Supporting Information

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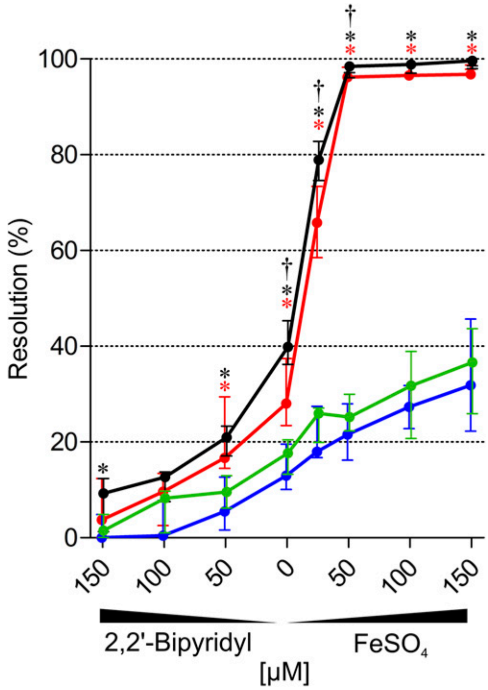

Fig. S1. Impact of iron availability on resolution frequency of the four reporter strains. Shown are the resolution frequencies versus iron concentrations for the four *V. cholerae* reporter strains Vc_res_TRIVET *irgA::tpc* (black), Vc_res1_TRIVET *irgA::tpc* (red), Vc_res_TRIVET1 *irgA::tpc* (green), and Vc_res1_TRIVET1 *irgA::tpc* (blue) with variation of the res or res1 cassette and pTRIVET or pTRIVET1 harboring different TetR-controlled promoter elements upstream of *tnpR*. Resolution was determined after 8 h in LB supplemented with varying amounts of 2,2′-bipyridyl or $FeSO_4$ as indicated to mimic low and high iron concentrations, respectively. Data are presented as the median with interquartile range from at least six independent experiments. Significant differences between resolution frequencies at a given iron concentration are indicated as follows: cross symbol [comparison of Vc_res_TRIVET *irgA::tpc* (black) and Vc_res1_TRIVET *irgA::tpc* (red)], black asterisk [comparison of Vc_res_TRIVET *irgA::tpc* (black) and Vc_res_TRIVET1 *irgA::tpc* (green)], and red asterisk [comparison of Vc_res1_TRIVET *irgA::tpc* (red) and Vc_res1_TRIVET1 *irgA::tpc* (blue)], respectively ($P < 0.05$, using a Mann–Whitney *U* test).

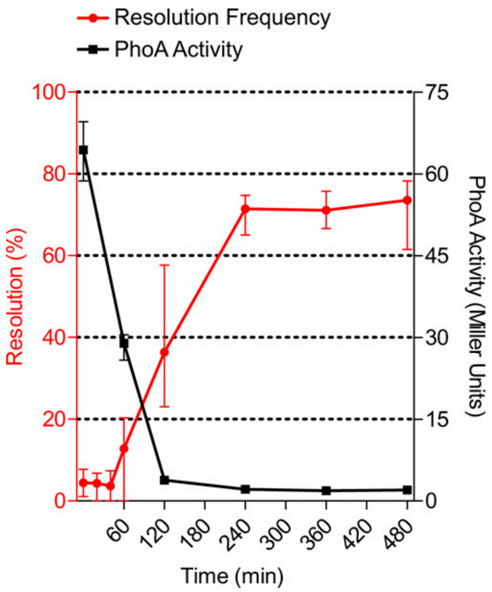

Fig. S2. Kinetics of resolution frequency and alkaline phosphatase (PhoA) activity. Vc_res1_TRIVET *irgA::tpc* was grown to late logarithmic phase in low iron medium (LB supplemented with 150 μM 2,2′-bipyridyl) to prevent resolution and then shifted to high iron medium (LB supplemented with 25 μM $FeSO_4$) and assayed for resolution (red) or PhoA activity (black) at the time points indicated on the *x* axis. Data are presented as the median with interquartile range from at least six independent experiments.

