). Based on this evidence, we named this previously unknown sRNA 'TarA' for ToxT-Activated RNA. TarA contains a putative binding site for the ubiquitous regulatory protein Hfq and lacks an open reading frame; thus, its function is likely to be regulatory.

#### *TarA* is highly conserved among *V. cholerae* strains

To determine whether TarA has homology to any other sRNA species, we performed searches with its sequence from the classical *V. cholerae* strain O395 using both the RNAfam database and BLAST (Touzet and Perriquet, 2004). The former search indicated that TarA has no homology to any known sRNA family. However, the latter

search indicated that TarA has a very high degree of sequence conservation among *V. cholerae* (Table 2, Fig. 3). TarA sequences from eight different *V. cholerae* strains were obtained from the BLAST search; two of these are classical biotype *V. cholerae*, two are El Tor biotype *V. cholerae* and four are non-O1 *V. cholerae*. The two classical strains have identical TarA sequences and the sequences of the two El Tor strains are also identical



**Fig. 2.** TarA is produced under ToxT-inducing conditions. Wild-type and  $\Delta$ *toxT* strains were grown under inducing and non-inducing conditions (LB pH 6.5, 30°C and LB pH 8.5, 37°C respectively) and total RNA was collected at indicated time points. Equal amounts of RNA from each sample (as determined by OD<sub>260</sub>) were subjected to Northern blot analysis, using an oligonucleotide complementary to the putative sRNA sequence as a probe. Methylene blue staining was used to confirm load amounts (data not shown).

	Hfq?
O395	GGUUUAGCACUCCCC_UAUAGUGCCUAAUCCAUAUC <u>UAUUAA</u> CUUGGUUACAUAUUCUUCCUGUGACACUUUGCCCCUUCGGUUUGGGGCUUUUUUUU
Z17561	GGUUUAGCACUCCCC_UAUAGUGCCUAAUCCAUAUC <u>UAUUAA</u> CUUGGUUACAUAUUCUUCCUGUGACACUUUGCCCCUUCGGUUUGGGGCUUUUUUUU
N16961	GGUUUAGCACUCCCCUUAUAGUGCCUAAUCCAUAUC <u>UAUUAA</u> CUUGGUUACAUAUUCUUCCUGUGACAUUUUGCCCCUUCGGUUUGGGGCUUUUUUUU
H1	GGUUUAGCACUCCCCUUAUAGUGCCUAAUCCAUAUC <u>UAUUAA</u> CUUGGUUACAUAUUCUUCCUGUGACAUUUUGCCCCUUCGGUUUGGGGCUUUUUUUU
SCE4	GGUUUAGCACUCCCC_UAUAGUGCCUAAUCCAUAUC <u>UAUUAA</u> CUUGGUUACAUAUUCUUCCUGUGACACUUUGCCCCUUCGGUUUGGGGCUUUUUUUU
SCE200	GGUUUAGCACUCCCC_UAUAGUGCCUAAUCCAUAUC <u>UAUUAA</u> CUUGGUUACAUAUUCUUCCUGUGACACUUUGCCCCUUCGGUUUGGGGCUUUUUUUU
SCE256	GGUUUAGCACUCCCC_UAUAGUGCCUAAUCCAUAUC <u>UAUUAA</u> CUUGGUUACAUAUUCUUCCUGUGACACUUUGCCCCUUCGGUUUGGGGCUUUUUUUU
SCE226	GGUUUAGCACUCCCC_UAUAGUGCCUAAUCCAUAUC <u>UAUUAA</u> CUUGGUUACAUAUUCUUCCUGUGACACUUUGCCCCUUCGGUUUGGGGCUUUUUUUU

**Fig. 3.** Comparison of *tarA* sequences among *V. cholerae* strains. Sequences from strains indicated in Table 2 are aligned, with bold letters indicating mutations. The box indicates a conserved potential Hfq binding site.

(Table 2 and Fig. 3). However, the El Tor sequences have a single nucleotide insertion and two substitutions that differ from the classical sequences. The non-O1 *V. cholerae* strains, commonly known as environmental *V. cholerae* to distinguish them from the O1 epidemic strains, carry the VPI but are not capable of causing cholera. These environmental *V. cholerae* strains have TarA sequences apparently derived from the classical biotype or vice versa. Three of these four non-O1 strains have TarA sequences identical to the classical TarA sequences; the fourth non-O1 strain has a single nucleotide change from the classical TarA sequences at a position that differs from the changes observed between classical and the El Tor biotype strains.

