Figure S1

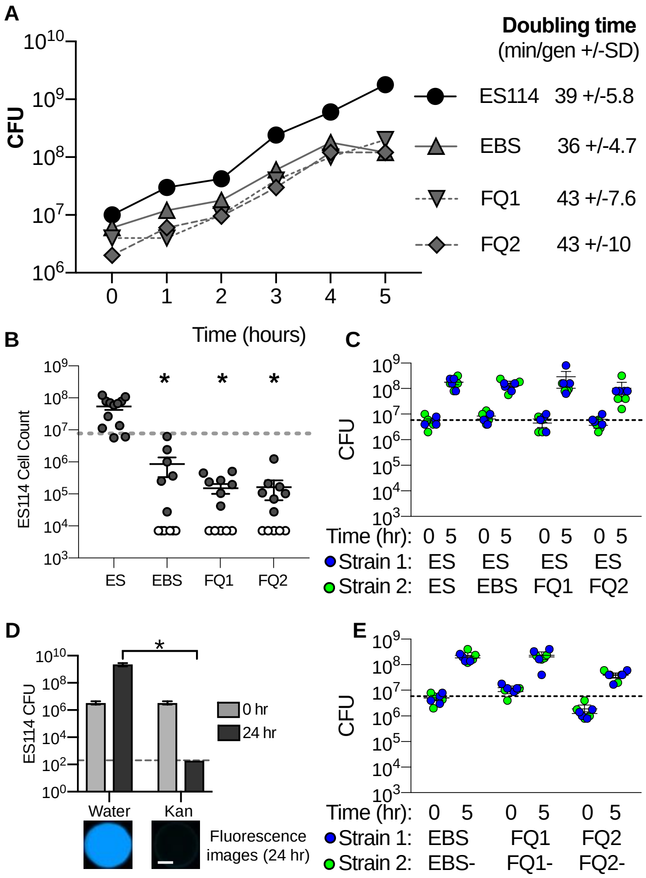


Figure S1. Competitive *V. fischeri* strains eliminate ES114 in a contact-dependent manner. (A) Growth curves of ES114 and lethal squid 1 (EBS) and squid 2 (FQ1 and FQ2) isolates; growth rates calculated with data from 2 to 4 h for all strains. (B) Flow cytometry cell count data of GFP-tagged ES114 after 5 h co-incubation with lethal squid isolates. Dashed line indicates average ES114 CFU at 0 h. White circles indicate cell counts at or under the limit of detection (7140 cells) and asterisks indicate statistical difference for ES114 co-incubated with other strains compared to with itself at 5 h using a student's t-test ($p<0.01$). (C) CFU counts for each co-incubation spot for co-incubations of ES114 (blue) with lethal squid isolates (green) where strains were physically separated by a 0.22 µm filter preventing direct cell-cell contact but still allowing diffusion of molecules. (D) CFU counts for each co-incubation spot for co-incubations of ES114 at 0 h (light gray) and after 24 h (dark gray) when incubated with water or kanamycin separated by a 0.22 µm filter. Fluorescence microscopy images were taken at 24 h; scale bar = 2 mm. Asterisk indicates statistical difference for ES114 incubated in water compared to kanamycin using a students t-test ($p=0.0004$). The dashed line indicates the limit of detection (200 CFUs) for the assay. (E) CFU counts for each co-incubation spot for co-incubations of lethal wild-type strains (blue) with the vasA_2 mutant derivative strain. Error bars indicate the standard error of the mean for biological replicates. Each experiment was performed at least three times and either combined data are shown (B and D, n=12) or a representative experiment is shown (A, n=1; C and E, n=4).

Figure S2

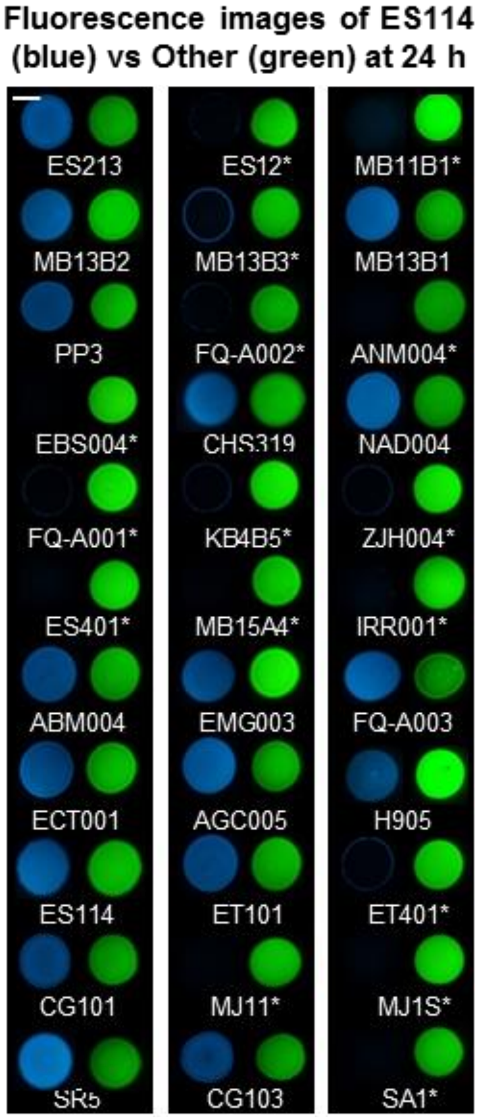


Figure S2. Co-incubations between *V. fischeri* isolates and ES114. Fluorescent microscopy images of co-incubations of GFP-tagged ES114 (blue) with 32 RFP-tagged *V. fischeri* isolates (green) taken at 24 h. Strains were mixed in a 1:5 ratio outnumbering ES114. Scale bar = 2 mm. RFP-tagged co-incubated strains are listed below the image pair. If ES114 (blue) is observed and not inhibited (ex. ES213), then the co-incubated strain is designated as non-lethal. If ES114 is not observed (ex. with ES12 or MB11B1), or if ES114 is only observed as outgrowth of survivors around the colony edge (ex: with MB13B3) then the co-incubated strain is designated as lethal.