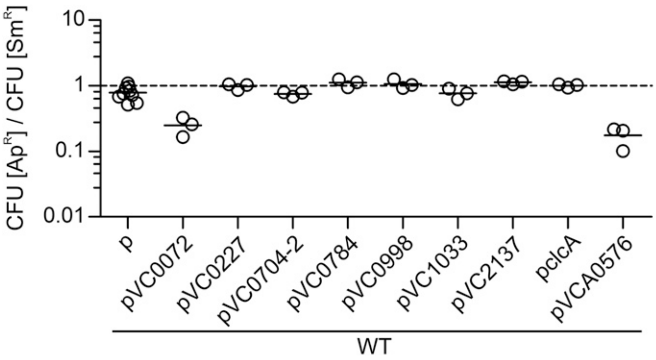

Fig. S3. Maintenance of pBR322 and derivatives in the absence of antibiotic selection. The stability of pBR322 (p, Ap^R) and derivatives pVC0072, pVC0227, pVC0704-2, pVC0784, pVC0998, pVC1033, pVC2137, pclcA, and pVCA0576 in *V. cholerae* WT is indicated as the ratio of cfu [Ap^R]/cfu [Sm^R] after 16 h incubation in LB-Sm. A ratio of 1 (dashed line) indicates optimal plasmid stability. Horizontal bars indicate the mean of each dataset ($n \geq 3$).

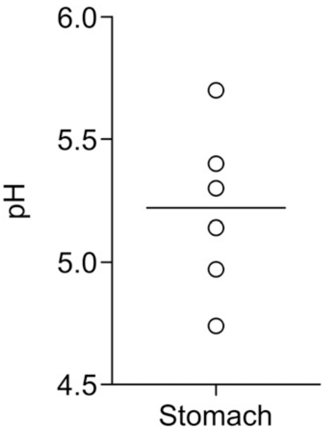

Fig. S4. Stomach content of infant mice is acidic. Stomach content of 5-d-old CD-1 mice was resuspended in 1 mL of saline, and the pH was measured by a pH meter (Metrohm 827 pH laboratory). Each circle represents the pH from an individual stomach content, and the horizontal bar indicates the median of the dataset ($n = 6$).

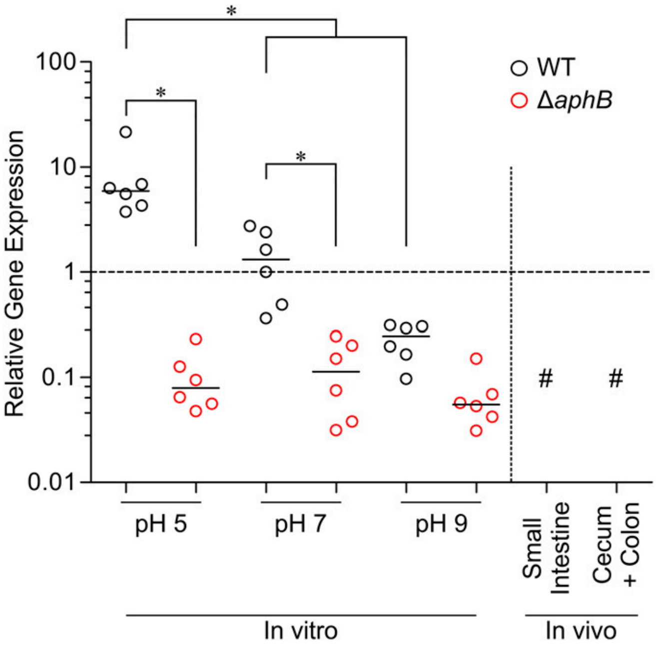

Fig. S5. Transcriptional regulation of *clcA* validated by qRT-PCR. Relative expression levels of *clcA* were determined by qRT-PCR for WT (black circles) and Δ*aphB* (red circles) grown in vitro using LB broth adjusted to pH 5, pH 7, or pH 9, as well as in vivo for WT colonizing the murine gastrointestinal tract (small intestine and cecum with colon). Each circle represents a biological replicate, horizontal lines highlight the median of each dataset, and an asterisk indicates significant differences ($n = 6$, $P < 0.05$, using a Kruskal–Wallis test followed by post hoc Dunn's multiple comparisons). The dashed line indicates a relative *clcA* expression of 1. As *clcA* mRNA transcripts were not detected in six out of six independent in vivo samples, the in vivo expression levels were set to the limit of detection (0.1 with respect to the WT pH 7 data set) indicated by the number sign (#).