To determine whether the degree of TarA sequence conservation among different *V. cholerae* strains is a general feature of the VPI, we compared the DNA sequences 99 bp directly upstream of the *tarA* start site in the above strains to the sequence in O395. This analysis indicated that the *tarA* sequence was preferentially conserved over the surrounding intergenic sequence (Table 2). The degree of conservation of the 99 bp directly upstream of *tarA* varies to a wide degree, even between strains that have identical *tarA* sequences. For example, classical O395 and environmental SCE 256 have 99/99 identical base pairs within the *tarA* gene but only 68/99 identical base pairs within the upstream sequence. These upstream 99 bp include both the promoter and the two toxboxes that are required for expression of *tarA*; notably, the toxbox sequences are 100% conserved in every strain. We have also observed TarA expression in the El Tor strain N16961 under ToxT-inducing conditions (data not shown). These results strongly suggest that TarA conveys an evolutionary advantage to both environmental and epidemic *V. cholerae* and that its regulation by ToxT is highly conserved.

#### *TarA* regulates *ptsG* mRNA levels in *V. cholerae*

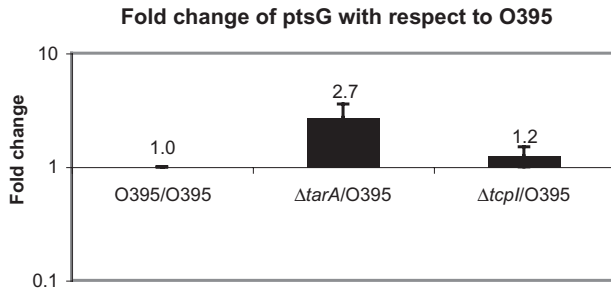
To identify potential regulatory targets of TarA, we probed microarrays of the *V. cholerae* genome with RNA isolated from classical strain O395 and its  $\Delta tarA$  derivative. The  $\Delta tarA$  strain features a markerless deletion of the

91-nucleotide RNA sequence with the promoters left intact. The whole-genome expression data were analysed using the Significance Analysis of Microarrays (SAM) program (Tusher *et al.*, 2001) using  $\geq 2.0$ -fold differences in gene expression and a  $\leq 1\%$  false discovery rate (FDR) as cut-off values. A gene annotated as *ptsG* (VC2013 in the N16961 genome sequence) was upregulated by greater than twofold in the  $\Delta tarA$  strain compared with wild type. This is consistent with a previous microarray study (Bina *et al.*, 2003), in which VC2013 RNA was shown to be upregulated in  $\Delta toxT$  *V. cholerae* relative to wild type. This pattern of regulation – with VC2013 expression being negatively controlled by ToxT – is in contrast to that of nearly all other known ToxT-regulated genes, such as the toxin co-regulated pilus (*tcp*) and the cholera toxin (*ctxAB*), which are positively controlled by ToxT. That *ptsG* may be the target of sRNA regulation in *V. cholerae* is consistent with the fact that *ptsG* in *Escherichia coli* is also regulated by a small RNA, SgrS (Vanderpool and Gottesman, 2004).

To confirm the microarray result, we used quantitative real-time polymerase chain reaction (qRT-PCR) comparing *ptsG* mRNA levels in wild type and *tarA* mutant *V. cholerae* strain O395. Consistent with the microarray experiments, we observed an approximately threefold increase in *ptsG* mRNA in mutant cells compared with wild type (Fig. 4), supporting the hypothesis that TarA acts to decrease the levels of *ptsG* mRNA. To be sure that regulation was due to the specific absence of *tarA* and not due to a polar effect on *tcpI*, a  $\Delta tcpI$  strain was also tested. *ptsG* transcript levels in the  $\Delta tcpI$  strain closely resemble those seen in wild-type O395, ruling out this possibility.

