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Supplemental Data

Genes Induced Late in Infection Increase Fitness of *Vibrio cholerae* after Release into the Environment

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Supplemental Experimental Procedures

Reagents.

Bacterial strains and plasmids are listed in Table S1. *V. cholerae* AC53 was used as a wildtype strain. For genetic manipulations *E. coli* strains DH5α, DH5αλpir and SM10λpir were used. Unless stated otherwise, bacteria were grown in LB at 37°C with aeration. M9 was prepared using Difco™ M9 Minimal Salts according to the instructions. Supplements were used in the following final concentrations: streptomycin (Sm, 100μg/ml), Kn (50μg/ml), ampicillin (Ap, 50μg/ml in combination with other antibiotics or 100μg/ml), Gly (0.5%), Glc (0.2%), GlcNAc (0.2%), crab shell chitin (1%), succinate (0.2%), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 40μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5mM) and betaine monohydrate (betaine, 50mM).

DNA manipulations and strains contructions

Polymerase chain reaction (PCR), DNA Purification, DNA Sequencing.

Chromosomal DNA was prepared using the DNeasy® Tissue Kit, whereas PCR products or digested plasmid DNA were purified using the Qiaquick® Gel extraction Kit or Qiaquick® PCR Purification Kit (Qiagen). PCRs for sequencing and subcloning were carried out using the Easy-A® High-Fidelity PCR Cloning enzyme (Stratagene), for all other reaction the Taq DNA Polymerase (NEB) was used. Automated DNA sequencing was performed with an automated ABI 3130XL DNA sequencer at the Tufts University Core Facility.

Construction of suicide plasmids and mutant strains.

All suicide plasmids using pCVD442 to generate in-frame deletions were constructed in a similar manner. PCR fragments of ~800bp upstream and downstream of each deletion were generated using the oligonucleotide pairs x-y-up and x-y-down, in which x stands for the gene and y for the restriction site/enzyme used (Table S1). Purified PCR fragments were treated with the respective restriction enzymes and ligated into a SacI/XbaI-digested pCVD442. After transformation of the ligation mix into DH5αλpir, Ap^R colonies were characterized by PCR and/or restriction analysis (data not shown).

The plasmid pGPVC1593-VC2370-VC2697 was generated by amplifying three PCR fragments using the oligonucleotide pairs VC1593-SacI-up/ VC1593-3'-KpnI, VC2370-5'-KpnI/ VC2370-3'-EcoRI and VC2697-5'-EcoRI/ VC2697-3'-XbaI. The purified PCR fragments were treated with the respective restriction enzymes indicated by the name of the oligonucleotide, ligated into pGP704 digested with SacI/ XbaI and transformed into DH5αλpir. Ap^R colonies were characterized by PCR and/ or restriction analysis (data not shown).

Once the right construct was identified the suicide plasmid was transformed into SM10λpir to allow conjugation into *V. cholerae*, which was achieved by crosstreaking O/N colonies of recipient and donor on a LB plate and incubation at 37°C for ~6h. Transfer into *V. cholerae* and integration on the chromosome was selected by isolating Sm^R/Ap^R colonies. In the case of pCVD442 derivatives after one passage of LB in the absence of antibiotics sucrose selection was used to obtain Ap^S colonies. Correct insertions or chromosomal deletions were confirmed by PCR (data not shown).

Construction of expression plasmids.

All expression plasmids using pMMB67EH were constructed in a similar manner. PCR fragments including the Shine Dalgarno sequence of the respective gene were generated using the oligonucleotide pairs x-5'-y and x-3'-y, in which x stands for the gene and y for the restriction site/enzyme used (Table S1). Purified PCR fragments were treated with the respective restriction enzymes and ligated into an appropriate digested pMMB67EH. After transformation of the ligation mix into DH5α, Ap^R colonies were characterized by PCR and/or restriction analysis (data not shown).

Construction of *V. cholerae* transcriptional fusion library.