Figure S3

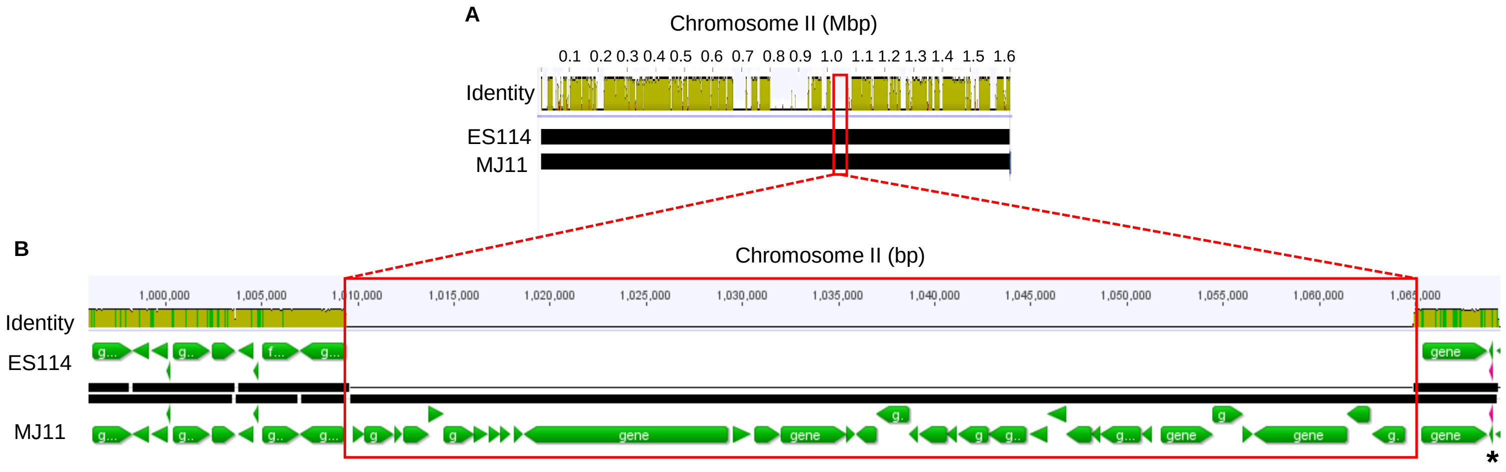


Figure S3. **Comparison of chromosome II for ES114 and MJ11**. Geneious R8 software was used to align the genomes of ES114 and MJ11 and visualized using Mauve. (A) Alignment of chromosome II sequences for ES114 (NC_006841.2) and MJ11 (NC_011186.1). Regions with high identity (yellow) indicate conserved genes and low identity (white) are strain specific. (B) Enlargement of T6SS2-encoding genomic island that is absent in ES114 but present in MJ11 shows it is near a tRNA gene (asterisk).

Figure 4

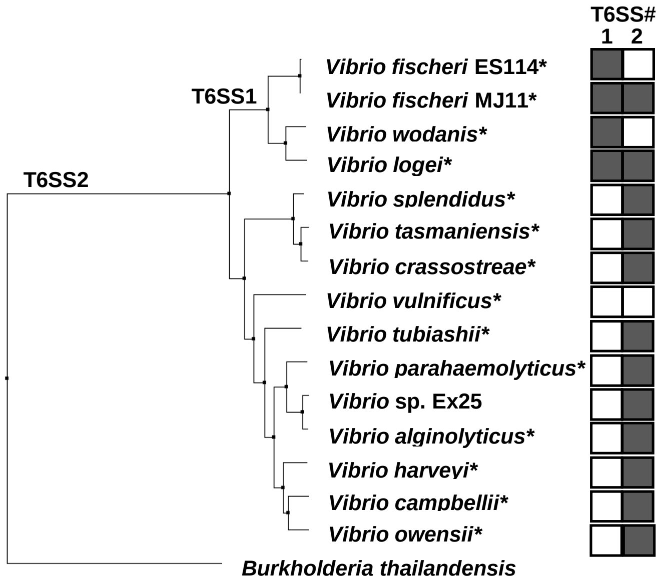


Figure S4. T6SS2 is broadly conserved among *Vibrio* species. Hsp60 percent identity tree for fully-sequenced representative genomes of *Vibrio* species. Filled boxes indicate presence of T6SS1 or T6SS2 IcmF homolog based on >60% identity (Table S2). Genomes were also examined for conserved genetic structure of T6SS. Asterisks indicate species found associated with an animal host. Note this figure only identifies homologs of T6SS1 and T6SS2 from *V. fischeri* and does not include other more distantly related T6SSs that may be in these representative genomes.

Figure S5

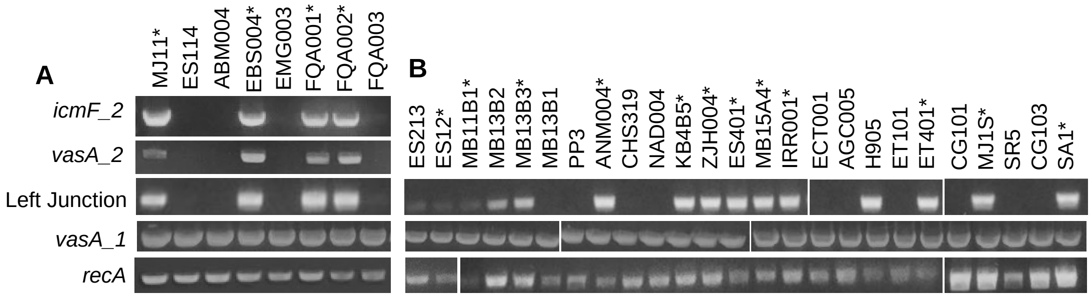
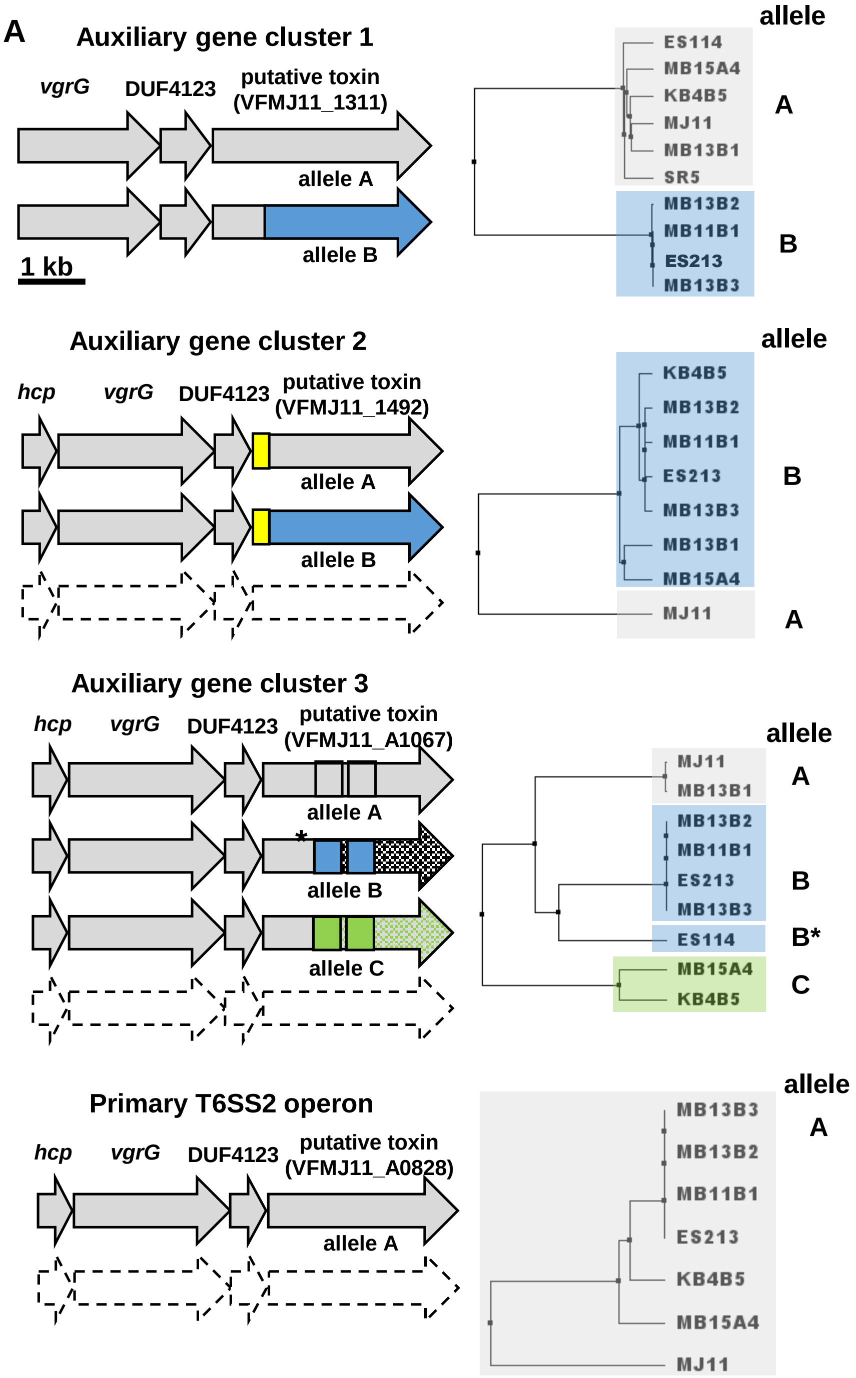