#### *Growth of V. cholerae in minimal glucose medium is limited in cells overexpressing TarA*

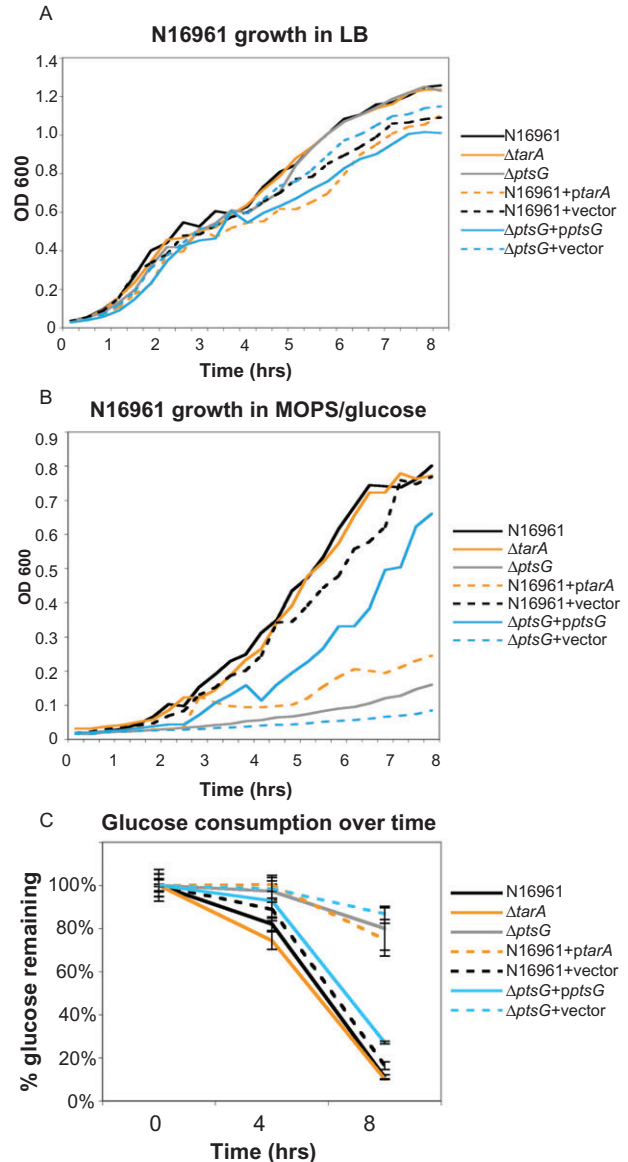
In *E. coli*, SgrS acts to reduce *ptsG* mRNA levels and to limit glucose uptake under conditions where flux through the glycolytic pathway may be disrupted, thereby allowing the accumulation of potentially lethal phospho-glucoside compounds (Vanderpool and Gottesman, 2004). When *E. coli* is grown with the glucose analogue alpha-methylglucoside ( $\alpha$ MG), the compound is phosphorylated and



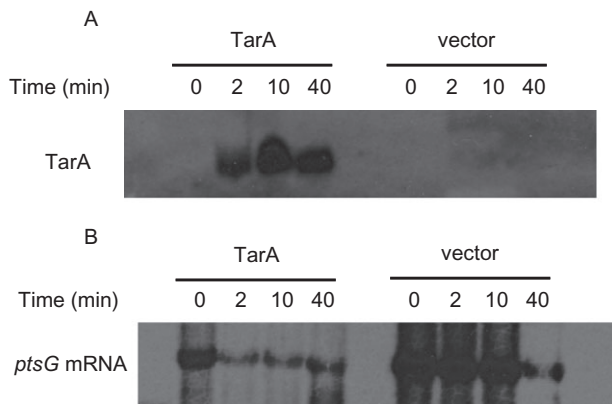
**Fig. 4.** Confirmation of *ptsG* regulation by qRT-PCR analysis. Cultures of the listed strains were grown under ToxT-inducing conditions for 7 h and total RNA was isolated using TRIzol. cDNA was prepared from equivalent amounts of RNA for each sample, and cDNA was used for SYBR Green qRT-PCR. Numbers indicate fold change in transcript level between strains, calculated using the  $\Delta\Delta CT$  method and *recA* transcript levels as an internal control.

taken up by PtsG, but phospho- $\alpha$ MG cannot be catabolized by the normal glucose metabolic pathway and therefore accumulates. Under such conditions, *sgrS* expression is induced and the resulting degradation of *ptsG* mRNA reduces further uptake of  $\alpha$ MG. Mutants lacking *sgrS* are highly sensitive to  $\alpha$ MG (Vanderpool and Gottesman, 2004). We tested whether  $\Delta tarA$  *V. cholerae* exhibited enhanced sensitivity to  $\alpha$ MG relative to wild type, which would be a physiological consequence of the fact that TarA regulates *ptsG* mRNA in *V. cholerae*. The two strains were grown in LB supplemented with  $\alpha$ MG in concentrations ranging from 1% to 10% weight by volume; we did not see a consistent effect of this glucose analogue on  $\Delta tarA$  *V. cholerae* at any of the concentrations tested.

Failing to identify an  $\alpha$ MG effect, we tested another *ptsG*-related hypothesis. We reasoned that constitutive overexpression of TarA, which would remove the RNA from its normal regulation by ToxT, would affect the growth physiology of *V. cholerae*, leading to diminished growth in glucose-containing minimal media if indeed *ptsG* mRNA levels are regulated by TarA. Overexpression of TarA in LB-grown *V. cholerae* had a slight effect on culture growth rate, although the final optical density achieved by this culture was close to wild type. Growth of  $\Delta tarA$  and wild-type *V. cholerae* carrying the vector alone was unaffected compared with wild type (Fig. 5A). However, after dilution from LB into minimal MOPS/0.5% glucose media, the cells overexpressing TarA were severely deficient for continued growth and resembled the  $\Delta ptsG$  strains, while the other strains grew similarly after a lag phase of approximately 3 h (Fig. 5B). The  $\Delta ptsG$  strain complemented with a constitutively expressed copy of *ptsG* exhibited partially restored growth in this medium. Analysis of glucose remaining in the supernatants of these cultures revealed similar levels of glucose for approximately 4 h, after which the culture overexpressing TarA and that of the  $\Delta ptsG$