The library of transcriptional *tnpR* fusions in AC53 was generated essentially as described (Osorio et al., 2005). First, pRes and pRes1 were mobilized into AC53 by conjugation, followed by allelic exchange to generate strains AC53res and AC53res1. Second, the library of random *tnpR* fusions designated as pIVET5nlibrary was conjugated into AC53res and AC53res1 by 24 independent matings. Unresolved strains were isolated by selection for Ap^R, Kn^R and Sm^R colonies. By combining ~1000 colonies from each mating, 12 library pools were generated.

Fusion strain reconstructions and quantification of resolution.

Unresolved *V. cholerae* fusion strains were reconstructed as described (Osorio et al., 2005). To quantify resolution, strains were first grown on LB-Sm/Kn/Ap plates O/N and diluted in LB to OD_{600}=0.002. To determine the *in vitro* resolution, 2ml were incubated for 8h, whereas the *in vivo* resolution at different time points was determined by intragastrically inoculating anesthetized 5-day-old CD-1 mice with 50µl. Mice were euthanized at 5, 7 or 24h post-infection, and *V. cholerae* were recovered as described above. The amount of resolution *in vitro* and *in vivo* was determined by plating appropriate dilutions on LB-Sm/Kn and LB-Sm/Ap plates. Results were expressed as % resolution, calculated as the Sm^R/Kn^S CFU [Sm^R/Ap^R CFU minus Sm^R/Kn^R CFU] divided by Sm^R/Ap^R CFU.

Table S1. Bacterial strains and plasmids used in this study

strain or plasmid	relevant genotype/ resistance	source
E. coli strains		
DH5α	F-Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	(Kolter et al., 1978) (Hanahan, 1983)
DH5αλpir	F-Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* λ::*pir*	(Kolter et al., 1978) (Hanahan, 1983)
SM10λpir	*thi recA thr leu tonA lacY supE RP4-2-Tc::Mu λ::pir*	(Miller and Mekalanos, 1988)
V. cholerae strains		
AC51	spontaneous Sm^r mutant of *V. cholerae* C6709-1 (O1 El Tor Ogawa, clinical isolate from Peru), *hapR*$^+$, Sm^r	(Wachsmuth et al., 1993)
AC53	spontaneous Sm^r mutant of *V. cholerae* E7946 (O1 El Tor Inaba, clinical isolate from Bahrain), *hapR*$^+$, Sm^r	(Miller et al., 1989) (Vance et al., 2003)
AC53res	insertion of res-*neo-sacB*-res in *lacZ* of AC53	This study
AC53res1	insertion of res1-*neo-sacB*-res1 in *lacZ* of AC53	This study
AC53*lacZ*	insertion of a pGP704 derivative in *lacZ* of AC53	This study
ΔVC0201	deletion of VC0201 in AC53	(Lombardo et al.)
Δ*tcpA*	deletion of *tcpA* in AC53 using pHT3	This study
ΔVC612	deletion of VC0612 leaving AA: MACG	This study
ΔVC612-3	double deletion of VC0612 and VC0613 leaving AA: MSACG	This study
ΔVC1926	deletion of VC1926 in AC53 leaving AA: MPWKP	This study
ΔVCA0601	deletion of VCA0601 in AC53 leaving AA: VRRTVVRSCRQRQQLQPAVLMRLKEKSMAS	This study
ΔVCA0686	deletion of VCA0686 in AC53 in AC53 leaving AA: MPWDS	This study
ΔVCA0744	deletion of VCA0744 in AC53 leaving AA: MTACE	This study
ΔVCA0920	deletion of VCA0920 in AC53 leaving AA: MMAC	This study
ΔVCA0980	deletion of VCA0980 in AC53 leaving AA: MCA	This study
ΔVC1593-ΔVC2697-ΔVC2370	triple deletion in AC53 of VC1593 leaving AA: MMACPRC, of VC2697 leaving AA:MVNRACADR and of VC2370 leaving AA: MH	This study
Plasmids		
pMMB67EH	IncQ broad-host-range cloning vector, Ap^r	(Morales et al., 1991)
pCVD442	*oriR6K mobRP4 sacB*, Ap^r	(Donnenberg and Kaper, 1991)
pGP704	*oriR6K mobRP4*, Ap^r	(Miller and Mekalanos, 1988)
pRes	pGP704 with *lacZ*::res-*neo-sacB*-res, Ap^r, Kn^r	(Osorio et al., 2005)
pRes1	pGP704 with *lacZ*::res1-*neo-sacB*-res1, Ap^r, Kn^r	(Osorio et al., 2005)
pIVET5nlibrary	library of random *V. cholerae* genomic fragments ligated into pGOA1193, pGOA1194 and pGOA1195 to create transcriptional fusions to *tnpR*	(Osorio et al., 2005)
pSL481	pIVET5*ctxA*::*tnpR*^{135}, Ap^r	(Lee et al., 1999)