Figure S5. Distribution of the T6SS1 and T6SS2 among *V. fischeri* isolates. Strains were screened for the presence/absence of structural T6SS2 genes (*icmF_2* and *vasA_2*), the genomic island's left junction using primers specific to the left flanking gene and the first gene encoded in the genomic island, a structural T6SS1 gene *vasA_1*, and housekeeping gene *recA*, which is present in all strains. Asterisks indicate lethal strains.

Figure S6



Allele A genes share >93% ID and allele B genes share >99.9% seq identity. Allele A and Allele B sequences share ~60% sequence identity. The first 791 bp share >93% sequence identity and after 791 the two alleles share ~48% sequence identity. A Pfam search returned no significant predicted functional domains for either allele.

Allele B genes share >93% seq identity. Allele A and Allele B sequences share ~60% sequence identity. The first 155 bp share >94% sequence identity and after 155 bp the two alleles share ~58% sequence identity. A Pfam search returned a conserved LysM domain at the N terminus of both alleles (yellow). Homologs of auxiliary gene cluster 2 were not detected in ES114 or SR5.

Allele A sequences share >99.9% sequence identity. Allele B sequences are 100% identical and B* allele for ES114 is 93% identical to the other B alleles, however it is distinct and has a stop codon. Allele C sequences share 97% sequence identity. All three alleles have two DUF2235 domains that are uncharacterized alpha/beta hydrolase domains (boxes). The three alleles share a highly conserved N-terminus sequence (>94% ID), while the DUF2236 domains are least similar (78-85% ID) and a more conserved C-terminus domain (88-94% ID). Homologs of auxiliary gene cluster 3 were not detected in SR5.

All sequences share >96% identity. No significant predicted functional domains were identified from a Pfam search. The T6SS2 operon was not detected in ES114, SR5, or MB13B1.

B Predicted Compatibility Table

Strain	Group	A1	A2	A3	P	killer
ES213	1	B	B	B	A	No
MB11B1	1	B	B	B	A	Yes
MB13B2	1	B	B	B	A	No
MB13B3	1	B	B	B	A	Yes
MB13B1	2	A	B	A	NP	No
KB4B5	3	A	B	C	A	Yes
MB15A4	3	A	B	C	A	Yes
ES114	4	A	NP	B*	NP	No
MJ11	5	A	A	A	A	Yes
SR5	6	A	NP	NP	NP	No

NP indicates the gene cluster was not detected either bioinformatically or with PCR.

C

Fluorescence Images (24 h)

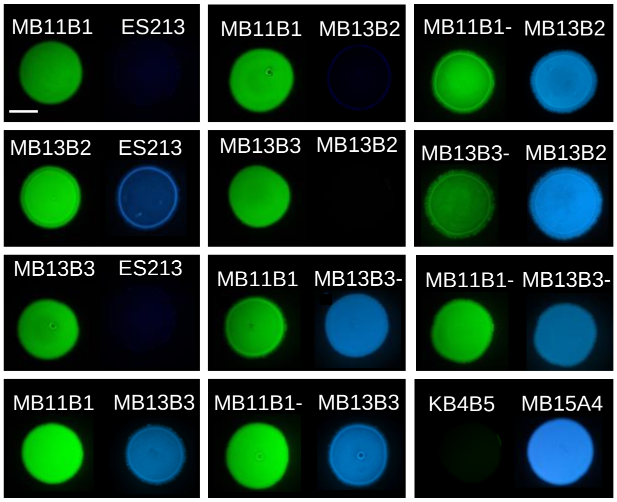


Figure S6. T6SS toxin genotypes of 10 *V. fischeri* isolates. (A) Putative T6SS toxin alleles of 10 *V. fischeri* strain draft genomes for auxiliary gene clusters 1 (A1), 2 (A2), 3 (A3) and the primary T6SS2 operon (P). Protein sequences from the *V. fischeri* MJ11 genome were used to perform a tblastn search for homologs which were aligned using clustal omega, and an average distance tree was built using jalview. (B) Predicted compatibility table showing 6 compatibility groups based on toxin alleles; NP (not present) indicates absence of a gene cluster. (C) Fluorescence microscopy images for pairwise co-incubations of Group 1 and Group 3 strains taken at 24 h; "-" indicates *vasA_2* mutants; scale bar is 2 mm.