**Fig. 5.** Overexpression of TarA results in severely reduced glucose uptake. A. LB growth. Indicated strains were grown overnight in LB media, subcultured into fresh LB and grown to mid-exponential phase, then washed with PBS and diluted into fresh LB to OD = 0.04. Cultures were grown in a 96-well plate at 30°C with constant aeration, and the OD<sub>600</sub> was measured every 20 min. Growth assays were performed at least three times, and a representative growth curve is shown. B. MOPS/glucose growth. Strains were prepared as in (A), but diluted into MOPS/0.5% glucose instead of LB and then grown as in (A). Growth assays were performed at least three times, and a representative growth curve is shown. C. Strains were grown overnight in LB, subcultured into LB and grown to mid-exponential phase, then washed and diluted into MOPS/0.5% glucose, and grown in flasks. At specified time points, supernatants were collected and the glucose concentration was tested using a tetrazolium blue-reducing sugar assay. Calculated glucose concentrations were divided by the starting concentration to determine the amount of glucose remaining in the media. This experiment was performed three times, and a representative assay is shown. Error bars indicate the standard deviation among triplicate samples in the representative experiment.



**Fig. 6.** Induction of TarA reduces *ptsG* transcript levels. Strains AR059 and AR060, containing an IPTG-inducible copy of TarA and an empty vector control, respectively, were grown overnight in LB. Cultures were diluted 1:200 into fresh LB, grown until OD ~0.4, and stimulated with IPTG. Thirty minutes after induction, cultures were washed and resuspended in fresh media lacking IPTG. RNA was collected at indicated time points (from the start of induction) using TRIzol, and equal amounts of RNA for each sample were subjected to Northern blot analysis. Methylene blue staining was used to confirm load amounts (not shown). Blots were probed with oligonucleotides complementary to TarA (A) and to the *ptsG* transcript (B).

mutants nearly stopped removing glucose from the medium while the others continued to do so (Fig. 5C). Thus, the poor growth of wild-type cells overexpressing TarA correlated with decreased levels of glucose uptake, similar to cells lacking *ptsG*.

To more carefully examine the association between TarA and *ptsG* mRNA levels, we placed *tarA* expression under control of an IPTG-inducible promoter in pHDB3 and followed steady-state levels of *ptsG* mRNA message upon TarA induction.  $\Delta tarA$  *V. cholerae* carrying this plasmid were grown in LB to mid-logarithmic levels before addition of IPTG to induce *tarA* expression. Thirty minutes after IPTG induction, cells were collected, washed, resuspended in fresh media and allowed to continue growing for another 30 min. RNA was harvested from cells at 0, 2, 10 and 30 min after addition of IPTG, and then at 10 and 30 min after the IPTG was washed out. RNA was analysed by Northern blotting using a probe specific for TarA or *ptsG* mRNA.

Prior to induction (0 min) TarA was undetectable while *ptsG* mRNA was abundant. Within 2 min after induction by IPTG, TarA became detectable and the amount of *ptsG* mRNA diminished. This continued throughout the course of the experiment, with TarA expression remaining at high levels and *ptsG* mRNA detectable at levels considerably lower than those seen prior to induction (Fig. 6). Cells carrying the cloning vector alone expressed no detectable TarA and very high levels of *ptsG* mRNA. The levels of *ptsG* mRNA were higher in the strain carrying only the cloning vector than in the cells carrying the *tarA* clone

without induction, which could be due to undetectable yet functional levels of TarA being expressed even prior to addition of IPTG. There is a slight decrease in *ptsG* mRNA levels in the vector control after 30 min of induction and 10 min of washout ( $t = 40$  time point), which we attribute to transcript turnover as the cells near stationary phase.

