SUPPORTING INFORMATION

A Novel Type V TA System Where mRNA for Toxin GhoT
is Cleaved by Antitoxin GhoS

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SUPPLEMENTARY METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids are listed in Supplementary Table 1. Luria-Bertani (LB) at 37°C was used except where indicated. To form pBS(Kan)-ghoS, the coding region along with 33 bp upstream of the ghoS start codon was amplified using Pfu DNA polymerase and using primers GhoS-f-KpnI and GhoS-r-Sacl (Supplementary Table 3), and the PCR product was cloned into vector pBS(Kan) at the KpnI and SacI sites. Plasmid pBS(Kan)-ghoS was verified by DNA sequencing using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with forward primer pBS(Kan)-f and reverse primer pBS(Kan)-r (Supplementary Table 3).

Protein expression and purification. The full-length GhoS (YjdK) gene was sub-cloned into the RP1B bacterial expression vector, which contains an N-terminal Thio6-His6-tag and Tobacco Etch Virus (TEV) cleavage site. The plasmid was transformed into E. coli BL21-Gold (DE3) Competent Cells (Agilent). The expression of uniformly 13C/15N-labeled and 15N-labeled protein was carried out by growing freshly transformed cells in M9 minimal medium containing 4 g/L [13C]-D-glucose and/or 1 g/L 15NH4Cl (Cambridge Isotope Laboratory) as the sole carbon and nitrogen sources, respectively.

For the purification, the pellets were resuspended in lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 5 mM imidazole, Complete tabs-EDTA free (Roche)). The cells were lysed using high-pressure homogenization using a C3 Emulsiflex high-pressure cell homogenizer (Avestin), and the cell debris was removed by centrifugation (45,000 × g, 50 min, 4°C). The supernatant was filtered through a 0.22-µm membrane (Millipore) and loaded onto a HisTrap HP column (GE Healthcare). His6-tagged proteins were eluted using a 5-500 mM imidazole gradient. The fractions containing GhoS were identified by SDS-PAGE and pooled. The His6-tag was removed using proteolytic cleavage by overnight incubation with TEV protease (dialysis buffer: 20 mM Tris pH 7.5, 500 mM NaCl; 4°C). Cleavage was verified by SDS-PAGE. GhoS was further purified using a Ni-NTA column (Qiagen) to separate cleaved protein from the protease (itself his6-tagged) and the cleaved tag. The protein was purified in a final step using size exclusion chromatography (Superdex 76 26/60, GE Healthcare; buffer: 20 mM NaPO4 pH 6.0, 100 mM NaCl, 0.5 mM TCEP). Comparison with SEC standards revealed that GhoS is a monomer in
solution. The hydrodynamic radius of native GhoS was measured using a 802 DLS dynamic light scattering instrument (Viscotek Corporation). GhoS was concentrated to 1.5 mg/ml in 20 mM NaPO₄ pH 6.0, 100 mM NaCl, and 0.5 mM TCEP and filtered through a 0.22-µm filter. The hydrodynamic radius was determined using OmniSize 3.0 software. We measured a radius of hydration \( R_h \) of 1.8 nm, which correlates perfectly to a ~12.7 kDa globular protein, further confirming that GhoS is a monomer in solution.

**GhoS substitutions and circular dichroism.** Mutagenesis of *ghoS* was carried out using the QuickChange Mutagenesis Kit (Agilent Technologies) using the manufacturer’s protocols; all constructs were verified by sequencing. The GhoS variants were produced and purified identically to the wild-type protein. Circular dichroism (CD) spectra for native GhoS and the GhoS variants were acquired using a Jasco J-815 CD spectrometer equipped with a Peltier temperature control system. CD measurements were carried out at protein concentrations of ~20 µM in sample buffer (20 mM sodium phosphate, pH 6.0, 100 mM NaCl, 0.5 mM TCEP). UV wavelength scans from 205 to 260 nm were performed at 25°C in a 0.2 cm path length cuvette using a scan rate of 20 nm/min. The GhoS variants were purified using protocols identical for native GhoS. mRNA cleavage assays with native GhoS and variant proteins were carried out as described except the PCR products were purified using the PureLink PCR purification kit (Invitrogen).

**Purification of His₆-GhoS for EMSA assays.** His-GhoS with six histidines and 10 additional cloning artifacts at the N-terminus was purified via pCA24N-*ghoS*⁵². BW25113/pCA24N-*ghoS* was grown in 1L LB with chloramphenicol and was induced with 1 mM IPTG at a turbidity at 600 nm of 0.5 for 15 h at room temperature. Cells were collected and were resuspended in 25 mL lysis buffer (50 mM potassium phosphate buffer, pH 8.0, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted twice by a French Press (Thermo Electron Corporation). Ni-NTA resin (Qiagen) was used according to the manufacturer’s protocol. Purified protein was dialyzed against buffer (50 mM potassium phosphate buffer, pH 7.6, 50 mM NaCl and 8% glycerol) twice at 4°C overnight, the protein was concentrated using a 5 kDa cut-off centrifugal concentrator (Millipore), and the protein concentration was measured by using a Pierce BCA assay kit (Pierce).
Electrophoretic mobility shift assays (EMSA). EMSA were performed as described previously. The promoter region of the ghoST operon (199 bp) (PghoST) was amplified by PCR using Pfu DNA polymerase from genomic DNA with primers PghoS-f and PghoS-r (Supplementary Table 3). The PCR products were gel purified with a QIAquick Gel Extraction Kit (Qiagen), and were labeled with biotin using the Biotin 3'-end DNA Labeling Kit (Pierce). For binding reactions, biotin-PghoS was incubated with purified His-GhoS protein either with or without unlabeled PghoS DNA for 2 h at room temperature. Different binding reaction conditions were performed with the nonspecific competitor DNA (poly dI-dC) and NP-40 and either with or without glycerol, CaCl₂, MgCl₂ and BSA. Samples were run on a 6% DNA retardation gel (Invitrogen) at 100V in 0.5× TBE (10 mM Tris borate at pH 8.3 and 2 mM EDTA) for 90 min. DNA was then transferred to a nylon membrane at 390 mA for 45 min then UV cross-linked at 302 nm. Chemiluminescence was performed with the LightShift Chemiluminescent EMSA Kit (Thermo Scientific) and samples were detected with a CCD imager (Typhoon 9410 Imager).