Table S1. Strains and plasmids used in this study

Strain or plasmid	Genotype/resistance	Source
E. coli strains		
DH5αλpir	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *hsdR17*(r_K^- m_K^+) λ*pir*RK6	(54)
XL-1	F′::Tn*10 proA* + *B* + *lac^q*Δ(*lacZ*)*M15*/*recA1 endA1 gyrA46* (Nal^r) *thi hsdR17* (r_K^- m_K^+) *supE44 relA1 lac*	New England Biolabs
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*RK6	(55)
V. cholerae strains		
WT	Spontaneous Sm^R mutant of E7946 (O1 El Tor Inaba), Sm^R	(56, 57)
Δ*lacZ*	Deletion of *lacZ* in WT, LacZ$^-$, Sm^R	This study
Δ*clcA*	Deletion of *clcA* (VCA0526) in WT, Sm^R	This study
Δ*aphB*	Deletion of *aphB* (VC1049) in WT, Sm^R	This study
irgA::tpc	Insertion of *tetRphoAcat* downstream of *irgA* in WT, Sm^R, Cm^R	This study
Vc_res *irgA::tpc*	Insertion of res cassette in *lacZ* in *irgA::tpc*, Sm^R, Cm^R, Km^R	This study
Vc_res1 *irgA::tpc*	Insertion of res1 cassette in *lacZ* in *irgA::tpc*, Sm^R, Cm^R, Km^R	This study
Vc_res_TRIVET *irgA::tpc*	Insertion of P$_{tetA}$ controlled *tnpR* in Vc_res *irgA::tpc*, Sm^R, Cm^R, Km^R	This study
Vc_res1_TRIVET *irgA::tpc*	Insertion of P$_{tetA}$ controlled *tnpR* in Vc_res1 *irgA::tpc*, Sm^R, Cm^R, Km^R	This study
Vc_res_TRIVET1 *irgA::tpc*	Insertion of P$_{tet1}$ controlled *tnpR* in Vc_res *irgA::tpc*, Sm^R, Cm^R, Km^R	This study
Vc_res1_TRIVET1 *irgA::tpc*	Insertion of P$_{tet1}$ controlled *tnpR* in Vc_res1 *irgA::tpc*, Sm^R, Cm^R, Km^R	This study
clcA::tpc	Insertion of *tetRphoAcat* downstream of *clcA* in WT, Sm^R, Cm^R	This study
Vc_res1 *clcA::tpc*	Insertion of res1 cassette in *lacZ* in *clcA::tpc*, Sm^R, Cm^R, Km^R	This study
Vc_res1_TRIVET *clcA::tpc*	Insertion of P$_{tetA}$ controlled *tnpR* in Vc_res1 *clcA::tpc*, Sm^R, Cm^R, Km^R	This study
Δ*aphB clcA::tpc*	Insertion of *tetRphoAcat* downstream of *clcA* in Δ*aphB*, Sm^R, Cm^R	This study
Δ*aphB* Vc_res1 *clcA::tpc*	Insertion of res1 cassette in *lacZ* in Δ*aphB clcA::tpc*, Sm^R, Cm^R, Km^R	This study
Δ*aphB* Vc_res1_TRIVET *clcA::tpc*	Insertion of P$_{tetA}$ controlled *tnpR* in Δ*aphB* Vc_res1 *clcA::tpc*, Sm^R, Cm^R, Km^R	This study
Vc_res	Insertion of res cassette in *lacZ* in WT, Sm^R, Km^R	This study