#### Base complementarity between TarA and the 5' region of the *ptsG* mRNA

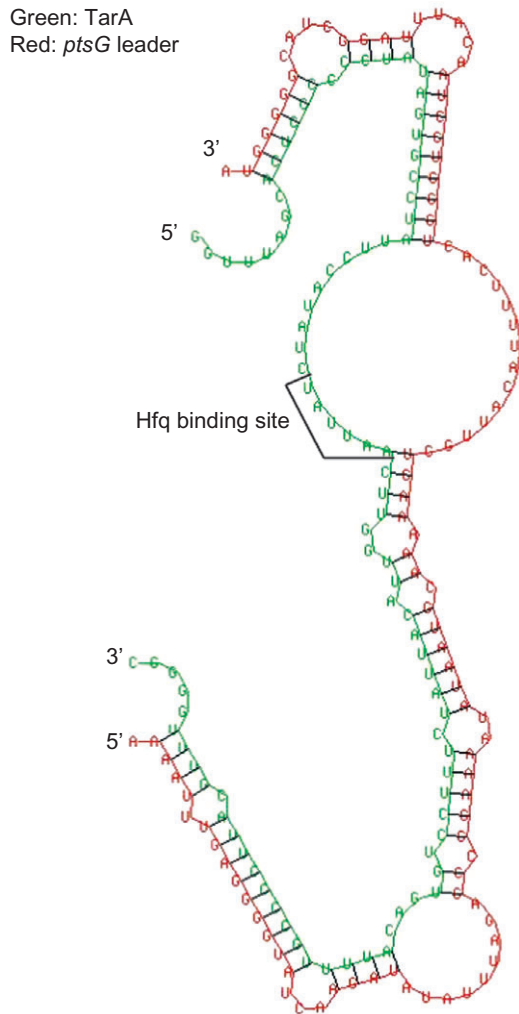
In *E. coli*, SgrS controls *ptsG* transcript levels in part by associating with the 5' end of the *ptsG* mRNA and causing its degradation in a process that depends on Hfq and RNase E (Wadler and Vanderpool, 2007; Maki *et al.*, 2008). We used the alignment prediction program RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/welcome.html>) to identify potential areas of complementarity between TarA and the leader sequence of the *ptsG* transcript that would be expected for a similar mechanism to be operating in *V. cholerae*. This program produced a structure with a favourable free energy ( $-54.4 \text{ kcal mol}^{-1}$ ) and a predicted unpaired region, which includes the putative Hfq binding site (Fig. 7). For purposes of comparison, we tested the potential complementarity of SgrS and the 100 bp directly upstream of *ptsG* in *E. coli* with the same program, which yielded a similarly composed structure with a minimal free energy of  $-49.2 \text{ kcal mol}^{-1}$  (data not shown).

#### TarA is unstable in the absence of Hfq

Given the putative Hfq binding site in the TarA sequence, we hypothesized that TarA interacts with Hfq. It is well established that Hfq-interacting small RNAs are markedly less stable in the absence of Hfq (Sledjeski *et al.*, 2001; Moller *et al.*, 2002; Masse *et al.*, 2003; Moll *et al.*, 2003; Morita *et al.*, 2004; Kawamoto *et al.*, 2005). To explore a potential TarA/Hfq interaction, we assessed TarA levels by Northern blot analysis of RNA from wild type and *hfq* mutant *V. cholerae*. Cells were grown for 7 h in ToxT-inducing conditions prior to RNA isolation and blotting. As predicted, the *hfq* mutant had significantly reduced TarA RNA in comparison with the wild-type strain O395 (Fig. 8).

## Discussion

Here we describe a small, non-coding regulatory RNA, TarA, whose transcription is directly activated by the major *V. cholerae* virulence activator protein ToxT. Activation of a regulatory RNA by ToxT adds another level to the complex *V. cholerae* virulence regulatory cascade and contributes to our understanding of ToxT-dependent gene activation. Based on our previous analyses of other ToxT-activated genes, the promoter architecture of *tarA* most closely



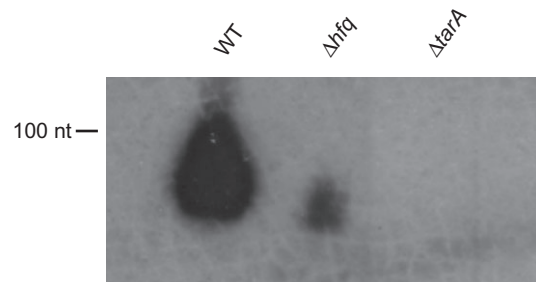
**Fig. 7.** Alignment of TarA and *ptsG* upstream sequences. The program RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/welcome.html>) was used to predict potential base-pairing between TarA and the *ptsG* leader sequence (defined here as 100 bp upstream of the AUG, including the +1 A). The TarA sequence is shown in green and the *ptsG* leader sequence in red. The putative Hfq binding site is indicated. The minimum predicted free energy for this alignment is  $-54.4 \text{ kcal mol}^{-1}$ .

resembles that of *tcpA* (Withey and DiRita, 2006). Furthermore, the degree of activation conferred by ToxT to the two genes is very similar – 41-fold for *tarA* and 44-fold for *tcpA*. The two *tarA* toxboxes have 7/7 and 6/7 of the toxbox core determinants conserved (Matson *et al.*, 2007), the highest match to consensus we have observed for any operon having two toxboxes. However, while the degree of ToxT activation of *tarA* is very high, it is not higher than the ToxT activation of *tcpA*, which has a poorer match to consensus at one toxbox. This suggests that the toxbox sequences may have evolved to be maximally efficient at any given locus, such that surrounding

sequence that is not conserved in the consensus alignment plays a more important role than previously thought.