pSL479	pIVET5*tcpA*::*tnpR*[135], Ap[r]	(Lee et al., 1999)
pHT3	Δ*tcpA* in pCVD442	(Thelin and Taylor, 1996)
pVC0201	VC0201 of AC53 in pMMB67EH, Ap[r]	This study
pVC0612	VC0612 of AC53 in pMMB67EH, Ap[r]	This study
pVC1926	VC1926 of AC53 in pMMB67EH, Ap[r]	This study
pVCA0601	VCA0601 of AC53 in pMMB67EH, Ap[r]	This study
pVCA0686	VCA0686 of AC53 in pMMB67EH, Ap[r]	This study
pVCA0744	VCA0744 of AC53 in pMMB67EH, Ap[r]	This study
pVCA0920	VCA0920 of AC53 in pMMB67EH, Ap[r]	This study
pVCA0980	VCA0980 of AC53 in pMMB67EH, Ap[r]	This study
pGPVC1593-VC2370-VC2697	VC1593, VC2370 and VC2697 of AC53 in pGP704, Ap[r]	This study
pΔVC0612	ΔVC0612 of AC53 in pCVD442, Ap[r]	This study
pΔVC0612-3	ΔVC0612-3 of AC53 in pCVD442, Ap[r]	This study
pΔVC1593	ΔVC1593 of AC53 in pCVD442, Ap[r]	This study
pΔVC1926	ΔVC1926 of AC53 in pCVD442, Ap[r]	This study
pΔVC2697	ΔVC2697 of AC53 in pCVD442, Ap[r]	This study
pΔVC2370	ΔVC2370 of AC53 in pCVD442, Ap[r]	This study
pΔVCA0601	ΔVCA0601 of AC53 in pCVD442, Ap[r]	This study
pΔVCA0686	ΔVCA0686 of AC53 in pCVD442, Ap[r]	This study
pΔVCA0744	ΔVCA0744 of AC53 in pCVD442, Ap[r]	This study
pΔVCA0920	ΔVCA0920 of AC53 in pCVD442, Ap[r]	This study
pΔVCA0980	ΔVCA0980 of AC53 in pCVD442, Ap[r]	This study

Table S2: Oligonucleotides used for PCR

name	sequence[a]
VC0612-SacI-up	TTTGAGCTCGCAATATCGCAGTATTCAGTA
VC0612-SphI-up	TATGCATGCCATTGTGCTTTCCTTTTAAACCA
VC0612-SphI-down	TATGCATGCGGTTAATCCATTTTCGGGCACT
VC0612-XbaI-down	TTATCTAGAATAAAGTTGCCTTTTTCATCGA
VC0613-SacI-up	AAAGAGCTCACGGGGAGGAGAACCAATGAA
VC0613-SphI-up	AAAGCATGCACTCATAAACCATCCTTATACA
VC1593-SacI-up	TTTGAGCTCTTCATACGGCGGGTTCTCT
VC1593-SphI-up	TTTGCATGCCATCATTCTTTTTATTCATCACAT
VC1593-SphI-down	TTTGCATGCCCAAGATGTTAAAAAACAAACT
VC1593-XbaI-down	TATTCTAGATAGGGACACAAACGTATGCT
VC1926-SacI-up	AAAGAGCTCCAAGCGGCGAAACTGGCAGT
VC1926-NcoI-up	AATCCATGGCATGGTTAGTCCATATTGAGAGGT
VC1926-NcoI-down	AAACCATGGAAACCCTGAAACCATCCCGCGTT
VC1926-Xba-down	AAATCTAGACACCAAAGCGACCTTGTAT
VC2697-SacI-up	TTAGAGCTCACTTCGTTGGTGTTCATGTT