Figure S7

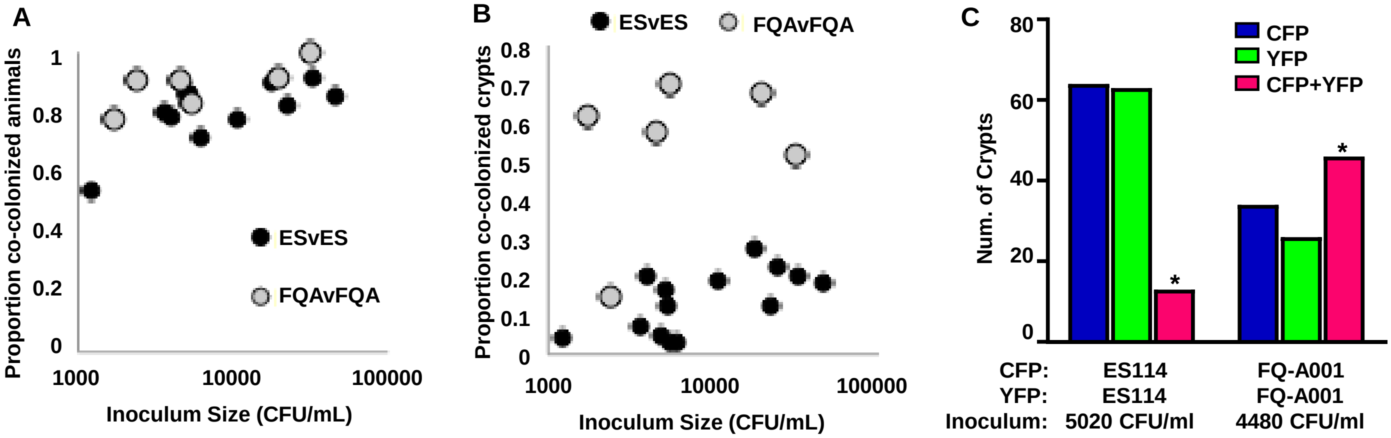


Figure S7. Inoculum size correlates with co-colonized animals. (A) Scatter plot showing calculated frequency of co-colonized squid light organs for animals exposed to ES114 differentially expressing CFP or YFP (ESvES) and animals exposed to FQA001 differentially expressing CFP or YFP (FQAvFQA) at various inoculum sizes. The frequencies of co-colonized animals were determined by dividing the number of animals with both YFP- and CFP-positive infections by the total number of animals in the group. (B) Scatter plot showing the calculated frequency of co-colonized crypts. Proportions of co-colonized crypts were determined by dividing the number of crypts that were positive for both CFP and YFP by the total number of crypts that were CFP positive. (C) Number of crypts that were scored as CFP only, YFP only, or CFP+YFP for competitions using CFP- and YFP-tagged ES114 (n = 29 animals) or CFP- and YFP-tagged FQ-A001 (n = 22 animals) at indicated inoculation sizes. The proportion of CFP+ YFP+ crypts between the different competitions were compared using a two-proportion z-test and an asterisk indicates $p < 0.001$.

Table S1. Type VI Secretion System 2 Genes in MJ11 Genome

VFMJ11#	Vas name	Other name	Predicted Function	% AA identity between T6SS1 and T6SS2 gene clusters
A0803		Flanking Gene		
A0804				
A0805	VasC	ImpI, TagH*,		24% to VFMJ11_1079
A0806	VasD	SciN*, TssJ*, EvpL*	*(core component)*	28% to VFMJ11_1078
A0807	VasE	ImpJ, SciO*, TssK*, EvpM*	*(core component)*	34% to VFMJ11_1077
A0808	VasF	ImpK, TssL‡, IcmH‡, DotU‡, SciP*, EvpN*	*(core component)*	30% to VFMJ11_1076
A0809			Conserved hypothetical protein	
A0810			Hypothetical protein	
A0811			Conserved hypothetical protein	
A0812			Conserved hypothetical protein	
A0813			Hypothetical protein	
A0814			Putative lipoprotein	
A0815			M23 peptidase domain protein	
A0816	VasL	ImpA, SciA*, EvpK* ((((TssA)	ImpA-related N-terminal family protein *(core component)*	Absent in T6SS1
A0817	VasK/IcmF	ImpL, TssM‡ , SciS*, EvpO*	Membrane transport protein … *(core component)*	24% to VFMJ11_1075
A0818			Putative transcriptional regulator	
A0819	VasB	ImpH, TssG*, AciB*, EmpG*	Baseplate *(core component)*	33% to VFMJ11_1083
A0820	VasA	ImpG, TssF‡, SciC*, EvpF*	Baseplate *(core component)*	34% to VFMJ11_1084
A0821	VasS		Lysozome-related protein *(core component)*	37% to VFMJ11_1085
A0822	VipB	ImpC, TssB‡	Outer sheath *(core component)*	67% to VFMJ11_1086
A0823	VipA	ImpB, TssC‡	Outer sheath *(core component)*	57% to VFMJ11_1087
A0824	VasJ	ImpA, DapB, SciA, EvpK	*(core component)*	25% to VFMJ11_1088
A0825			Serine-threonine protein kinase	
A0826			Conserved hypothetical protein	
A0827			Conserved hypothetical protein	
A0828			Conserved hypothetical protein	
A0829			Conserved hypothetical protein	
A0830	VgrG	TssI‡, VgrS*	Spiked tip *(core component)*	Absent in T6SS1
A0831	Hcp	TssD‡, SciK*, SciM*, EvpC*	Inner tube *(core component)*	Absent in T6SS1
A0832	VasG	ClpV, SciG*, TssH*, EvpH*	*(core component)*	53% to VFMJ11_1082
A0833			Nitric Oxide reductase regulator	

A0834			Putative lipoprotein	
A0835		OmpA	OmpA family protein	
A0836			Putative lipoprotein	
A0837			Conservative hypothetical protein	
A0838		Flanking Gene	Chitodextrinase	
A0839	tRNA-Gly		Aminoacyl-tRNA biosynthesis	

‡ Cianfanelli, F.R., Monlezun, L. and Coulthurst, S.J., 2016. Aim, load, fire: the type VI secretion system, a bacterial nanoweapon. *Trends in microbiology*, *24*(1), pp.51-62.
*Cascales, E., 2008. The type VI secretion toolkit. *EMBO reports*, *9*(8), pp.735-741.

Supplemental Table S2. Distribution of *V. fischeri* T6SS2-encoded proteins among *Vibrio* spp.