TarA joins a growing list of regulatory sRNAs in *V. cholerae*. Four sRNAs, Qrr1, 2, 3 and 4, are involved in regulating the quorum-sensing response, together with Hfq protein, by negatively affecting the stability of *hapR* mRNA (Lenz *et al.*, 2004). Another three sRNAs, CsrB, C, D, are also involved in quorum sensing; these act to inhibit activity of CsrA protein, which subsequently leads to expression of the Qrr sRNAs (Lenz *et al.*, 2005). Another sRNA is RyhB, which is involved in regulating iron-containing proteins (Davis *et al.*, 2005). RyhB acts with Hfq to destabilize the mRNAs encoding these proteins. While many other sRNAs have been described in *E. coli*, very few of these are conserved in *V. cholerae*. The exceptions are SsrA, also known as tmRNA, RnpB, Spf and Ffs (Livny and Waldor, 2007). Recently, large numbers of sRNAs have been identified in *V. cholerae* using bioinformatics and parallel sequencing methods (Vogel *et al.*, 2003; Livny *et al.*, 2008). One of these, IRG7, also contributes to regulation of carbon utilization by negatively regulating a mannitol transport gene, *mtIA* (Liu *et al.*, 2009). TarA was not identified in either of these studies.

Our results suggest that a target of negative regulation by TarA in *V. cholerae* is *ptsG*, encoding a glucose transporter in the phosphotransferase system. In *E. coli*, *ptsG* is negatively regulated by SgrS, an sRNA whose mechanisms of action have been the subject of a significant amount of research. Expression of *sgrS* is activated by a conserved regulatory protein called SgrR, in response to glucose-phosphate stress (Vanderpool and Gottesman, 2004). SgrS is a bifunctional molecule, postulated to inhibit PtsG through two distinct mechanisms. One is by acting as a regulatory RNA directly by participating in a ternary complex with Hfq and the *ptsG* mRNA through specific base-pairing between the two RNA molecules.



**Fig. 8.** TarA is unstable in the absence of Hfq. Wild type,  $\Delta tarA$  and  $\Delta hfq$  strains were grown under inducing conditions (LB pH 6.5, 30°C) and total RNA was collected after 7 h of growth. Equal amounts of RNA from each sample (as determined by  $OD_{260}$ ) were subjected to Northern blot analysis, using an oligonucleotide complementary to TarA as a probe. Methylene blue staining was used to confirm load amounts (data not shown).

The site on *ptsG* mRNA that is base-paired to SgrS is in the 5' end of the transcript at the Shine–Dalgarno sequence (Vanderpool and Gottesman, 2004), blocking translation and eventually leading to degradation of *ptsG* mRNA through a mechanism that requires RNase E (Maki *et al.*, 2008). We think it likely that TarA also acts in a similar fashion, given the base complementarity between TarA and *ptsG* RNA, overlapping the latter's Shine–Dalgarno site. AU-rich sequences within a predicted unpaired region of TarA may also correspond to an Hfq-binding domain (Fig. 7) (Link *et al.*, 2009). Our observation that TarA is less stable in an *hfq* mutant lends support to our hypothesis that Hfq and TarA interact. The second mechanism by which SgrS regulates PtsG is through a small protein, SgrT, encoded at the 5' end of the same RNA that includes SgrS in *E. coli* and in a variety of other species (Wadler and Vanderpool, 2007) (Horler and Vanderpool, 2009). SgrT is hypothesized to act post-translationally on PtsG function (Wadler and Vanderpool, 2007). TarA does not have an open reading frame and is consequently much shorter than SgrS. Another distinction between SgrS and TarA is that, unlike functional SgrS homologues in many other Gram-negative bacteria (Horler and Vanderpool, 2009), a homologue of the SgrR regulatory protein is not found encoded next to *tarA* in the VPI. As sequence homology to *sgrR* was used to locate SgrS functional homologues in other enteric bacteria, the fact that an SgrS homologue was not predicted in *V. cholerae* is not surprising.