Vc_res1	Insertion of res1 cassette in *lacZ* in WT, Sm^R, Km^R	This study
Vc_res::pLOFtpc	Transposon mutant library with random chromosomal insertions of the *tetRphoAcat* in Vc_res, Sm^R, Cm^R, Km^R	This study
Vc_res1::pLOFtpc	Transposon mutant library with random chromosomal insertions of the *tetRphoAcat* in Vc_res1, Sm^R, Cm^R, Km^R	This study
Vc_res_TRIVET::pLOFtpc	Integration of TetR-controlled (P$_{tetA}$) *tnpR* downstream of *lacZ* in Vc_res::pLOFtpc, Sm^R, Cm^R, Km^R	This study
Vc_res1_TRIVET::pLOFtpc	Integration of TetR-controlled (P$_{tetA}$) *tnpR* downstream of *lacZ* in Vc_res1::pLOFtpc, Sm^R, Cm^R, Km^R	This study
Vc_res_TRIVET1::pLOFtpc	Integration of TetR-controlled (P$_{tetO}$) *tnpR* downstream of *lacZ* in Vc_res::pLOFtpc, Sm^R, Cm^R, Km^R	This study
Vc_res1_TRIVET1::pLOFtpc	Integration of TetR-controlled (P$_{tetO}$) *tnpR* downstream of *lacZ* in Vc_res1::pLOFtpc, Sm^R, Cm^R, Km^R	This study
Plasmids		
pCVD442	Suicide vector, OriR6K, *sacB*, Ap^R	(25)
p	pBR322 origin, Ap^R, Tc^R	(58)
pAC1000	Cm^R	(59)
pTrc99A-Km	pBR322 origin, Km^R	(60)
pGOA1193	pIVET5n tnpR, *oriR6K mobRP4 lacZ tnpR*, Ap^R	(17)
pGP704	*oriR6K mobRP4*, Ap^R	(55)
pCVD442irgA::tpc	pCVD442 with *irgA::tetRphoAcat*, Ap^R	This study
pCVD442clcA::tpc	pCVD442 with *clcA::tetRphoAcat*, Ap^R	This study
pCVD442lacZ	Suicide vector for deletion of *lacZ*, OriR6K, *sacB*, Ap^R	This study
pRes	pSL111 with res-*neo-sacB*-res cassette, Km^R	(17)
pRes1	pSL111 with res1-*neo-sacB*-res1 cassette, Km^R	(17)
pLOFKm	Tn*10* based delivery plasmid, Km^R, Ap^R	(24)
pLOF::tpc	*tetRphoAcat* cassette in pLOF, Ap^R, Cm^R	This study
pTRIVET	P$_{tetA}$ controlled *tnpR* in pGP704, Ap^R	This study
pTRIVET1	P$_{tet1}$ controlled *tnpR* in pGP704, Ap^R	This study
pTrc-tpc	pTrc99A with *tetR::phoA::cat*, Km^R	This study
pVC0072	VC0072 of E7946 in pBR322, Ap^R	This study
pVC0227	VC0227 of E7946 in pBR322, Ap^R	This study
pVC0704-2	VC0704, VC0703 and VC0702 of E7946 in pBR322, Ap^R	This study
pVC0784	VC0784 of E7946 in pBR322, Ap^R	This study
pVC0998	VC0998 of E7946 in pBR322, Ap^R	This study
pVC1033	VC1033 of E7946 in pBR322, Ap^R	This study
pVC2137	VC2137 of E7946 in pBR322, Ap^R	This study

