Supporting information

Article Title:

Development of an Orthogonal Inhibitor Screening Platform and Identification of 2′,4′-Dihydroxychalcone Targeting HlyU, a Master Virulence Regulator in *Vibrio vulnificus*

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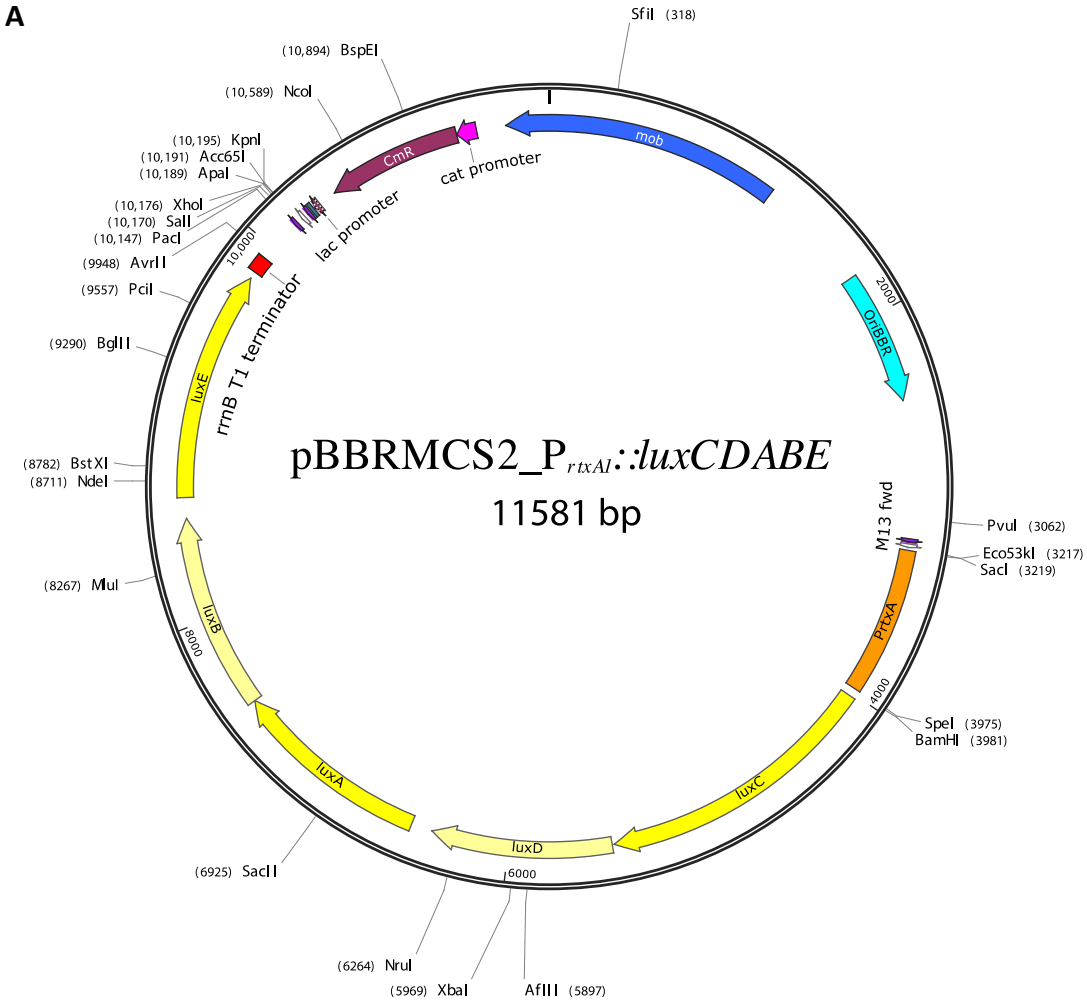
1. Supplementary Figures