Western blot analysis. BW25113/pCA24N-ghoS was grown to a turbidity at 600 nm of 0.1 in LB with chloramphenicol, then 0.5 mM IPTG was added to induce ghoS. When the turbidity reached 1, cells were washed and resuspended in fresh LB with chloramphenicol (to remove IPTG). The stressors tested were H₂O₂ (20 mM), heat (50°C), ampicillin (100 µg/mL), tetracycline (10 µg/mL), salt (6% NaCl), and rifampin (200 µg/mL). After 15 min and 30 min, samples were collected and processed with 1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich), and the Western blot was performed with primary antibodies raised against a His tag (Cell Signaling Technology) and horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Millipore).

NMR spectroscopy. NMR experiments were acquired at 298K on a Bruker Avance II 500 MHz spectrometer. In addition, a 3D ¹⁵N-resolved [¹H,¹H]-NOESY and a 3D ¹³C-resolved [¹H,¹H]-NOESY spectra were recorded on a Bruker Avance II 800 MHz spectrometer. Both spectrometers are equipped with a TCI HCN z-gradient cryoprobe. Proton chemical shifts were referenced to an internal 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS).

Chemical shift assignments and structure calculation. The following spectra were used to achieve the
sequence-specific backbone and side-chain assignments of all aliphatic residues: 2D \[^{1}H,^{15}N\] HSQC, 2D \[^{1}H,^{13}C\] HSQC, 3D HNCA, 3D HNCACB, 3D (HBHA)CBCA(CO)NH, 3D (H)CC(CO)NH, 3D HBHA(CBCACO)NH, 3D \(^{15}N\)-resolved \[^{1}H,^{1}H\]-NOESY, 3D \(^{13}C\)-resolved \[^{1}H,^{1}H\]-NOESY, and 3D HC(C)H-TOCSY (12 ms CC spin-lock). 2D \[^{1}H,^{1}H\]-NOESY, 2D \[^{1}H,^{1}H\]-TOCSY and 2D \[^{1}H,^{1}H\]-COSY spectra of GhoS in D\(_2\)O solution after complete H/D exchange of the labile protons were used for the assignment of the aromatic side chains. TopSpin 2.1 (Bruker) was used for data acquisition and processing. NMR spectra were analyzed using CARA (http://cara.nmr.ch). The following spectra were used for structure calculation: 3D \(^{15}N\)-resolved \[^{1}H,^{1}H\]-NOESY (mixing time of 80 ms), 3D \(^{13}C\)-resolved \[^{1}H,^{1}H\]-NOESY (mixing time of 80 ms), and 2D \[^{1}H,^{1}H\]-NOESY (mixing time 80 ms, D\(_2\)O solution). The amino acid sequence, the chemical shift assignment and the NOESY spectra were input for the automated NOESY peak picking and NOE assignment method of ATNOS/CANDID/CYANA. The results of ATNOS/CANDID/CYANA were refined by manual peak adjustment and additional calculations in CYANA\(^{29-31}\). The 20 conformers from the final CYANA cycle with the lowest residual CYANA target function values (of 200 calculated) were energy-minimized in a water shell with the program CNS 1.3. The structure quality was assessed by the programs WHATCHECK\(^{53}\), AQUA\(^{54}\) and NMR-PROCHECK\(^{54}\) which are part of the iCing suite (http://nmr.cmbi.ru.nl/cing/iCing.html), and MOLMOL\(^{55}\). Chemical shift assignments of GhoS were deposited in the BioMagResBank (BMRB) under accession number 18086 and coordinates were submitted to the PDB under accession code 2LLZ. 99.25% of the residues are in most favored regions and additionally allowed regions, 0.7% in generously allowed regions, and 0.05% in disallowed regions of the Ramachandran diagram. Furthermore, the ensemble has excellent geometry with no violations of distance restraints greater than 0.5 Å and no dihedral angle violations greater than 5°. With the exception of the N-terminal residues (-2) to 4 and the C-terminal residues 96 to 98, the GhoS structure is well-defined. The root-mean-square-deviation value about the mean coordinate positions of the backbone atoms for residues 5 to 95 is 0.36 ± 0.08 Å.

RNA isolation and whole-transcriptome studies. Cultures of \textit{E. coli} BW25113/pCA24N-ghost and BW25113/pCA24N were grown in LB medium from turbidity at 600 nm of 0.05 to 0.2, then induced with
1 mM IPTG for 90 min. After breaking the harvested cells using a bead beater (Biospec, Bartlesville, OK), total RNA was isolated with the RNeasy Mini Kit (Qiagen, P/N 74104) and converted into cDNAs through a reverse transcription reaction with poly(A) RNA controls. The cDNAs were then digested with DNase I (Amersham Biosciences) to produce 50- to 200-bp fragments, labeled with Biotin-ddUTP using the Enzo BioArray Terminal Labeling Kit (Affymetrix, P/N 900181), and the labeled cDNAs were hybridized at 45°C for 16 h to the *E. coli* GeneChip Genome 2.0 array (Affymetrix, P/N 511302). The gene expression data are accessible through GEO accession number GSE36779.