Table S1. Cont.

Strain or plasmid	Genotype/resistance	Source
pclcA	VCA0526 of E7946 in pBR322, Ap^R	This study
pVCA0576	VCA0576 of E7946 in pBR322, Ap^R	This study
pΔaphB	ΔVC1049 of E7946 in pCVD442, Ap^R	This study
pΔclcA	ΔVCA0526 of E7946 in pCVD442, Ap^R	This study

Table S2. List of oligonucleotides

Name of primer	Sequence (5′ to 3′)*
VC0072-5′-NheI	AAAGCTAGCATAGCCGACATGAAATTAAAGTGTGCTT
VC0072-3′-SalI	AAAGTCGACCTACGCTTTCTCTTTGAGTTG
VC0227-5′-BamHI	AAAGGATCCAATAGATGTAAAATGCAGCACGCCAGAT
VC0227-3′-SalI	AATGTCGACTCTTTTAATTAAATATCACTCTCTAAC
VC0704-5′-BamHI	AAAGGATCCACTAGTTACGCTATTAGGTTATTTGTCGG
VC0702-3′-SalI	AAAGTCGACGATGAGTTGAAGTGAATGAGAGC
VC0784-5′-BamHI	AATGGATCCAATAGTACCGAACACACAACAGACACAA
VC0784-3′-SalI	AAAGTCGACGCGAATGCTAACCCTTTGACTTA
VC0998-5′-EcoRV	AATGATATCTAGTTTTGCCAGCCAGTCTATAGTTTT
VC0998-3′-SalI	AAAGTCGACGGAGCTACACTCGCCCATTCA
VC1033-5′-BamHI	AAAGGATCCAATAACTCCAAGGTGTAAGCTGATCAC
VC1033-3′-SalI	AAAGTCGACCGAACCTCCTAGTTAAACGACC
VC2137-5′-EcoRV	AAAGATATCTAACAAACGGTGCAACCACAACTTC
VC2137-3′-SalI	AAAGTCGACATTAATGCCAATATATTGACTATGGT
clcA-5′-EcoRV	AAAGATATCTAGATGCAGCGTATAATTCCGGCAC
clcA-3′-BamHI	AAAGGATCCTTATGAGTTGGGGCTCTGCGG
VCA0576-5′-SphI	AAAGCATGCATAGTACTCAACATGGAAATTGCAGGTA
VCA0576-3′-SalI	AAAGTCGACAAGCCCAGTGCTAGACTATGTG
tetR-5′-BamHI	AATGGATCCTAGAGTGTCAACAAAAATTAGGAATTA
tetR-3′-KpnI	TTAGGTACCATCACGGAAAAAGGTTATGCT
phoA-5′-KpnI	TTTGGTACCTTTTTAATGTATTTGTACATGGAGAA
phoA-3′-XbaI	AAATCTAGACATTAAGTCTGGTTGCTAACAGCA
cat-5′-XbaI	AAATCTAGATAAGCTTGATGAAAATTTGTTTGA
cat-3′-BamHI	TTTGGATCCTTCTTCAACTAACGGGGCA
tetRphoAcat-5′-NotI	TATGCGGCCGCCCTAGGTAATTAGGATCCTAGAGT
tetRphoAcat-3′-NotI	TTTGCGGCCGCCCTAGGTCTCATCCGCCAAAACAGCCAA