VC2697-SphI-up	TTAGCATGCACGATTCACCATAAATCCGTT
VC2697-SphI-down	AAAGCATGCGCTGATCGGTAAACAAAAAGTGT
VC2697-XbaI-down	AATTCTAGATCGTGAAAAGGGTTGGGCT
VC2370-SacI-up	TTTGAGCTCAGCAGCAGAGTATTCAAGCG
VC2370-SphI-up	TTAGCATGCATTCAGCGGTGTTATTGA
VC2370-SphI-down	AAAGCATGCACTAAAACTGTGCGGTTCGCC
VC2370-XbaI-down	AAATCTAGAAATAAACGTGGTTTTGTCGC
VCA0601-SacI-up	ATTGAGCTCTGGCACCATCCACCAAAA
VCA0601-NheI-up	TTTGCTAGCCATACTCTTCTCCTTAAGCCGCA
VCA0601-NheI-down	AATGCTAGCTAGTGGAAAGAGGCAATAGGA
VCA0601-XbaI-down	AATTCTAGATGATAGGCATAAAAGACATACGA
VCA0686-SacI-up	TTTGAGCTCTTCTGCGGTATACATGGGCA
VCA0686-NcoI-up	TTTCCATGGCATGGTCAAACCTTATTATTGAGT
VCA0686-NcoI-down	TTTCCATGGGATTCTTAGGAGACATCACCGT
VCA0686-XbaI-down	TTTTCTAGACTTAGGTGCTTTATACAACTCTT
VCA0744-SacI-up	TTAGAGCTCACCATTACAGCGGTACTGATGTT
VCA0744-SphI-up	TTAGCATGCAGTCATGTTCTATTCCTTATGGTT
VCA0744 SphI-down	TTTGCATGCGAGTAATTTTTCTCTCTCCATAA
VCA0744 XbaI-down	TTATCTAGAGCGGCCTCTTTTCTTGATGGTT
VCA0920-SacI-up	AAAGAGCTCCAACAATCTATTGAGTGTGACAA
VCA0920-SphI-up	AAAGCATGCCATCATGGCGAAGCGTTTATCCT
VCA0920-SphI-down	AATGCATGCTAAAGTAGAAAAAGCAAAAGAGGA
VCA0920-XbaI-down	AAATCTAGAGAAATGATGAAAGCTCTCGGT
VCA0980-SacI-up	AAAGAGCTCAGGTGAAGGGTTGGCTGAAA
VCA0980-SphI-up	AAAGCATGCCATGTGACCTCCATTAAACCAGA
VCA0980-SphI-down	AATGCATGCTAAATACAAAGAATTTCAAGCTGT
VCA0980-XbaI-down	AAATCTAGAGCAGCGCGGTCATAAACACT
VC0201-5'-XbaI	TTTTCTAGATTGTCCGTATTCTTGGATGA
VC0201-3'-SphI	TTAGCATGCAGACGAACATTCCATAGACTCA
VCA0920-5'-XbaI	TATTCTAGACAAAACTCGCCACACAGGAT
VCA0920-3'-SphI	TTTGCATGCTTTTTCTACTTTAGCGAGCGA
VCA0980-5'-XbaI	TTTTCTAGAGCCTTCTGGTTTAATGGAGGT
VCA0980-3'-SphI	TTTGCATGCCTTGAGTCACAGCTTGAAATTCT
VC1593-3'-KpnI	TTTGGTACCTCAGCACTGAATCGGTAAATCT
VC2370-5'-KpnI	TTAGGTACCAGGAGAATGCATGCCTGAATTTCTCTCTGA
VC2370-3'-EcoRI	TTTGAATTCTTAGTGGTTTGGTTGATAAATTGAGGT
VC2697-5'-EcoRI	AAAGAATTCAAAGGAACGGATTTATGGTGAA
VC2697-3'-XbaI	TTTTCTAGATTTTGTTTACCGATCAGCAATCA
VC0612-5'-XbaI	AAATCTAGAAAAGGAAAGCACAATGAAATACGG
VC0612-3'-SphI	AAAGCATGCCCGAAAATGGATTAACCTAGA
VC1926-5'-EcoRI	TTAGAATTCACACCTCTCAATATGGACTA