**RNA sequencing and mutagenesis of ghoT mRNA.** To identify the GhoS cleavage sites within *ghoT* (207 nt, primers shown in Supplementary Table 7), a modified RNA sequencing method was used. Instead of using RNase III-fragmented RNA samples, RNA sequencing was performed starting from 500 ng of GhoS-cleaved *ghoT* transcripts with an Ion PGM sequencer (Life Technologies). Using NextGENe version 2.2.0 (SoftGenetics) to align the 593,148 sequence reads of the cleaved *ghoT* locus, the four most abundant reads (28-nt, 51-52 nt, 64-66 nt and 116-117 nt) were identified; to retrieve these sequences, the .fastq file was uploaded to Galaxy (https://main.g2.bx.psu.edu/#), processed with FASTQ Groomer, and filtered with Filter FASTQ by length and quality. The regions with decreased levels of counts were considered cleaved. The cleaved positions were confirmed via alignment with the full-length *ghoT* coding sequence. Alignment of the 10-nt regions in the cleavage sites revealed a conserved pattern of 5’-UNNU(A/C)N(A/G)(A/U)A(A/U)-3’.

To confirm the GhoS cleavage sites in *ghoT* mRNA, plasmids were constructed with modified *ghoT* sequences for the *in vitro* cleavage assay. PCR was performed using pBS(Kan)-*ghoT* (Supplementary Table 1) as the template with different sets of primers (Supplementary Table 3). The PCR products were recovered, subjected to DpnI digestion and transformed into *E. coli* TG1 (Supplementary Table 1). The plasmids prepared from the resulting transformants were confirmed by sequencing and designated as pBS(Kan)-*ghoTm1*, pBS(Kan)-*ghoTm2*, pBS(Kan)-*ghoTm3*, and pBS(Kan)-*ghoTm4* (Supplementary Table 1). For construction of pBS(Kan)-*ghoTm1m2*, PCR was performed using pBS(Kan)-*ghoTm1* as the
template with primers *ghoT*-m2f/*ghoT*-m2r (Supplementary Table 3), and the plasmid was confirmed by sequencing. RNA secondary structure was predicted using MFold software using default parameters.

**Persister revival assay.** BW25113Δ*ghoT/pCA24N-ghoT* cells were diluted to a turbidity at 600 nm of 0.05 in LB containing chloramphenicol, grown for 2.5 h, then 1 mM IPTG was added for 2 h to produce GhoT. The cells were washed with 25 mL of 0.85% NaCl, adjusted to a turbidity of 1.0 in 25 mL of LB, and treated with 100 µg/mL ampicillin to form persister cells (untreated cells did not receive ampicillin). After incubation for 2 h, all samples were washed with 25 mL of 0.85% NaCl, washed again with LB, then diluted to a turbidity of 0.05 in LB, and cell growth was monitored every 30 min.

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**SUPPLEMENTARY RESULTS**

**Supplementary Table 1.** Bacterial strains and plasmids used in this study. Chloramphenicol (30 or 34 µg/mL) and kanamycin (50 µg/mL) were used to maintain pCA24N-based plasmids and pBS(Kan)-based plasmids, respectively.

| Bacterial strains/Plasmids | Description | Source |
|---------------------------|-------------|--------|
| **E. coli K12 strains**   |             |        |
| TG1                       | supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, (rK⁻ mK⁻), F' [traD36 proAB° lacF° lacZΔM15] | Stratagene |
| BL21(DE3)                 | F'ompT hsdS6(rB⁻ mB⁻) gal dcm lon (DE3) | Novagen |
| BW25113                   | lacPrrnB714ΔlacZW316 hsdR514ΔaraBAD ΔrhaBADΔD78 | 27 |
| BW25113 ΔghoT             | BW25113 ΔghoT ΔKm | 27 |
| BW25113 ΔghoS             | BW25113 ΔghoS ΔKm | 27 |
| BW25113 ΔyahH             | BW25113 ΔyahH ΔKm | 27 |
| BW25113 ΔyhfQ             | BW25113 ΔyhfQ ΔKm | 27 |
| BW25113 ΔybhT             | BW25113 ΔybhT ΔKm | 27 |
| BW25113 ΔyciG             | BW25113 ΔyciG ΔKm | 27 |
| BW25113 ΔymdF             | BW25113 ΔymdF ΔKm | 27 |
| BW25113 ΔtrpL             | BW25113 ΔtrpL ΔKm | 27 |
| BW25113 ΔralR             | BW25113 ΔralR ΔKm | 27 |
| BW25113 ΔkilR             | BW25113 ΔkilR ΔKm | 27 |
| BW25113 ΔhisL             | BW25113 ΔhisL ΔKm | 27 |
| BW25113 ΔpheL             | BW25113 ΔpheL ΔKm | 27 |
| BW25113 ΔygaQ             | BW25113 ΔygaQ ΔKm | 27 |
| BW25113 ΔtnaC             | BW25113 ΔtnaC ΔKm | 27 |
| BW25113 ΔyheV             | BW25113 ΔyheV ΔKm | 27 |
| BW25113 ΔyahH ΔKm         | BW25113 ΔyahH ΔKm | this study |
| BW25113 ΔtrpL ΔKm         | BW25113 ΔtrpL ΔKm | this study |
| BW25113 ΔhisL ΔKm         | BW25113 ΔhisL ΔKm | this study |
| BW25113 ΔtnaC ΔKm         | BW25113 ΔtnaC ΔKm | this study |
| **Plasmids**              |             |        |
| pCA24N                    | CmR; lacF°; pCA24N | 52 |
| pCA24N-mqsR               | CmR; lacF°; pCA24N P_T5-lac::mqsR° | 52 |
| pCA24N-ghoS               | CmR; lacF°; pCA24N P_T5-lac::ghoS + | 52 |
| pCA24N-ghoT               | CmR; lacF°; pCA24N P_T5-lac::ghoT + | 52 |
| pBS(Kan)                  | KmR; pBS(Kan) | 50 |
| pBS(Kan)-ghoS             | KmR; pBS(Kan)P_lac::ghoS + | this study |
| pBS(Kan)-ghoSX            | KmR; pBS(Kan)P_lac::ghoS + with stop codon introduced at Y16 | this study |
| pBS(Kan)-ghoT             | KmR; pBS(Kan)P_lac::ghoT + | this study |
Cm\(^R\) and Km\(^R\) are chloramphenicol and kanamycin resistance, respectively.
**Supplementary Table 2.** Fold change in the persistence of the BW25113 Keio Collection mutants whose genes encode mRNA that lack the primary MqsR cleavage site 5’-GCU relative to wild-type BW25113 upon producing MqsR (using 1 mM IPTG for 2 h) from pCA24N-mqsR after exposing to ampicillin (100 µg/mL) for 2 h. The possibility of a polar effect was evaluated for the most important mutants by eliminating the kanamycin resistance cassette (∆kan) that replaced the gene that was knocked out and retesting for an effect on persistence.