tetRphoAcat-5′-SacI	ATAGAGCTCAGAGTGTCAACAAAAATTAGGAATTAA
tetRphoAcat-3′-SalI	TTTGTCGACTTCTTCAACTAACGGGGCA
tnpR-5′-BglII	CGACCCGGGAGATCTCAATTGTTCGAATTTAGGATACATTTTTAT
tnpR-3′-XbaI	TTTTCTAGATTAAGTTGGGTAACGCCAGGGT
lacZ-5′-SacI	TTTGAGCTCTGATTTACCGCCGCTGCCAA
lacZ-3′-NheI	TTTGCTAGCTTATTGTGGGTGATGACGCTTT
irgA-SphI-1	AATGCATGCTCCGAGTAAACCGCAAACACTT
irgA-SacI-2	AATGAGCTCGTATCCCGCCGCAGTGACCA
irgA-SalI-3	AAAAGTCGACCGAACTATTCCATGTGT
irgA-XbaI-4	AATTCTAGACGTGAGGTTTGGCGCTTA
clcA-SphI-1[†]	ATAGCATGCAGAAATTAACGTGGCGACTAC
clcA-SacI-2[†]	TAAGAGCTCACTAATGACACCTATTGATGAAT
clcA-SacI-1[‡]	AATGAGCTCTAAGTCCACATAAGGCTGATACT
clcA-SalI-2[‡]	TAAGTCGACACTAATGACACCTATTGATGAAT
clcA-SalI-3[†,‡]	TAAGTCGACAAGTCAATTTGCCTAAAGTAAAG
clcA-XbaI-4[†,‡]	TATTCTAGATGACCAGATAAGTGGCTAAG
aphB-SacI-1	TATGAGCTCGAGCAATTCTTTGGCAGCAT
aphB-SalI-2	TTTGTCGACCGTTTCTGACACATTATGTTG
aphB-SalI-3	AAAGTCGACAAAGCATTTCAATAGAAAAAGGGC
aphB-XbaI-4	TATTCTAGAGACGGTTTGTTGACGAGAGC
P_{tetA}-5′-3′	CTAGCCAGAGAGCCTTAAGGCTCTCTTTTTTCTAATTTTTGTTGACACCCTATCAGTGATAGAGTTATTTTACCACTCCCTATCAGTGATAGA
P_{tetA}-3′-5′	GATCTCTATCACTGATAGGGAGTGGTAAAATAACTCTATCACTGATAGGGTGTCAACAAAAATTAGAAAAAAGAGAGCCTTAAGGCTCTCTGG
P_{tet1}-5′-3′	CTAGCAGAGAGCCTTAAGGCTCTCTTTTTTCTAATTTTTGTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACATCA
P_{tet1}-3′-5′	GATCTGATGTGCTCAGTATCTCTATCACTGATAGGGATGTCAATCTCTATCACTGATAGGGACAAAAATTAGAAAAAAGAGAGCCTTAAGGCTCTCTG
phoA-5′-probe	TTTTTAATGTATTTGTACATGGAGAA
phoA-3′-probe	GTGATCTGCCATTAAGTCTGGTT
seqPrimer-cat	GAATTGTCAGATAGGCCTAATG
qPCR-clcA-5′	GAAGGTCCTACAGTACAA
qPCR-clcA-3′	CAGGGTATAGCGAAATTG
qPCR-irgA-5′	ATACCGATAACCAGTACC
qPCR-irgA-3′	CTTTCGTGTTTACCTTCC
qPCR-VCr001-5′[§]	AGGGAGGAAGGTGGTTAAGT
qPCR-VCr001-3′[§]	CGCTACACCTGAAATTCTACCC