Unlike *sgrS* in *E. coli*, *tarA* expression is not controlled by the glucose-phosphate response regulator SgrR, but rather by ToxT, the activator responsible for expression of the major virulence factors of *V. cholerae*. The high degree of similarity between the *tarA* and *tcpA* toxboxes suggests that the two are expressed synchronously during infection. *tarA* makes a slight contribution to the fitness of *V. cholerae* during infection, as competition experiments between wild type and *tarA* mutant *V. cholerae* in the infant mouse model resulted in a small but consistent colonization defect of the *tarA* mutant (average competitive index = 0.45). Cells expressing elevated PtsG during infection could be at a competitive disadvantage if glucose is not the preferred *in vivo* carbon source. This hypothesis is under investigation. An alternative explanation for how the link between *tarA* expression and virulence gene activation may have evolved comes from work demonstrating that *V. cholerae* in stool samples of cholera patients store glucose in the form of glycogen granules, and that glycogen biosynthesis genes are expressed late during infection (Schild *et al.*, 2007). Perhaps *tarA* expression by ToxT during infection, with a subsequent reduction in *ptsG* mRNA, prepares the cells for the apparent metabolic conversion to glycogen production by reducing the amount of glucose transported into the glycolytic pathway.

## Experimental procedures

### *V. cholerae* strains and plasmids

Strains used in this work were classical *V. cholerae* O395, El Tor strain N16961 and derivatives (listed in Table S1). The  $\Delta tarA$ ,  $\Delta ptsG$  and  $\Delta hfq$  strains were constructed from O395 and N16961 by mating with *E. coli* SM10 $\lambda$ pir using the pKAS32 suicide vector as previously described (Skorupski and Taylor, 1996). The deletion was confirmed by PCR using primers flanking the gene. Strains were grown in LB medium at 37°C (overnight cultures), in LB medium with a starting pH of 6.5 at 30°C (inducing conditions for classical strains), or under AKI conditions (inducing conditions for El Tor strains). Strains were maintained in LB/20% glycerol at –70°C. Antibiotics were used in the following concentrations: ampicillin: 100  $\mu\text{g ml}^{-1}$ , streptomycin: 100  $\mu\text{g ml}^{-1}$  and chloramphenicol: 30  $\mu\text{g ml}^{-1}$ .

Strains used for  $\beta$ -galactosidase assays were either O395 or an O395  $\Delta toxT$  derivative (VJ740, Champion *et al.*, 1997) carrying the respective *tarA::lacZ* fusion in plasmid pTL61T (Linn and St Pierre, 1990). All constructs were cloned between the HindIII and XbaI sites of pTL61T. For constitutive and inducible expression of TarA, plasmids pKK177-3RI (Opdyke *et al.*, 2004) and pHDB3 (Ulbrandt *et al.*, 1997) were used, respectively. In pKK177-3RI, the *tarA* sequence alone was cloned between the EcoRI and HindIII sites, creating plasmid pJM292. The *tarA* sequence preceded by the P<sub>LacO</sub> operator sequence was cloned between the BamHI and HindIII sites of pHDB3 to generate pAR6. These plasmids, and the empty vector controls, were electroporated into wild-type O395 and N16961 (pKK177-3RI and pJM292) and the corresponding  $\Delta tarA$  strains (pHDB3 and pAR6). For *ptsG* complementation, the *ptsG* sequence preceded by a ribosome binding site was cloned between the EcoRI and XbaI sites of pUC19, generating plasmid pAR7. This plasmid and the empty vector control were then electroporated into N16961  $\Delta ptsG$ . All plasmid sequences were confirmed by the University of Michigan DNA sequencing core.

### $\beta$ -Galactosidase assays

Strains were cultured overnight in LB medium at 37°C, then subcultured at a 1/50 dilution into inducing medium and grown for 3 h at 30°C with vigorous aeration. Bacteria were then placed on ice and chloramphenicol was added to 0.5 mg ml<sup>-1</sup>.  $\beta$ -Galactosidase assays were performed using the basic procedure of Miller (Miller, 1972) as previously described (Withey and DiRita, 2005b).

### DNA manipulations

Plasmids were purified using the Promega Wizard Plus Miniprep kit. PCR was performed using *Taq* DNA polymerase (Roche) as specified by the manufacturer. Restriction enzymes were purchased from New England Biolabs and used as specified by the manufacturer.