| Name  | Fold-change | b-number | Operon     | Polar effect | Relative Position | Fold-change ∆kan |
|-------|-------------|----------|------------|--------------|------------------|-----------------|
| yahH  | 4.9         |          |            |              |                  | 8.4             |
| ybfQ  | -4.3        | b4514    |            | None         |                  |                 |
| ybhT  | 1.4         | b0762    |            | None         |                  |                 |
| yciG  | -1.9        | b1529    | yciGFE     | Possible     | 86 bp upstream of yciF |                 |
| ymdF  | 2.4         | b4518    |            | None         |                  |                 |
| trpL  | 7.7         | b1264    |            | Possible     | 92 bp upstream of trpE | 3.8             |
| ralR  | -1.8        | b1348    | ralR-ydaC  | Yes          | overlapped       |                 |
| kilR  | 2           | b1352    | kilR-ydaE  | Yes          | overlapped       |                 |
| hisL  | -14.3       | b2018    |            | Possible     | 146 bp upstream of hisG | -103.0          |
| pheL  | 2           | b2598    |            | Possible     | 99 bp upstream of pheA |                 |
| ygaQ  | -3          | b4462    |            | None         |                  |                 |
| tnaC  | 3.3         | b3707    |            | Possible     | 221 bp upstream of tnaA | -4.0            |
| yheV  | 1.8         | b4551    |            | Possible     | 95 bp upstream of slyD |                 |
| ghoT  | -14.7       | b4559    | ghoS-ghoT  | None         |                  |                 |
**Supplementary Table 3.** Oligonucleotides used for RT-PCR, qRT-PCR, mutant verification, cloning (restriction sites are underlined and in bold), site directed mutagenesis (target mutated nucleotides are underlined), and EMSA. f indicates forward primer and r indicates reverse primer.

| Name       | Sequence (5' to 3')                                                        |
|------------|---------------------------------------------------------------------------|
| **RT-PCR and qRT-PCR**                                                                 |
| ghoS-f     | CCATCATCTTATTCTCTCAGTGGTCT                                               |
| ghoS-r     | TAAGTCTAAGCATTGAGCCTGATT                                                 |
| ghoT-f     | TGGTGTGAACATATCCTTTGTC                                                  |
| ghoT-r     | TAATGCCACAGGCAAGACTCA                                                    |
| ralR-f     | AAACCATGTCCATTTTGTTG                                                    |
| ralR-r     | TCCAGTGTTCTGTTTATTC                                                    |
| ompA-f     | CACCTGCGTGTTTGCCTACCS                                                 |
| ompA-r     | ACCATTTCAAAGCCAACATC                                                    |
| ompF-f     | AAGCGCAATTCTGCGGACAT                                                  |
| ompF-r     | TGCCACGGTAACTGTTTC                                                     |
| purA-f     | GGGGCTGCTTATGAGAATAAAGT                                                |
| purA-r     | TCAACCACCATAGAAGTCAGGAT                                                |
| purM-f     | CGGTGTGTTGATTGGACGCGG                                                   |
| purM-r     | GAGCACGACCCGAGATTT           |
| purH-f     | GCCGACGCACTTTGGCGGC                                                     |
| purH-r     | CTTCAGCGGCTCCATCCATCATCT                                                |
| purE-f     | GCAATAATCGCGCGGGTGGTC                                                  |
| purE-r     | GTTTATCGGGGTTGCGGTGAG                                                  |
| pyrI-f     | GAAGCTATTAAACGCGGCAGG                                                   |
| pyrI-r     | TTTGATTCAGATCTTTGCGGCC                                                  |
| pyrB-f     | AGGCGGCAGAATCATGGAAGCAAAC                                                |
| pyrB-r     | CCCAGCGCGGTCATAGATG                                                    |
| carA-f     | GCCGGTGGGGGAAGTCTGTT                                                   |
| carA-r     | CTTCAGCGGCTCCATGGTGTC                                                  |
| carB-f     | CCTGATTCTGGGTGCGGGC                                                    |
| carB-r     | ATGTCGCGCGGTTGGAGT                                                    |
| **Deletion mutant verification**                                                     |
| GhoS-veri-f| CTCCTATATGAGAATCATCAATCGGGG                                             |
| GhoS-veri-r| GACAAGGATATGGTCCACACCAATCA                                               |
| GhoT-veri-f| GACCTCAACATATGACAGTGGTAGAC                                              |
| GhoT-veri-r| GCTTCGTTTCACTGTTCCGCAAATCC                                                |
| **Cloning and verification**                                                         |
| GhoS-f-KpnI | GTCTAGGGTACCCAGAGCGTTAATATGAGTCATATTATGGAAGGTTAAAA |
| GhoS-r-SacI | GAGCAGAGCTCCTACTTGGAAAACTGACGGAATAGTCATC |
| pBS(Kan)-f | GCTTCTGAGTGCTGATACGCGCT                                                |
| pBS(Kan)-r | GTGATGGTTCACGTAGTGGGCGCATCG                                             |
| GhoS-f-NdeI | GGCGCGCCCATATGGGAAGGTTAAAAACAAG                                       |
GhoS-r-\text{XhoI} \quad \text{CCGTTT} \text{CTCGAG} \text{TCATTGTAAACACGCG}