Figure S1.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(10,176) XhoI
(10,170) SalI
(10,147) PacI
(9948) AvrII
(9557) PciI
(9290) BglII
(8782) BstXI
(8711) NdeI
(8267) MluI
(6925) SacII
(6264) NruI
(5969) XbaI
AflII (5897)
(3975) SpeI
(3981) BamHI
(3217) Eco53kI
(3219) SacI
(3062) PvuI
CmR
cat promoter
mob
lac promoter
rrnB T1 terminator
luxE
luxB
luxA
luxD
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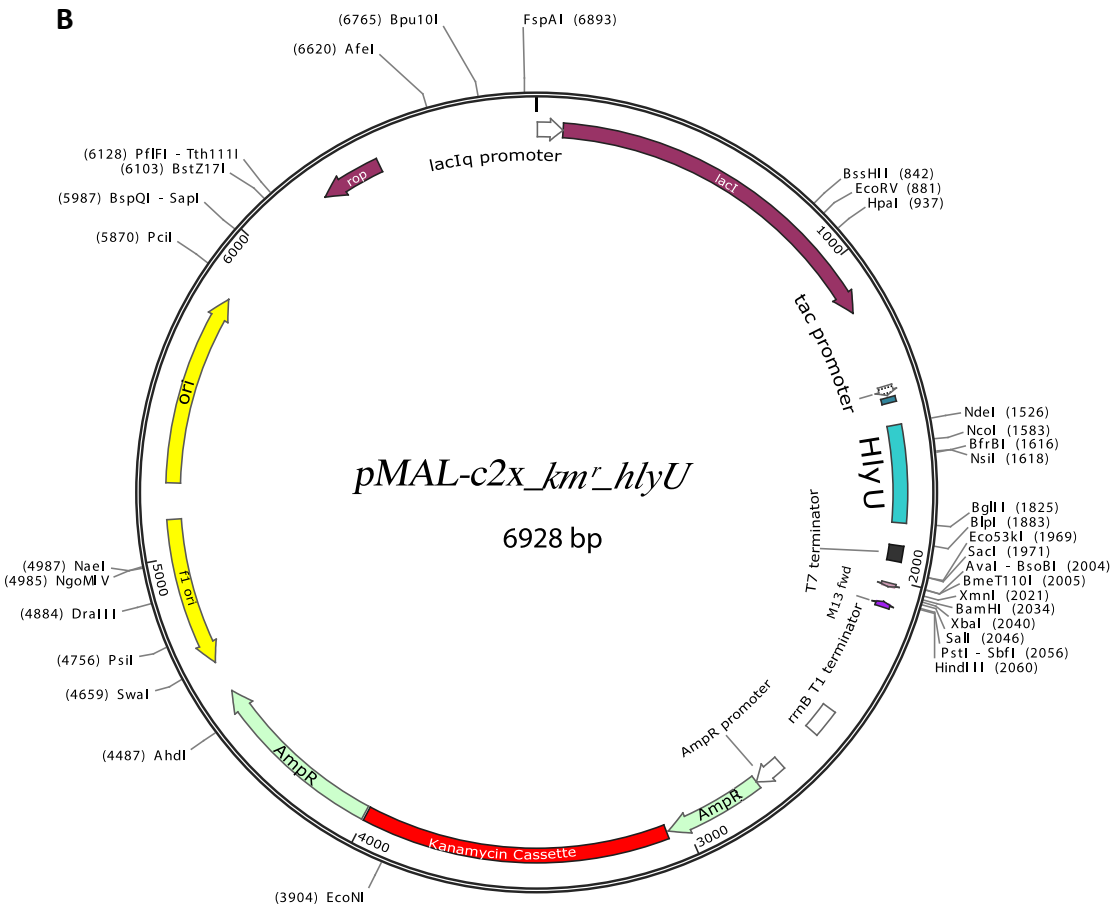


Figure S1. Plasmid maps. (**A**) The plasmid pBBRMCS2_P*rtxA1*::*luxCDABE* map was achieved by inserting the P*rtxA1* promoter in the pBBR1MCS2-*lux* plasmid backbone [1]. The scorable reporter P*rtxA1*-*luxCDABE*-*cm*^{r} cassette (~8 kb; reporter fragment) was PCR amplified from pBBRMCS2_P*rtxA1*::*luxCDABE* plasmid and integrated at an innocuous site of *E. coli* K-12 strain MG1655 genome and the strain was designated as *E. coli*-P*rtxA1*::*lux_cm*^{r}. (**B**) HlyU virulence transcription factor encoding gene *hlyU* was cloned at SalI and SacI into pMAL-c2X expression vector [2] to obtain pMAL-c2*X_hlyU* plasmid (*amp*^{r}). The plasmid was modified and the kanamycin resistance marker was inserted at the ScaI site of ampicillin to inactivate the ampicillin resistance gene (*km*^{r}, *amp*^{s}). The pMAL-c2X_*hlyU_km*^{r} underwent electroporation in the *E. coli*-P*rtxA1*::*lux_cm*^{r} strain to develop the stable inhibitor screening platform.