***Vibrio* species**	**MJ11_A0817 homolog**	**% ID[a]**	**MJ11_A0818 homolog**	**% ID[a]**	**Ref.[b]**
*V. fischeri**	ACH63505.1	100%	ACH63887.1	100%	(1)
*V. logei**	WP_017020851.1	80%	WP_065611695.1	89%	(2)
*V. wodanis**	WP_061004504.1	80%	WP_061004503.1	87%	(3)
*V. proteolyticus**	WP_040902488.1	66%	GAD67006.1	78%	(4)
*V. jasicida**	WP_038878560.1	64%	WP_045410168.1	56%	(5)
*V. crassostreae**	WP_017064525.1	63%	OEE92195.1	76%	(6)
*V. tasmaniensis**	WP_065104332.1	63%	WP_012600854.1	77%	(7)
*V. splendidus**	WP_054543291.1	63%	KPL96954.1	76%	(8)
*V. campbellii**	WP_005532464.1	63%	WP_005427005.1	74%	(9)
*V. harveyi**	WP_045491053.1	63%	AIV08715.1	74%	(10)
V. alginolyticus†*	WP_065645837.1	63%	EAS76637.1	73%	(8)
V. parahaemolyticus†*	WP_053807876.1	63%	WP_005480668.1	72%	(11)
V. antiquarius	WP_006741088.1	63%	EDN58559.1	73%	(12)
*V. owensii**	WP_041052795.1	62%	KIF49344.1	74%	(13)
*V. tubiashii**	WP_004749068.1	62%	WP_004743867.1	71%	(14)
V. azureus	WP_021710015.1	61%	WP_021710014.1	71%	(15)
*V. caribbeanicus**	WP_009603313.1	60%	WP_009603314.1	72%	(16)
*V. coralliilyticus**	WP_045985165.1	58%	EEX32044.1	75%	(17)
*V. neptunius**	WP_045976861.1	58%	WP_045976860.1	75%	(18)

[a] Percent identity based on BlastP results using VFMJ11_A0817 (IcmF_2) and VFMJ11_A0818 as sequence query.

[b] Reference for host association or isolation

* Indicates species is associated with a marine host

† Indicates species is associated with human host

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18. Thompson FL, *et al.* (2003) *Vibrio neptunius* sp nov., *Vibrio brasiliensis* sp nov and *Vibrio xuii* sp nov., isolated from the marine aquaculture environment (bivalves, fish, rotifers and shrimps). *Int J Syst Evol Micr* 53:245-252.

Table S3. Type VI Secretion System Auxiliary Gene Clusters in MJ11 Genome

VFMJ11#	Vas name	Other name	Auxiliary gene cluster	% AA identity between MJ11 and ES114
1309	VgrG	TssI‡, VgrS*	Auxiliary cluster I	93% to VF_1229
1310		DUF4123 domain protein	Auxiliary cluster I	93% to VF_1230
1311		Conserved hypothetical	Auxiliary cluster I	94% to VF_1231
1312		Conserved hypothetical	Auxiliary cluster I	79% to VF_1232
1313		Conserved hypothetical	Auxiliary cluster I	77% to VF_1233
1314		Conserved hypothetical	Auxiliary cluster I	82% to VF_1232
1315		Conserved hypothetical	Auxiliary cluster I	87% to VF_1232
1316		Conserved hypothetical	Auxiliary cluster I	78% to VF_1233
1495	Hcp	TssD‡, SciK*, SciM*, EvpC*	Auxiliary cluster II	Absent in ES114
1494	VgrG	TssI‡, VgrS*	Auxiliary cluster II	Absent in ES114
1493		DUF4123 domain protein	Auxiliary cluster II	Absent in ES114
1492		LysM domain protein	Auxiliary cluster II	Absent in ES114
A1070	Hcp	TssD‡, SciK*, SciM*, EvpC*	Auxiliary cluster III	100% to VF_A0954
A1069	VgrG	TssI‡, VgrS*	Auxiliary cluster III	99% to VF_A0953
A1068		DUF4123 domain protein	Auxiliary cluster III	97% to VF_A0952
A1067		DUF2235 domain protein	Auxiliary cluster III	86% to VF_A0950
A1066		DUF2931 domain protein	Auxiliary cluster III	72% to VF_A0945

Table S4. Impact of T6SS2 on proportion of co-colonized animals

	ES v FQ1 Trial 1	ES v FQ1- Trial 1	ES v FQ1 Trial 2	ES v FQ1- Trial2
Co-colonized animals	24	20	24	22
Total Squid	30	24	26	27
Proportion	0.80	0.92	0.83	0.81
P value by z test	0.107		0.424	

Table S5. Statistical analysis of co-colonized crypts

	Trial 1		Trial 2	
Strains in inoculum	ES114vFQA	ESvFQA-	ES114vFQA	ESvFQA-
CFP+ crypts	370	68	470	87
CFP+YFP+ crypts	10	12	10	17
Proportion	0.027	0.176	0.021	0.195
p-value by z-test	<0.001		<0.001	
Proportion needed for power = 0.8	0.105		0.099	
Effect size	0.369		0.4106	

Supplemental Table S6. General information about *Vibrionaceae* strains used in this study.

Strain[1]	**Collection Description**		**Source or Reference**	**NCBI GenBank Accession Numbers**			
	Geography	**Ecology**		***recA***	***mdh***	***katA***	***pyrC***
ABM004[2]	Oahu, HI, USA (Maunalua Bay)	*Euprymna scolopes* (squid light organ)	This study	MF076795	MF076808	MF076821	MF076834
AGC005[3]	State College, PA, USA	Aquarium seawater containing *E. scolopes* collected from Maunalua Bay	“”	MF076800	MF076813	MF076826	MF076839
ANM004[3]	Oahu, HI, USA (Maunalua Bay)	*E. scolopes* (squid light organ)	“”	MF076798	MF076811	MF076824	MF076837
CG101	Australia	*Cleidopus gloriamaris* (fish light organ)	(Lee 1994)	HQ595306	EU907966	EU907990	JF509856
CG103	“”	“”	“”	HQ595307	HQ595322	HQ595331	JF509855
CHS319[2]	Oahu, HI, USA (Maunalua Bay)	*E. scolopes* (squid light organ)	This study	MF076801	MF076814	MF076827	MF076840
EBS004[2]	“”	“”	“”	MF076797	MF076810	MF076823	MF076836
ECT001[2]	“”	“”	“”	MF076804	MF076817	MF076830	MF076843
EMG003[2]	“”	“”	“”	MF076796	MF076809	MF076822	MF076835
ES12	Oahu, HI, USA (Kaneohe Bay)	“”	(Boettcher and Ruby 1994)	HQ595309	HQ595323	HQ595332	JF509862
ES114; ATCC 700601	“”	“”	(Boettcher and Ruby 1990)	VF_0535[5]	VF_0276[5]	VF_A0009[5]	VF_A0412[5]
ES213	Oahu, HI, USA (Maunalua Bay)	“”	(Boettcher and Ruby 1994)	HQ595310	EU907971	EU907995	JF509863
ES401	“”	“”	(Lee 1994)	HQ595311	HQ595324	HQ595333	JF509864
ET101	Victoria, Australia (Crib Point)	*Euprymna tasmanica* (squid light organ)	(Nishiguchi 2002)	HQ595312	HQ595325	HQ595334	JF509865
ET401	Townsville, Australia (Magnetic Island)	“”	(Nishiguchi 2002)	HQ595313	HQ595326	HQ595335	JF509866