\textbf{Site-directed mutagenesis}

\begin{align*}
\text{GhoS-X-f} & \quad \text{CTTATGTGTGTTTCTGATTAGCCATCATCTTTATTCATTCC} \\
\text{GhoS-X-r} & \quad \text{CACTGAGGAATAAGATGATGGGTATAAAGAAACACATAAG} \\
\text{ghoT-m1} & \quad \text{ATGGCATATTCTCTCAGCCGCAATTTTTATGTGATTGGT} \\
\text{ghoT-m1} & \quad \text{ACCAATCACATAAAAATTTGCGCGTTTAGAGAATAGTGCCAT} \\
\text{ghoT-m2} & \quad \text{ATCTCACATGAGAAAAACACCCGCGGTGTTTTACTTAGACTTGCAATTCC} \\
\text{ghoT-m2} & \quad \text{CAGGAATGCACTAAAGTAACGCACCGCGGTGTTTTATTCATCTGAGAT} \\
\text{ghoT-m3} & \quad \text{ACTATTTGTTGTTTCTTTTGATTAGCCATCATCTTTATTCATTCC} \\
\text{ghoT-m3} & \quad \text{GTTCACACACATCACATCGCCGTATATTTTGAGAATAATG} \\
\text{ghoT-m4} & \quad \text{TGAGAAAAACACATATTCCGACCCCTAGTGCACTTCGGTGGG} \\
\text{ghoT-m4} & \quad \text{CCGACCAGGAATGCACTAGGGTGCGAAATAATGTGTTTTGCTCA} \\
\text{ghoST-f} & \quad \text{GGTAAAAACAGGCCGCAATTTCTTTATGATTTGATTTAATG} \\
\text{ghoST-r} & \quad \text{GGGAGAAATTCATATCATACATCAATGATGCTAATCTTAAAGAAGCATGAGAATAAGATCG} \\
\text{ghoST-f} & \quad \text{CCATCATCATTCTCAGTTTCATTAGCAATTAGATCATATGTGATATGATATTTCATTCC} \\
\text{ghoST-r} & \quad \text{GGAGAAAATTCATATCATACATCAATGATGCTAATCTTAAGAACACACTGAGGAATAAGATCG} \\
\text{EMSA} & \quad \text{TCATTCTCCTCAGGTGATGATGCGAC} \\
\text{PghoST-f} & \quad \text{AATATGACTCTCAATTAAAACGCTCT} \\
\end{align*}
**Supplementary Table 4.** NMR and refinement statistics for the GhoS protein structure.

|                                | GhoS               |
|--------------------------------|--------------------|
| **NMR distance and dihedral constraints** |                    |
| Distance constraints          |                    |
| Total NOE                     | 2479               |
| Intra-residue                 | 554                |
| Inter-residue                 |                    |
| Sequential \((i - j) = 1\)    | 638                |
| Medium-range \((i - j) < 4\)  | 569                |
| Long-range \((i - j) > 5\)    | 718                |
| Hydrogen bonds                |                    |
| Total dihedral angle restraints|                    |
| \(\phi\)                     | 45                 |
| \(\psi\)                     | 45                 |

| **Structure statistics**       |                    |
| Violations (mean and s.d.)     |                    |
| Distance constraints (Å)       | 0.02 ± 0.005       |
| Dihedral angle constraints (º) | 0.13 ± 0.06        |
| Max. dihedral angle violation (º)| 0.98 ± 0.45       |
| Max. distance constraint violation (Å) | 0.47 ± 0.06       |

| Deviations from idealized geometry |                    |
| Bond lengths (Å)                  | 0.012 ± 0.001     |
| Bond angles (º)                   | 1.31 ± 0.04       |
| Improps (º)                       | 1.53 ± 0.06       |

| Average pairwise r.m.s. deviation** (Å) |      |
| Heavy (residues 5-95)                | 0.36 ± 0.08      |
| Backbone (residues 5-95)             | 0.71 ± 0.05      |

**Pairwise r.m.s. deviation was calculated among 20 refined structures.**
Supplementary Table 5. DALI results for GhoS.

| PDB ID | Z-SCORE | NRES | %ID | PDB DESCRIPTION                      |
|--------|---------|------|-----|--------------------------------------|
| 3ic1   | 6.3     | 370  | 4   | succinyl-diaminopimelate desuccinylase |
| 3isz   | 6.2     | 369  | 3   | succinyl-diaminopimelate desuccinylase |
| 3nrb   | 6.2     | 280  | 7   | formyltetrahydrofolate deformylase   |
| 2i0x   | 5.6     | 82   | 19  | uncharacterized protein              |
| 1u0s   | 5.6     | 86   | 7   | chemotaxis histidine kinase          |
| 2bmb   | 5.5     | 513  | 13  | pyrophosphokinase/dihydropteroate synthase |
| 1bxj   | 5.5     | 104  | 10  | uncharacterized protein              |
| 2zn1   | 5.1     | 147  | 12  | HTH-type transcriptional regulator  |
| 1vk8   | 5.3     | 94   | 7   | putative thiamine biosynthesis/salvage protein |
| 2ko1   | 5.3     | 88   | 6   | ACT domain from GTP pyrophosphokinase |
| 2nzc   | 5.2     | 80   | 8   | uncharacterized protein              |
| 3oq2   | 5.1     | 101  | 8   | CAS2 endoribonuclease               |
| 3mco   | 5.1     | 386  | 14  | pyrophosphokinase                   |
| 3o1l   | 5.1     | 285  | 9   | formyltetrahydrofolate deformylase  |
| 1ru1   | 5.1     | 143  | 16  | pyrophosphokinase                   |
**Supplementary Table 6. Summary of the qRT-PCR results.** Conditions and strains are indicated along with the cycle number (Ct) for each sample including that for the target genes as well as that of the housekeeping gene, *rrsG*, which was used to normalize the data.