VC1926-3'-BamHI	TTTGGATCCTTTAATCTGAGACCTAGACT
VCA0601-5'-EcoRI	TTAGAATTCGATATTTGGCATTGAGAGCA
VCA0601-3'-BamHI	TTTGGATCCTATTGCCTCTTTCCACTAA
VCA0686-5'-XbaI	TTTTCTAGACTCAATAATAAGGTTTGACCAT
VCA0686-3'-SphI	TTTGCATGCGGTGATGTCTCCTAAGAATCTT
VCA0744-5'-EcoRI	TTAGAATTCATAAGGAATAGAACATGACT
VCA0744-3'-BamHI	TTTGGATCCTTTATGGAGAGAGAAAAATTACTCT
IVET-1	AAATCGTACGCCGACTAGAATGTC
IVET-2	ACGTCACCTTCCTCCACCTTCATC
IVET-3	CCTTCATCCTCAGCAAGTCCA
VC1593-qF	CATTATCGGGATGATAGCGGTC
VC1593-qR	CAACTGTGGAGTCAATGAGTGG
VC1926-qF	CTTACCCGGTTTAAGTGGACAG
VC1926-qR	GTACCGTGTCAATCAACCTCTC
VCA0601-qF	CCTGCTATCACTGATGCTGTTG
VCA0601-qR	CACACTGATACCAAGCACTAAGG
VCA0744-qF	GTGGTGTGGAATAAAGAGACGG
VCA0744-qR	TACCTGAGAAGTAAGGGTCGAG
VC0612-qF	GGTTACTCCTTCTATCACTCACC
VC0612-qR	GATAGACCAGAAATCGCCTGTC
VCA0686-qF	CCTACTCTAGCTTCTGTATGCTGG
VCA0686-qR	GAATGATGCTGATCAGTGAGCC
VCA0980-qF	CTTAGAGACAGTGAAATCGGCG
VCA0980-qR	TGCCTGATCTAGGGTCAGATAC
VC0620-qF	GCGTACAACCACCGACTTTATC
VC0620-qR	CTTCACCGTCAGACCACTTAAC
RPB2FD[b]	CTGTCTCAAGCCGGTTACAA
RPB2RV[b]	TTTCTACCAGTGCAGAGATGC

[a] restriction sites are underlined

[b] oligonucleotides for *rpoB* according to (Quinones et al., 2005)

Table S3: Comparison of the two pond water samples used in this study.

Analysis was performed by the Chemical Analysis Laboratory/Center for Applied Isotope Studies at the University of Georgia. Tested, but below detection (>10 ppt) in both samples: Al, B, Ba, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Zn

element/ anion/ cation	**pond water, Bangladesh (ppm)**	**pond water, Boston (ppm)**
Na	24.7	55.2
K	7.1	1.1
Ca	32.1	17.5
Mg	6.3	5.8
Si	1.8	below detection
Sr	0.12	0.12
P	0.2	0.4
NO_2^-	1.3	0.5
NH_4^+	0.1	0.1