*Restriction sites are underlined.

[†] Used for chromosomal *tpc*-cassette insertion.

[‡] Used for in-frame deletion mutagenesis.

[§] Oligonucleotides for 16S rRNA are according to ref. 61.

Table S3. In vivo repressed genes of *V. cholerae*

Operon*	Gene locus[†]	Annotation/gene symbol[†]
Cellular process and metabolism		
	VC0019	Valine-pyruvate transaminase (*avtA*)
	VC0072	Sensory box/GGDEF family protein
VC0100-99	VC0100	Thiosulfate sulfurtransferase (*glpE*)
	VC0227	3-Deoxy-D-manno-octulosonic-acid kinase
VC0418-22	VC0422	tldD protein
	VC0516	Phage integrase
	VC0537	Cysteine synthase B (*cysM*)
VC0704-2	VC0703	c-di-GMP phosphodiesterase A-like protein
	VC0711	clpB protein
VC0813-2	VC0812	Helicase-like protein
	VC0824	Thiol peroxidase (*tpx*)
VC1066-7	VC1067	Diguanylate cyclase
VC1132-9	VC1136	Imidazole glycerol phosphate synthase subunit HisH (*hisH*)
VC1288-7	VC1287	Glucosyltransferase MdoH
VC1449-51	VC1451	RTX toxin RtxA
	VC1492	Glutamate dehydrogenase
	VC1554	Glycerophosphoryl diester phosphodiesterase
	VC1758	Phage family integrase
	VC1765	Type I restriction enzyme HsdR
VC1769-6	VC1768	Type I restriction enzyme, S subunit
VC2020-19	VC2019	3-Oxoacyl-ACP synthase
	VC2033	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase
	VC2110	5-Methylaminomethyl-2-thiouridine methyltransferase
	VC2418	Thiol:disulfide interchange protein DsbC
VC2456-4	VC2454	GGDEF family protein
	VC2646	Phosphoenolpyruvate carboxylase
	VCA0316	Acetyltransferase
	VCA0436	Acetyltransferase
VCA0445-4	VCA0445	Antitoxin StbD
	VCA0490	GDXG family lipase
	VCA0549	phnA protein
	VCA0572	D-alanyl-alanine synthetase A (*ddl*)
	VCA0574	Acetyltransferase
	VCA0657	Glycerol-3-phosphate dehydrogenase (*glpD*)
	VCA0700	Chitodextrinase
	VCA0717	DNA helicase IV (*held*)
VCA0873-2	VCA0873	Cytochrome d ubiquinol oxidase subunit II
	VCA0875	D-serine dehydratase
	VCr005	23S ribosomal RNA (*23Sb*)
	VCr014	23S ribosomal RNA (*23Se*)
Transporter Systems		
	VC0784	Sodium/alanine symporter
	VC1033	Zinc/cadmium/mercury/lead-transporting ATPase (*zntA*)
	VC1235	Sodium/dicarboxylate symporter
VC1333-2	VC1332	Putative tricarboxylic transport membrane protein
VCA0107-20	VCA0114	Type VI secretion system protein ImpJ
VCA0122-4	VCA0123	VgrG protein
	VCA0220	Hemolysin secretion protein HylB
	VCA0454	Sulfate-binding protein
	VCA0526	Chloride channel protein
	VCA0576	Heme transport protein HutA
	VCA0628	SecA-like protein
Regulation		
VC1653-1	VC1653	Sensory box sensor histidine kinase/response regulator VieS
	VC1831	Sensor histidine kinase
VC2137-29	VC2137	Fis family transcriptional regulator
	VCA0523	CAI-1 autoinducer synthase
	VCA1078	LuxR family transcriptional regulator
Surface Structure		
VC0399-406	VC0403	MSHA biogenesis protein MshM
VC0410-12	VC0411	MSHA pilin protein MshD
VC0413-4	VC0414	MSHA biogenesis protein MshQ

Table S3. Cont.

Operon*	Gene locus[†]	Annotation/gene symbol[†]
	VC0845	Accessory colonization factor AcfD
	VC1329	Opacity protein-like protein
Motility and Chemotaxis		
	VC0512	Methyl-accepting chemotaxis protein
	VC0998	Pilus assembly protein FimV
VC2137-29	VC2134	Flagellar hook-basal body protein FliE (*fliE*)
	VCA0954	Chemotaxis protein CheV
Unknown		
	VC0177	Hypothetical protein
VC0178-81	VC0180	Hypothetical protein
VC0185-2	VC0184	Hypothetical protein
VCA0492-0	VC0492	Hypothetical protein
	VC0583	Pseudogene
	VC0846	Pseudogene
VC1183-4	VC1183	Hypothetical protein
	VC1224	Hypothetical protein
VC1556-5	VC1556	Hypothetical protein
VC1764-1	VC1764	Hypothetical protein
	VC1794	Hypothetical protein
	VC2338	Pseudogene
	VC2600	Hypothetical protein
	VC2739	Hypothetical protein
	VCA0097	Hypothetical protein
VCA0101-0	VCA0101	Hypothetical protein
VCA0105-6	VCA0106	Hypothetical protein
	VCA0163	Hypothetical protein
VCA0176-7	VCA0177	Hypothetical protein
	VCA0186	Hypothetical protein
	VCA0314	Hypothetical protein
	VCA0325	Hypothetical protein
	VCA0336	Hypothetical protein
	VCA0361	Hypothetical protein
VCA0369-70	VCA0370	Hypothetical protein
	VCA0374	Hypothetical protein
	VCA0376	Hypothetical protein
	VCA0395	Hypothetical protein
VCA0396-7	VCA0396	Hypothetical protein
	VCA0453	Hypothetical protein
VCA0497-8	VCA0498	Hypothetical protein
VCA0501-2	VCA0501	Hypothetical protein
VCA0580-78	VCA0579	Hypothetical protein
	VCA0731	Hypothetical protein
VCA0776-8	VCA0778	Hypothetical protein
	VCA0874	Hypothetical protein

*Operon prediction according to www.microbesonline.org/.

[†] Gene locus and annotation/gene symbol are according to the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/).