Fold changes in the transcription of various targets with and without GhoS (*ghoS*/*ghoS*) were calculated as described earlier 58:

\[
2^{-\left(\text{Ct}_{\text{target}}_{\text{ghoS}+}-\text{Ct}_{\text{rrsG}_{\text{ghoS}}}\right)} / 2^{-\left(\text{Ct}_{\text{target}}_{\text{ghoS}-}-\text{Ct}_{\text{rrsG}_{\text{ghoS}}}ight)}
\]

Values less than one are indicated as negative fold changes (i.e., transcription was repressed). The specificity of the qRT-PCR products was verified by a melting curve analysis. The *ghoS*+ strains were BW25113/pCA24N-*ghoS*, and the *ghoS* strains were BW25113/pCA24N. Means and standard errors are indicated.

| Conditions       | Strains                        | C<sub>t</sub> (target gene) | C<sub>t</sub> (housekeeping) | Fold change |
|------------------|--------------------------------|-----------------------------|-----------------------------|-------------|
| OD ~5            | BW25113 wild type              | *ghoS* 23.92 ± 1.75         | *rrsG* 13.93 ± 0.90         | 21 ± 2      |
|                  |                                | *ghoT* 28.35 ± 0.56         | *rrsG* 13.93 ± 0.90         |             |
| 1 mM IPTG, 60 min| BW25113/pCA24N-*ghoS*         | *ghoT* 31.84 ± 0.30         | *rrsG* 10.66 ± 0.53         | -5 ± 1      |
|                  | BW25113/pCA24N                |                            | *rrsG* 10.92 ± 0.37         |             |
|                  |                                | *ompA* 19.85 ± 0.11         | *rrsG* 11.89 ± 0.06         | -1.1 ± 0.2  |
|                  | BW25113/pCA24N                |                            | *rrsG* 11.91 ± 0.18         |             |
|                  |                                | *ompF* 15.88 ± 0.07         | *rrsG* 11.89 ± 0.06         | 1.2 ± 0.1   |
|                  | BW25113/pCA24N                |                            | *rrsG* 11.91 ± 0.18         |             |
|                  |                                | *ralR* 21.67 ± 0.48         | *rrsG* 11.89 ± 0.06         | -1.7 ± 0.4  |
|                  | BW25113/pCA24N                |                            | *rrsG* 11.91 ± 0.18         |             |
|                  |                                | *purA* 20.74 ± 0.41         | *rrsG* 11.89 ± 0.06         | -1.9 ± 0.5  |
|                  | BW25113/pCA24N                |                            | *rrsG* 11.91 ± 0.18         |             |
| 1 mM IPTG, 90 min| BW25113/pCA24N-*ghoS*         | *ghoS* 18.13 ± 0.10         | *rrsG* 13.93 ± 0.60         | 316.5 ± 0.6 |
|                  | BW25113/pCA24N                |                            | *rrsG* 14.17 ± 0.83         |             |
|                  |                                | *ghoT* 28.74 ± 0.21         | *rrsG* 13.93 ± 0.60         | -3.2 ± 0.6  |
|                  | BW25113/pCA24N                |                            | *rrsG* 14.17 ± 0.83         |             |
|                  |                                | *purM* 28.40 ± 0.71         | *rrsG* 14.28 ± 0.79         | -7.4 ± 0.5  |
|                  | BW25113/pCA24N                |                            | *rrsG* 14.79 ± 0.64         |             |
|                  |                                | *purH* 24.79 ± 0.64         | *rrsG* 13.55 ± 0.15         |             |

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| Genes  | Conditions | pCA24N-<i>ghoS</i> (mM) ± SE | pCA24N (mM) ± SE | ΔmM ± SE |
|--------|------------|-------------------------------|-----------------|-----------|
| **purE**<sup>rrsG</sup> | 1 mM IPTG, 90 min | 27.60 ± 0.02 | 25.30 ± 0.47 | -3.0 ± 0.5 |
| **pyrI**<sup>rrsG</sup> | 1 mM IPTG, 90 min | 27.70 ± 0.06 | 25.14 ± 0.05 | -2.3 ± 0.5 |
| **carA**<sup>rrsG</sup> | 1 mM IPTG, 90 min | 23.22 ± 0.02 | 21.48 ± 0.03 | -9.8 ± 0.6 |
| **carB**<sup>rrsG</sup> | 1 mM IPTG, 90 min | 27.20 ± 0.11 | 25.24 ± 0.24 | 2.3 ± 0.5 |
**Supplementary Table 7.** Oligonucleotides used for PCR to generate templates for T7 RNA polymerase *in vitro* transcription. f indicates forward primer and r indicates reverse primer. For the forward primers, the T7 promoter sequence is underlined and the bases incorporated into RNA during transcription are in bold.