Figure S2.

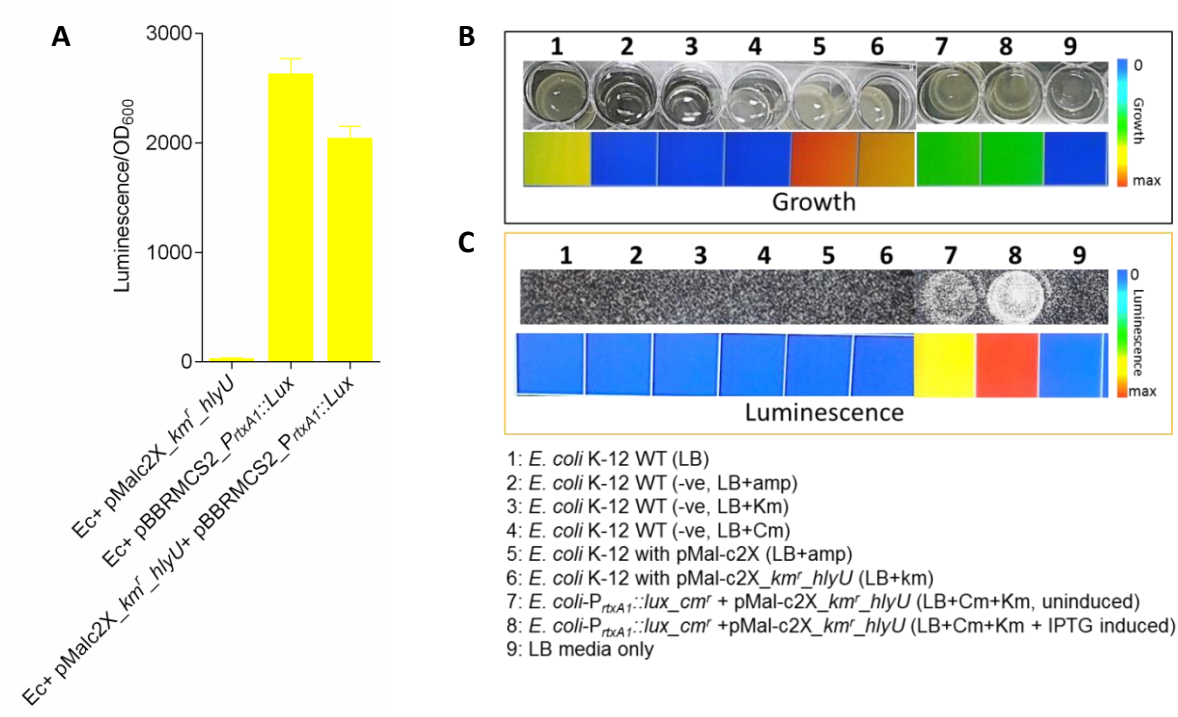


Figure S2. Multiple plasmid-based reporter system versus stable inhibitor reporter platform. Overnight grown culture was diluted in fresh LB medium at 1:500 ratio and supplemented with 1 mM IPTG and appropriate antibiotics (Cm and Km). The luminescence per unit OD600 was calculated after 5 h of incubation. (**A**) Quantitative expression of dual-plasmid inhibitor screening reporter system. Strain *E. coli* K-12 MG1655 is designated as 'Ec'. Two plasmids- based reporter platform became unstable and showed low luminescence either by plasmid instability or reduced antibiotic pressure to maintain the vertical transfer during assay. (**B, C**) An orthogonal stable inhibitor reporter platform. Verification of growth (**B**) and luminescence (**C**) of WT, empty/*hlyU* vector control and inhibitor screening reporter *E. coli* strains in various culture conditions are indicated as 1-9. Strain 7 showed luminescence signal due to the leaky expression of HlyU, which is enhanced upon IPTG induction (strain 8). The signal was expected to reduce when an inhibitor interacts and inhibit the DNA-binding activity of HlyU virulence transcriptional regulator.

Figure S3.