Fig. S1

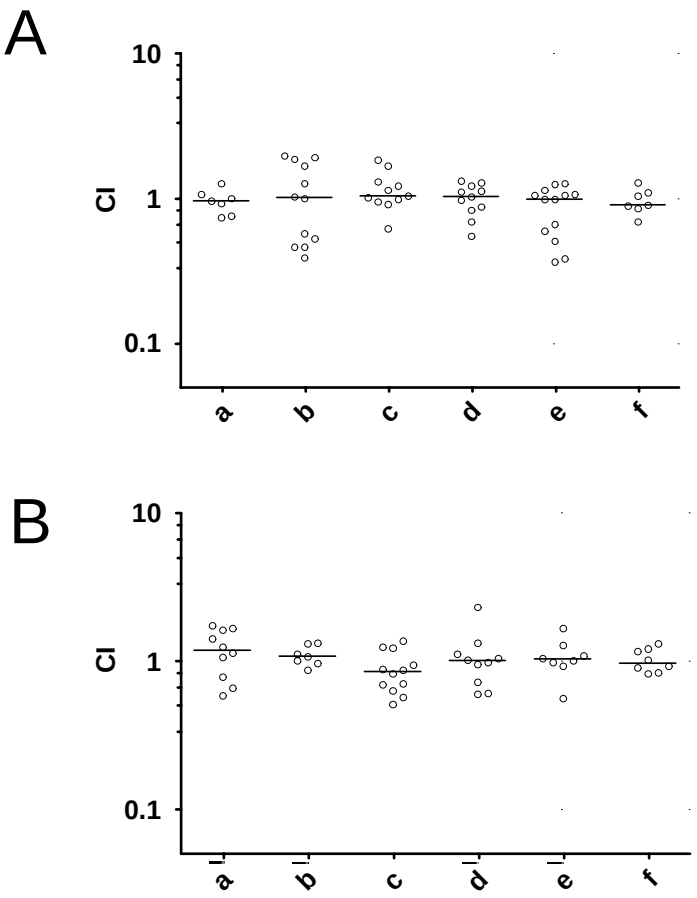

Figure S1. Late gene mutants not attenuated *in vivo*.

Results are shown as the CI using the infant mouse model for *in vitro* passaged bacteria (A) or *in vivo* passaged bacteria (B). Each circle represents the CI from a single assay. The horizontal bars indicate the median of each data set.

Abbreviations stand for the following competitions: a) ΔVC1926/AC53res1; b) ΔVCA0744/AC53res1; c) ΔVC0612-3/AC53res1; d) ΔVCA0686/AC53res1; e) ΔVCA0601/AC53res1; f) ΔVC1593-ΔVC2697-ΔVC2370/AC53res1 /AC53res1.

Supplemental References

Donnenberg, M.S., and Kaper, J.B. (1991). Construction of an eae deletion mutant of enteropathogenic Escherichia coli by using a positive-selection suicide vector. Infect. Immun. *59*, 4310–4317."

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. *166*, 557–580.

Kolter, R., Inuzuka, M., and Helinski, D.R. (1978). Trans-complementation-dependent replication of a low molecular weight origin fragment from plasmid R6K. Cell *15*, 1199–1208.

Miller, V.L., and Mekalanos, J.J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. J. Bacteriol. *170*, 2575–2583.

Morales, V.M., Backman, A., and Bagdasarian, M. (1991). A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene *97*, 39–47.

Thelin, K.H., and Taylor, R.K. (1996). Toxin-coregulated pilus, but not mannose-sensitive hemagglutinin, is required for colonization by Vibrio cholerae O1 El Tor biotype and O139 strains. Infect. Immun. *64*, 2853–2856.

Wachsmuth, I.K., Evins, G.M., Fields, P.I., Olsvik, O., Popvic, T., Bopp, C.A., Wells, J.G., Carrillo, C., and Blake, P.A. (1993). The molecular epidemiology of cholera in Latin America. J. Infect. Dis. *167*, 621–626.