| RNA | Transcript size (nt) | Primer Name | Sequence (5' to 3') |
|-----|---------------------|-------------|---------------------|
| *ghoT* | 207 | PT7-*ghoT*-F | TAATACGACTCATAAGGGAGACCAGGATAAGAAGAACATAA AAAATG |
|     |     | PT7-*ghoT*-R | CTAAAAGAGAGAAAAAGTAATGC |
| *ghoS* | 311 | PT7-*ghoS*-F | TAATACGACTCATAAGGGAGACCAGGAGCGTCTAAATATGGATAGGGAGCTATT |
|     |     | PT7-*ghoS*-R | TCATCAACTGTCATAATGGGAG |
| *atpE* | 189 | PT7-*atpE*-F | TAATACGACTCATAAGGGAGATGGAAAACCTGAATATGGATCTGC |
|     |     | PT7-*atpE*-R | CATCCACCAGACCATAACGATAA |
| *ompA* | 211 | PT7-*ompA*-F | TAATACGACTCATAAGGGAGACCGTGCTCAATGGATCTGGATTA |
|     |     | PT7-*ompA*-R | CGGCTGAGTTACAACGTCTTTGAT |
**Supplementary Table 8.** Representative sequences retrieved from groups of the most abundant sequence reads from RNA-Seq. The cleavage sites identified from the 3' -end (S1, S2, S3 and S4) or 5’-end (S5) of the sequences are shown. ∨ indicates the position of the cleavage site.

| Group | Length (nt) | Sequence (5’ to 3’)                                                                 | Cleavage site |
|-------|-------------|-------------------------------------------------------------------------------------|---------------|
| 1     | 29          | GGGAGACCAGGAAGGATAAGAAGAAGAAGCAAATA                                                | S1            |
| 2     | 52          | GGGAGACCAGGAAGGATAAGAAGAAGAAGCAATAAAAAATGGCATA                                       | S2            |
| 3     | 65          | GGAATAACCTGGCCAATGAGTCTGCCTGTGGCATTACTTTTTTCTCTCTTAGAGAAAAAAAAGA                    | S5            |
| 3     | 65          | GGGAGACCAGGAAGGATAAGAAGAAGAAGCAATAAAAAATGGCATA                                       | S3            |
| 4     | 116         | GGGAGACCAGGAAGGATAAGAAGAAGAAGCAATAAAAAATGGCATA                                       | S4            |

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Supplementary Table 9. Differential gene expression/mRNA degradation upon production of GhoS for 90 min with 1 mM IPTG via BW25113/pCA24N-ghoS vs. BW25113/pCA24N. The DNA microarray results were verified by qRT-PCR for the transcripts indicated.