FQ-A001	Oahu, HI, USA (Kaneohe Bay)	*E. scolopes* (squid light organ)	(Sun Miyashiro 2016)	KU756584	KU756585	KU756586	KU756587
FQ-A002[2]	“”	“”	This Study	MF076793	MF076806	MF076819	MF076832
FQ-A003[2]	“”	“”	“”	MF076794	MF076807	MF076820	MF076833
H905	“”	Planktonic	(Lee and Ruby 1992)	HQ595314	EU907972	EU907996	JF509867
IRR001[3]	“”	*E. scolopes* (squid light organ)	This Study	MF076799	MF076812	MF076825	MF076838
KB4B5	“”	“”	(Wollenberg and Ruby 2009)	JF509762	JF509787	JF509845	JF509873
LFI1238 (*V. salmonicida*)	Hammerfest, Norway	*Gadus morhua* (cod head kidney)	(Hjerde et al 2008)	VSAL_I0634[5]	VSAL_I0359[5]	VSAL_II0215[5]	VSAL_II0468[5]
MB11B1	Oahu, HI, USA (Maunalua Bay)	*E. scolopes* (squid light organ)	(Wollenberg and Ruby 2009)	JF509765	JF509789	JF509847	JF509876
MB13B1	“”	“”	(Wollenberg and Ruby 2009)	JF509766	JF509790	JF509848	JF509877
MB13B2	“”	“”	(Wollenberg and Ruby 2009)	JF509767	JF509791	JF509849	JF509878
MB13B3	“”	“”	(Wollenberg and Ruby 2009)	JF509768	JF509792	JF509850	JF509879
MB15A4	“”	“”	(Wollenberg and Ruby 2009)	JF509771	JF509793	JF509851	JF509882
MJ1S[2]	Japan	*Monocentris japonicus* (fish light organ)	(Bose Stabb 2011?)	MF076792	MF076805	MF076818	MF076831
MJ11	Japan	*Monocentris japonicus* (fish light organ)	(Ruby and Nealson 1976)	VFMJ11_0538[5]	VFMJ11_0264[5]	VFMJ11_A0023[5]	VFMJ11_A0452[5]
NAD004[2]	Oahu, HI, USA	*E. scolopes*	This Study	MF076802	MF076815	MF076828	MF076841

	(Maunalua Bay)	(squid light organ)					
PP3	Oahu, HI, USA (Kaneohe Bay)	Planktonic	(Lee and Ruby 1992)	HQ595317	HQ595329	HQ595338	JF509893
SA1	Banyuls sur Mer, France	*Sepiola affinis* (squid light organ)	(Fidopiastis et al 1998)	HQ595318	EU907986	EU908010	JF509894
SA6 (*V. logei*)	“”	“”	(Fidopiastis et al 1998)	JF509782	JF509796	JF509854	JF509895
SR5	“”	*Sepiola robusta* (squid light organ)	(Fidopiastis et al 1998)	HQ595319	EU907987	EU908011	JF509896
ZJH004[2]	Oahu, HI, USA (Maunalua Bay)	*E. scolopes* (squid light organ)	This Study	MF076803	MF076816	MF076829	MF076842

[1] All strains are *V. fischeri* unless otherwise noted.

[2] Sequences collected in this study by PCR and Sanger sequencing.

[3] Sequences collected in this study by next-generation sequencing via the Illumina platform.

Supplemental Table S7. Strains, Plasmids, Oligo table

Strains or Plasmids	Relevant characteristics	Source or Ref.
E. coli		
DH5α	F′/*endA1 hsdR17 glnV44 thi-1 recA1 gyrA relA1* Δ(*lacIZYA-argF*)*U169deoR*(f80*dlacI*Δ(*lacZ*)*M15*)	(Hanahan, 1983)
DH5αλ pir	*λpir* derivative of DH5α	(Dunn et al., 2005)
CC118λpir	Δ(*ara-leu*) *araD* Δ*lac74 galE galK phoA20 thi-1 rpsE rpsB argE*(Am) *recA λpir*	(Herrero et al., 1990)
V. fischeri[a]		
ANS2098	FQ-A001 with *vasA_2* disruption (Erm^R)	This study
ANS2099	FQ-A002 with *vasA_2* disruption (Erm^R)	This study
LAS003	EBS004 with *vasA_2* disruption (Erm^R)	This study
LAS005	FQ-A001 with *vasA_1* disruption (Erm^R)	This study
LAS006	MB11B1 with *vasA_2* disruption (Erm^R)	This study
LAS007	MB13B3 with *vasA_2* disruption (Erm^R)	This study
Plasmids		
pAS2038	*vasA_2* disruption vector; *oriV*$_{R6K\gamma}$, *oriT*, Erm^R	This study
pLS04	*vasA_1* disruption vector; *oriV*$_{R6K\gamma}$, *oriT*, Erm^R	This study
pSNS116	*vasAB_2* complementation vector; *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Kn^R	This study
pSNS119	*vipA_2-gfp* fusion vector; *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Kn^R	This study
pSCV38	P$_{tetA}$-*yfp*, P$_{tetA}$-*mCherry, oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Cm^R	Sun et al., 2016
pYS112	P$_{proD}$-*cfp*, P$_{tetA}$-*mCherry,oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Cm^R	Sun et al., 2016
pEVS104	conjugative helper, *oriV*$_{R6K\gamma}$, *oriT*, Kn^R	Stabb & Ruby, 2002
pAKD601	*lacIq* and IPTG-inducible promoter with optional GFP fusion, *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Kn^R	Dunn and Stabb, 2008
pEVS122	*oriV*$_{R6K\gamma}$, *oriT*, Erm^R	Dunn et al., 2005
pVSV102	*gfp+*, *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Kn^R	Dunn et al., 2006
pVSV208	*dsRed+*, *oriV*$_{R6K\gamma}$, *oriV*$_{pES213}$, *oriT*, Cm^R	Dunn et al., 2006
Oligonucleotides[b]		
AS1146	TAGGTACCCTGATGTTGAACGCTTATTAG	This study
AS1147	ATGCATGCAGATACTTGATTGTTATGCG	This study
AS1064	ATGGTACCCAAGCAGACCTACGTTTATTATGGG	This study
AS1066	ATGGTACCTTAGAAAAAAACTTCTCGAATATCAATGG	This study
AS1140-R	TATTAACTCCTACTACACATTAAACTG	This study
AS1141-R	ATGATTCAATATATTGTTAATAAACC	This study
AS1158	TGGCTCTGCATATAAATACGG	This study
AS1159	TCACCTTTAGCAAATGCAGG	This study
AS1204	GCGAATTCGAGCTCGGTACCAACGCTTAGATAACCAGTTACC	This study
AS1205	GACTCTAGAGGATCCCCGGGATAGATACGTATCAAAGTGCCC	This study
LS004	TTCGAAGGGTTCGCTTTTTTAG	This study
SNS56	GTGGATCCGAGCTCGGTACCAAGGATGAATTATGTCACGTGATG	This study
	AGTTCTTCTCCTTTTCCTCCTCCTGCTGCTGCGCTAGCTTCAGCCTTA	
SNS57	GCTTCTTCTTTAG	This study
SNS41	GTGGATCCGAGCTCGGTACCAGGAGTTAATAGTGAGCAATAGC	This study
SNS42	TCTCCTTTGCTAGCTCTAGATTAGTTACTGCCTACTATTTTAATTTTCG	This study
recA outer-F	GACGATAACAAGAAAAAAGCACTGG	Wollenberg 2012
recA outer-R	CGTTTTCTTCAATTTCWGGAGC	Wollenberg 2012
recA inner-F	TGARAARCARTTYGGTAAAGG	Wollenberg 2012
recA inner-R	GGAGCRGCATCAGTCTCTGG	Wollenberg 2012
mdh outer-F	AAGTAGCTGTTATTGGTGC	Wollenberg 2012