### Growth analysis and glucose quantification

Strains N16961, AR43, AR38, AR39, AR74, AR80 and AR81 were grown overnight in LB, then subcultured 1:100 into fresh



LB and grown to mid-log phase (6 h). OD<sub>600</sub> was measured for each culture, and  $1 \times 10^9$  bacteria of each strain were pelleted, washed in PBS and resuspended to a final OD<sub>600</sub> of 2 in PBS. These suspensions were then diluted 1:50 (final OD<sub>600</sub> = 0.04) into LB and MOPS minimal medium supplemented with 0.4% glucose (Neidhardt *et al.*, 1974), and dispensed into a 96-well plate. The plate was incubated at 30°C with constant shaking for 8 h in an Omega Polarstar plate reader, with OD<sub>600</sub> measured every 20 min. For glucose quantification, cultures were prepared as described and grown in 125 ml flasks on a ThermoFisher orbital shaker at 37°C. One millilitre of samples were taken at 0, 4 and 8 h of growth. Bacteria were pelleted, and 10-fold diluted supernatants were used in a tetrazolium blue-reducing sugar assay as described (Jue and Lipke, 1985). Glucose concentration was determined for each sample and divided by the starting concentration to determine the per cent glucose remaining. Assays were run in triplicate three times.

#### Northern blots

Whole-cell RNA was purified from O395 and VJ740 ( $\Delta$ toxT) *V. cholerae* grown in parallel under inducing and non-inducing conditions (Fig. 2), from strains AR59 and AR60 after addition of IPTG (Fig. 6), and from strains O395, AR15 and AR24 grown under inducing conditions (Fig. 8). At specific points (as detailed in figure legends for each experiment), the OD<sub>600</sub> of each culture was read and aliquots of each culture equal to 1 ml of a 0.7 OD<sub>600</sub> culture were removed. RNA purification was performed using the TRIzol reagent as specified by the manufacturer (Invitrogen). For TarA visualization, 7 µg of total RNA from each sample was separated by 8% polyacrylamide gel electrophoresis and transferred to an Amersham Hybond nylon membrane (GE Healthcare) (0.45 µ) with a Semi-Phor apparatus (Hofer). An oligonucleotide probe complementary to nt 12–36 of TarA was radiolabelled with [ $\gamma$ -<sup>32</sup>P]-ATP with T4 polynucleotide kinase (New England Biolabs). Probing conditions were as previously described (Sambrook *et al.*, 1989). To visualize *ptsG* transcript, 12.5 µg of each sample was run on a 1.2% agarose/formaldehyde gel and transferred to an Amersham Hybond nylon membrane (GE Healthcare). Membranes were probed with an oligonucleotide complementary to nt 176–208 of the *ptsG* transcript, labelled as above.

#### Microarray and qRT-PCR analysis

Cultures of O395 and  $\Delta$ tarA strains were grown overnight and then diluted 1:100 into LB pH 6.5 and grown at 30°C. Once cultures reached an OD<sub>600</sub> ~0.35, they were diluted 1:10 and grown until OD<sub>600</sub> ~0.35 and 2 ml aliquots of the samples were harvested. Cell pellets were resuspended in TRIzol (Invitrogen) and total RNA was isolated according to the manufacturer's protocol as described previously (Yildiz *et al.*, 2004). Whole-genome expression analysis was performed using 70-mer oligos representing the open reading frames present in the *V. cholerae* N16961 genome. cDNA synthesis, microarray hybridization and analysis of expression data were performed as described previously (Beyhan *et al.*, 2006) using two biological and four technical replicates (for each biological replicate).

RNA samples for qRT-PCR were DNase treated, run on an agarose gel to check quality and quantified by measuring the OD<sub>260</sub>. Approximately 2.5 µg of each sample was treated with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's specifications. For detection of transcripts, primers amplifying a 200 bp region in the middle of the mRNA were used with SYBR Green Master Mix (Stratagene) on a Stratagene MX3000P thermocycler. Primers were designed using the OligoPerfect tool (Invitrogen). Each test was performed in triplicate at least three times, and fold change in expression was calculated using the  $\Delta\Delta$ CT method (Pfaffl, 2001), with *recA* transcript levels used as controls between cDNA samples.

#### Infant mouse colonization assays

Four-day-old CD1 mice (Charles River) were inoculated intragastrically with approximately  $10^6$  bacteria. The inocula were 50:50 mixtures of the strain of interest (either *tarA*<sup>+</sup>/*lacZ*<sup>+</sup> *V. cholerae* or the *tarA*<sup>+</sup>/*lacZ*<sup>+</sup> control) and the control strain, which is a *lacZ*<sup>-</sup> O395 derivative. Inoculated mice were incubated at 30°C for 16 h, after which the mice were sacrificed and their intestines were removed and homogenized. Serial dilutions of the individual intestinal homogenates were plated on LB agar plates containing streptomycin and X-gal. Blue and white colonies were counted after overnight growth at 37°C. To determine the ratio of strains in the initial inoculum, the mixtures used to inoculate the mice were diluted into PBS and plated onto LB agar plates containing streptomycin and X-gal, and again the blue and white colonies were counted. The competitive index was calculated by dividing the *in vivo* blue : white ratio by the blue : white ratio of the initial inoculum.

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