| Gene b number | Fold change (GhoS/Vector) | Description |
|---------------|--------------------------|-------------|
| **Putative toxin/antitoxin pair** | | |
| ghoS b4128    | 26.0                     | antitoxin   |
| ghoT b4559    | 1.1                      | toxin       |
| **Purine synthesis and transport** | | |
| purM b2499    | -7.5                     | phosphoribosyl-aminoimidazole synthase |
| purH b4006    | -6.1                     | phosphoribosyl-aminoimidazole carboxamide formyltransferase |
| purE b0523    | -5.3                     | N5-carboxyaminoimidazole ribonucleotide mutase |
| cvpA b2313    | -4.6                     | protein required for colicin V, first gene in the purF operon |
| purC b2476    | -4.3                     | Amidophosphoribosyltransferase |
| purD b4005    | -4.3                     | phosphoribosylamine-glycine ligase |
| purK b0522    | -4.3                     | N5-carboxyaminoimidazole ribonucleotide synthase |
| purN b2500    | -7.5                     | phosphoribosyl-aminoimidazole synthase |
| purT b1849    | -4.3                     | glycine ribonucleotide transformylase 1 |
| **Pyrimidine synthesis and transport** | | |
| pyrI b4244    | -19.7                    | aspartate carbamoyltransferase |
| pyrB b4245    | -16.0                    | aspartate carbamoyltransferase, catalytic subunit |
| carA b0032    | -12.1                    | carbamoyl-phosphate synthase, small subunit |
| carB b0033    | -16.0                    | carbamoyl-phosphate synthase, large subunit |
| codA b0337    | -7.5                     | cytosine deaminase |
| codB b0336    | -10.6                    | cytosine permease |
| pyrC b1062    | -4.6                     | dihydroorotate, the third step in pyrimidine biosynthesis |
| pyrD b0945    | -9.2                     | dihydroorotate dehydrogenase |
| pyrL b4246    | -7.5                     | pyrBI operon regulatory leader peptide |
| uraA b2497    | -9.2                     | uracil permease; affects concentration dependence of pyr mutants |
| catF b0034    | -4.9                     | transcriptional activator of cai operon, next to carAB operon |
Supplementary Figure 1. The ghOST operon. (a) Schematic of the operon. There are 27 bp between ghoS and ghOT. Black arrow indicates the direction of transcription. The position of one forward primer (ghoS-f) and two reverse primers (ghoS-r and ghOT-r) are indicated by black arrows. (b) Results of reverse-transcription PCR analysis of the ghOST operon. cDNA was synthesized with reverse transcriptase using total RNAs from the BW25113 wild-type strain grown at 37°C during the exponential phase (turbidity was 0.5). Lanes 1 and 4 are cDNA synthesized from mRNA, lanes 2 and 5 are RNAs (used as a negative control), and lanes 3 and 6 are genomic DNAs (used as a positive control). Lane 1 indicates two genes were amplified from a single mRNA transcript using primers ghs0-f and ghOT-r. Lane 4, as a control, indicates ghs0 was present using primers ghs0-f and ghs0-r. (c) DNA sequence for ghOST. The putative transcription start site of ghOST operon is indicated by a black arrow, and the putative RBS sites are highlighted in grey. Forward primer (ghoS-f) and reverse primers (ghoS-r and ghOT-r) for RT-PCR are indicated via blue and red arrows, respectively, and the primers used for in vitro transcription of ghs0 and ghOT mRNAs are double underlined.
Supplementary Figure 2. GhoS is stable with stress. (a) Upper panel (Western blot) shows that there is no apparent degradation of antitoxin GhoS as detected with an anti-His tag antibody with different stressors. His-tagged GhoS (marked with arrows) was produced from pCA24N-ghoS, which has six histidine residues and ten other amino acids attached at the N-terminus (expected size of ~13.8 kDa). Lower panel shows the corresponding whole cell lysates as visualized by SDS-PAGE. Stressors include H$_2$O$_2$ (20 mM), heat (50°C), Amp (ampicillin, 100 µg/mL), Tet (tetracycline, 10 µg/mL), Salt (6% NaCl), Acid (pH 2.5), and Rif (rifampin, 200 µg/mL). Production of ghoS was induced via 0.5 mM IPTG at turbidity of 0.1 for 2 h. (b) Positive controls show the degradation of antitoxins MqsA (upper panel) and antitoxin DinJ (lower panel) upon H$_2$O$_2$ stress (20 mM). His-tagged MqsA and DinJ (marked with arrows) were produced from pCA24N-based plasmids.
Supplementary Figure 3. GhoS does not bind to its promoter. (A) EMSA shows that GhoS does not bind and shift its promoter (PghoST) under varying binding conditions. Biotin-labeled DNA was incubated with either 250-fold (lanes 2 to 5, and 10) or 375-fold excess of GhoS (lanes 6 to 9). Lane 10 also contains 50-fold excess of unlabeled DNA. For the binding reactions, all lanes contain poly dI-dC and NP-40, lanes 2 and 6 also contain glycerol, lanes 3 and 6 also contain glycerol and MgCl₂, lane 4 and 8 contain glycerol, MgCl₂ and CaCl₂, and lanes 5 and 9 also contain glycerol, MgCl₂, CaCl₂ and BSA. Each assay was performed three times. (B) EMSA shows that MqsA binds and shifts its promoter (PmqsR), as a positive control. Biotin-labeled DNA was incubated with either 50-fold (lanes 2 and 5), 100-fold (lane 3) or 200-fold (lane 4) excess of MqsA. Lane 5 also contains 50-fold excess of unlabeled DNA. For the binding reactions, lanes 1 to 5 contain poly dI-dC, NP-40 and MgCl₂.
**Supplementary Figure 4 GhoS is monomeric.** (a) Superdex 75 26/60 size exclusion chromatogram (SEC) for GhoS. A single peak is observed at 219 mL, an elution position expected for a monomer (the molecular weight of GhoS is 11.7 kDa). Inset, SDS-PAGE shows that the protein is highly pure. (b) The mass distribution histogram of wild-type GhoS from DLS experiments. The peak corresponds to a hydrodynamic radius of 1.8 nm, equivalent to a molecular weight of 12.7 kDa.
Supplementary Figure 5. Annotated 2D $[^1\text{H},^{15}\text{N}]$ HSQC spectrum of GhoS. Each assigned residue is labeled with its residue name (one letter code) and number in the primary sequence. NH$_2$ groups of asparagine and glutamine side chains are indicated by a solid line. The arginine side chain Hε-Nε peaks are labeled with asterisks.
Supplementary Figure 6. GhoS adopts a ferredoxin-like fold. (a) Stereo view of the backbone atoms (N, Ca, C’) of the 20 superimposed lowest-energy NMR structures. Three α-helices are in coral and five β-strands are in green; loops are in dark blue. (b) The hydrophobic cluster that stabilizes β1, α1, β3, α2 and β4. Hydrophobic residues necessary for the formation of this hydrophobic core are shown as yellow sticks and labeled. (c) The hydrophobic cluster that stabilizes β2, β2’ and α3. Hydrophobic residues necessary for the formation of this hydrophobic core are shown as coral sticks and labeled. (d) Overlay of GhoS (dark blue) and the CAS2 protein SSO1404 (coral). Residues important for SSO1404 catalytic activity and structurally homologous residues in GhoS are shown as sticks, with GhoS residues labeled. (e) SSO1404 with residues critical for endoribonuclease activity shown as sticks and labeled.
Supplementary Figure 7. GhoS is similar to CAS2 monomers. GhoS (blue) is shown with the 5 CAS2 proteins that have DALI Z-scores of 5.0 or higher. SSO1404 (PDBid 2I8E; Z-score = 6.1, coral), SSO8090 (PDBid 3EXC; Z-score = 5.8, teal), TT1823 (PDBid 1ZPW; Z-score = 5.6, pink), PF1117 (PDBid 2I0X, Z-score = 5.1, orange) and DvuCAS2 (PDBid 3OQ2, Z-score = 5.0, purple). The overlay of all six structures are shown on the right. While CAS2 proteins are functional dimers, GhoS is monomeric (see Supplementary Fig. 4).
Supplementary Figure 8. Full gels of the GhoS cleavage assays of Fig. 3 and Fig. 4.
**Supplementary Figure 9. GhoS RNase activity is specific.** Total RNA isolated from wild-type cells and treated with GhoS *in vitro* (left panel), as well as total RNA isolated from wt/pCA24N and wt/pCA24N-ghoS after 1 h induction with 1 mM IPTG (*in vivo*) (middle panel). Right panel: *E. coli* tRNAs (73 to 93 nt) treated with GhoS (*in vitro*).
Supplementary Figure 10. GhoT and GhoS affect early biofilm formation and motility. (a) Normalized biofilm formation for the wild-type strain, ΔghoS, and ΔghoT measured after 8 h and 24 h in LB medium at 37°C and 30°C, respectively. (b) Motility after 12 h of incubation at 37°C. A representative image is shown on a low-salt motility agar plate. Error bars indicate standard error of mean (n = 3). Significant changes are marked with an asterisk for P < 0.05.