mdh outer-R	CTTCGCCAATTTTGATATCG	Wollenberg 2012
mdh inner-F	GGCATTGGACAAGCGTTAGC	Wollenberg 2012
mdh inner-R	CGCCTCTTAGCGTATCTAGC	Wollenberg 2012
katA outer-F	TGTCCTGTTGCACATAACC	Wollenberg 2012
katA outer-R	CGCTTACATCAATATCAAG	Wollenberg 2012
katA inner-F	CGTGGTATTCCTGCAACATAC	Wollenberg 2012
katA inner-R	CCGATACCTTCACCATAAGC	Wollenberg 2012
pyrC outer-F	CTGATGATTGGCATTTACAC	Wollenberg 2012
pyrC outer-R	GCCACTCAACAGCTTCACC	Wollenberg 2012
pyrC inner-F	CACTTACGTGATGGTGATGTG	Wollenberg 2012
pyrC inner-R	GCCACTCAACAGCTTCACC	Wollenberg 2012

[a] For complete list of *V. fischeri* strains used in this study see supplemental Table S6.
[b] Restriction sites are underlined.

Supplemental Methods for Speare *et al.*

Media and growth conditions. *V. fischeri* strains were grown in LBS medium (1) at 24°C and *E. coli* strains were grown in either LB medium (2) or Brain Heart Infusion (Difco) at 37°C. Antibiotic selection for *V. fischeri* and *E. coli* strains were as described previously (3). Plasmids with the R6Kγ origin of replication were maintained in *E. coli* strain DH5αλpir (3) and plasmid pEVS104 (4) was maintained in strain CC118λpir (5). All other plasmids were maintained in *E. coli* strain DH5α (6).

Isolation of symbiotic *V. fischeri*. New *V. fischeri* isolates described in this study (Table S3) were isolated from *Euprymna scolopes* light organs. Briefly, adult *E. scolopes* squid were caught by dip-net in Kaneohe or Maunalua Bay, Oahu. After capture, animals were transported to a holding tank supplied with natural seawater. Adults were transported to Penn State where they were kept in an aquarium before anesthetizing, dissection, and plating of dilution series of light organ homogenate. Individual colonies were picked and re-streaked for purification.

Strain and plasmid construction. Bacterial strains, plasmids, and oligonucleotides used in this study are presented in Table S4. For mutant construction in *V. fischeri,* mutant alleles were mobilized on plasmids into recipients by triparental mating using CC118λpir pEVS104 as a conjugative helper. Potential mutants were screened for appropriate antibiotic resistance markers and verified using PCR. All primer design was based on the MJ11 genome sequence. To construct the *vasA_1* disruption mutant, approximately 1 kb of the *vasA_1* gene was PCR amplified using primers AS1204 and AS1205 from FQ-A001 gDNA. The resulting PCR product was cloned into the KpnI and SphI sites of plasmid pEVS122, resulting in the *vasA_1* disruption construct, pLS04.

The *vasA_1* disruption construct on pLS04 was moved into strain FQ-A001, resulting in strains LAS005. To construct the *vasA_2* disruption mutants, approximately 1 kb of the *vasA_2* gene was PCR amplified using primers AS1146 and AS1147 from FQ-A001 gDNA. The resulting PCR product was cloned into the KpnI and SphI sites of plasmid pEVS122, resulting in the *vasA_2* disruption construct, pAS2038. The *vasA_2* disruption construct on pAS2038 was moved into strains FQ-A001, FQ-A002, EBS004, MB11B1, and MB13B3 resulting in strains ANS2098, ANS2099, LAS003, LAS006, and LAS007, respectively.

To construct the VipA-GFP fusion expression vector, *vipA_2* was PCR-amplified from strain ES401 gDNA using primers SNS56 and SNS57. The forward primer includes 11 bp upstream of the *vipA_2* start codon to include the native ribosome binding site (RBS). The reverse primer excluded the native stop codon for *vipA_2* and a linker sequence was added (5' GCAGCAGCAGGAGGAGGA 3') for translational fusion of *vipA_2* to the *gfp* gene encoded in pAKD601 (7). The *vipA_2* PCR product was cloned into KpnI and NheI digested pAKD601 using the standard sequence-and ligation-independent cloning (SLIC) technique (8). The *vipA_2-gfp* fusion in the resulting plasmid (pSNS119) is located downstream of an IPTG-inducible promoter.

To construct a complementation vector for the *vasA_2* mutation, *vasAB_2* was PCR-amplified from strain FQ-A001 gDNA using primers SNS41 and SNS42. The forward primer includes 11 bp upstream of the start codon to include the native RBS. The reverse primer included the native stop codon to prevent a translational fusion to, or expression of, the downstream *gfp* gene on pAKD601. The resulting *vasAB_2* PCR product was cloned downstream of an IPTG-inducible promoter in plasmid pAKD601 (cut with KpnI and NheI) using the standard SLIC cloning technique (8), resulting in plasmid pSNS116.

Single-cell Fluorescence Microscopy. To visualize GFP-tagged T6SS2 sheath formation in V. fischeri cells, we used a single-cell fluorescence microscopy approach adapted from Basler et al., 2012 (9). Overnight cultures of *V. fischeri* wild-type FQ-A001, the *vasA_1* mutant (LAS05), or the *vasA_2* mutant (ANS2098) strains carrying the IPTG-inducible *vipA_2-gfp* fusion expression vector (pSNS119) were diluted 1:100 into fresh LBS medium supplemented with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and cultivated at 24°C with shaking for 2.5-3 hours to an OD600 of approximately 1.5. Cells from 5 µL of these cultures were spotted onto a thin pad of LBS with 2% agar and 0.5 mM IPTG, covered with a glass cover slip and imaged after two hours at room temperature. Fluorescence images were captured using an Olympus BX51 microscope outfitted with a Hammatsu C8484-03G01 camera and a 100X/1.30 Oil Ph3 objective lens. Images were captured using MetaMorph software. Contrast on images was adjusted uniformly across images by subtracting background using ImageJ software.

Contact-dependent Co-incubation Assay. To test for contact-dependent interactions, strains were prepared as described in the methods section, except strains were separated using a 0.22 µm nitrocellulose membrane. Specifically, 5 µl of each strain was spotted onto a membrane and allowed to dry. These membranes were placed directly on top of one another (alternating which strain was on the top and bottom membranes) and placed onto LBS agar plates and incubated at 24°C for 5 h. After 5 h, both membranes were removed from the plate and suspended in 3 mL LBS medium. Strain were quantified by plating serial dilutions for T0 and T5 onto selective LBS agar plates. For each experiment four independent cultures of each strain were assayed and each experiment was repeated three times.

Squid Colonization Assays. Overnight cultures of the indicated strains were diluted 1/100 into LBS supplemented with 2.5 µg/ml chloramphenicol and grown to OD_{600} ~ 1.0. For each inoculum, cultures were diluted into filter-sterilized seawater (FSSW) and sampled for CFU. For each treatment, 24-30 freshly hatched juvenile squid were exposed to the inoculum containing an even mix of YFP- and CFP- labeled strains (using pSCV38 and pYS112, respectively) at a final concentration ranging from 1600 to 8240 CFU/ml. Squid were exposed to this mixed inoculum for 20 h and then washed in fresh FSSW. After 44 h, animals were fixed in 4% paraformaldehyde/marine phosphate buffered saline (mPBS) for 24 h at 4°C, then washed exhaustively in mPBS. Animals were prepared for fluorescence microscopy by dissecting the ventral side of the mantle and removing the siphon to reveal the light organ. YFP, CFP, and differential interference contrast (DIC) images were taken using a Zeiss 780 confocal microscope (Carl Zeiss AG, Jena, Germany) equipped with a 10x or 40x water lens. Each crypt space was scored separately for CFP and YFP fluorescence.

Phylogenetic Analysis Details. A multi-locus phylogenetic analysis was performed using partial sequences of four loci: *recA*, *mdh*, *katA*, and *pyrC*. Published sequence data and newly amplified sequences of 35 total *Vibrio* isolates were collected, aligned with ClustalX 2.1 (10), analyzed via three independent runs of 2,000 samples each in ClonalFrame 1.2 (11), and visualized with a consensus network in Splitstree 4.12.2 (12) as described previously (13, 14). The resulting consensus network showed little evidence of phylogenetic incongruence (so-called “splits” represented by parallelograms visualized among nodes in the network) among these four partial loci. Because the ClonalFrame/Splitstree analysis revealed little evidence of phylogenetic incongruence among these four partial loci, for each isolate the four partial sequences were combined into a single concatenated sequence (ordered *recA mdh katA pyrC* – approximately 2880 nucleotides). Concatenated sequences were analyzed by jModelTest 2.1 v20160303 (15) via three information criteria methods (Akaike, Bayesian, and Decision Theory). The latter two methods calculated the lowest likelihood score for a transitional model with a gamma shape parameter and a proportion of invariable sites (TIM3+**Γ**+**I**) while a general time-reversible model with a gamma shape parameter and a proportion of invariable sites (GTR+**Γ**+**I**) was given the lowest likelihood score with the Akaike method.

TIM3+ **Γ**+**I** evolutionary model parameter estimates calculated by jModelTest were used by the software program PAUP*4.0b10 (16) to infer phylogenetic trees and bootstrap those trees via two methods: Maximum Parsimony (MP) and Maximum Likelihood (ML). ML phylogenetic inference and bootstrapping was performed by searching heuristically using simple addition and subtree pruning and regrafting for swaps, treating gaps as missing, and swapping on “best only” with 1000 replicates and 1000 bootstrap pseudoreplicates. MP phylogenetic inference and bootstrapping was performed by searching heuristically using simple addition and tree bisection reconnection for swaps, treating gaps as missing, and swapping on “best only” with 1000 replicates and 1000 bootstrap pseudoreplicates.

A Bayesian approach (Ba) to phylogenetic inference was also completed with the program MrBayes 3.1.2 (17) by setting the “nst” variable to “6” and the “rates” variable to “invgamma” (this approximates a GTR+**Γ**+**I** model); three heated chains were set using the “temp” variable to a value of 0.05 (to ensure appropriate chain swapping). Construction of the majority-rule consensus tree and statistical analysis of clade membership/presence was assessed by sampling an “appropriately stationary” posterior probability distribution. For the purposes of this study, an “appropriately stationary” distribution was defined, as recommended by Ronquist and colleagues (18), as an average standard deviation of split frequencies of less than 0.01 for 70% to 90% of samples between two, independent Metropolis-coupled Markov Chain Monte Carlo runs. Approximately 3,000,000 total generations were sampled every 100 generations for a total of 30,000 samples – 10,000 of these samples were discarded via the "burnin" variable in MrBayes. Majority-rule consensus trees drawn from the resulting 20,000-sample, stationary distribution were used for the assessment of the posterior probabilities of all clades. The above methods were independently repeated twice; all three separate Ba "replicates" showed nearly identical phylogenetic patterns of clades and posterior probabilities. Sequences associated with this analysis were submitted to the GenBank database and their accession numbers are listed in Table S3.

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