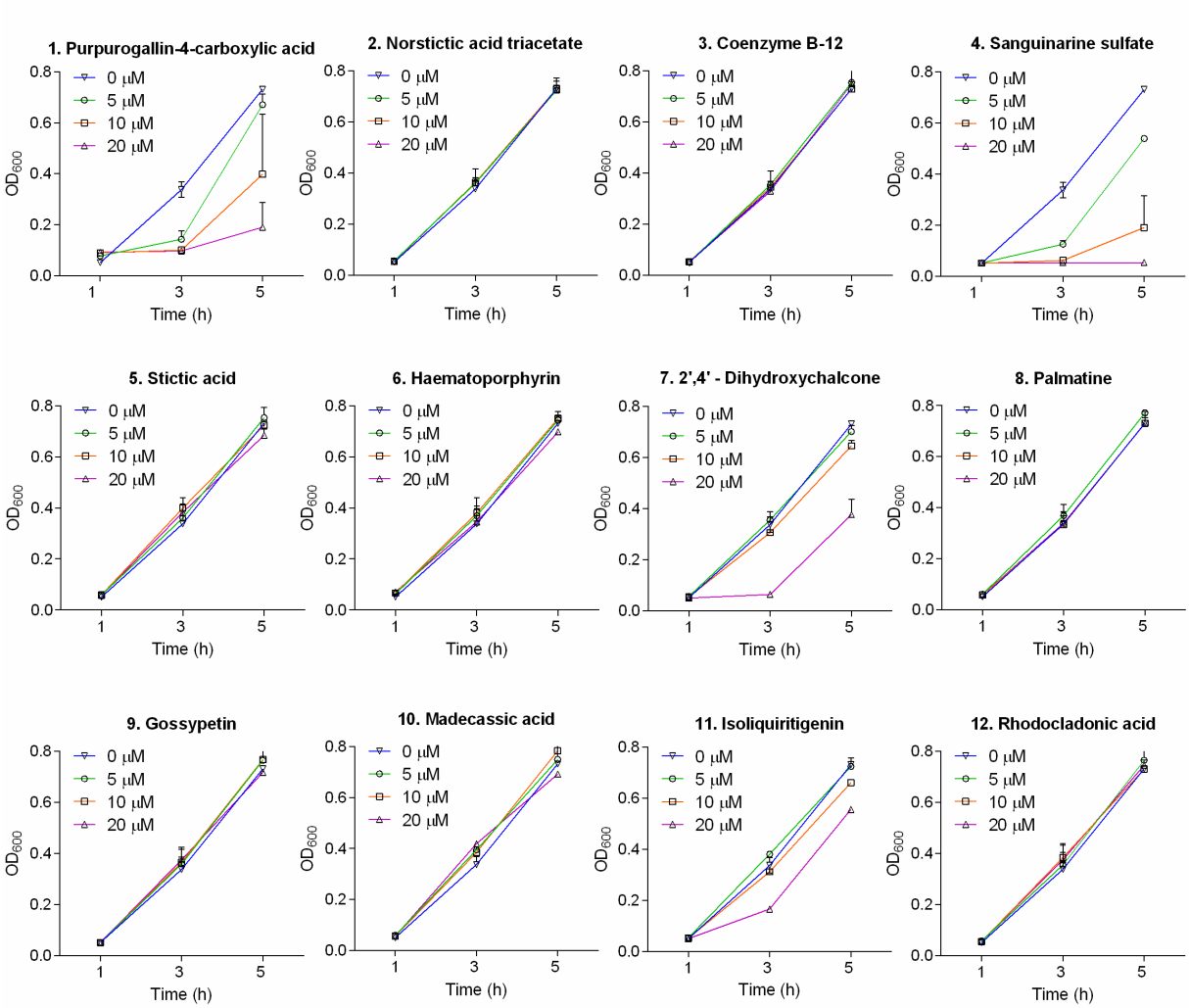


Figure S3. Screening of primary antivirulence hits for growth inhibition of *V. vulnificus.* The 12 antivirulence hits obtained from the *E. coli*-P$_{rtxA1}$::*lux_cm*^{r}+pMal-c2X_*km*^{r}_*hlyU* reporter were examined for dose-dependent growth inhibitory effects (secondary screening) on the target organism, *V. vulnificus.* Overnight grown culture of WT *V. vulnificus* was diluted by 1:100 in fresh LBS and incubated with 0, 5, 10, and 20 µM of antivirulence hits and OD_{600} was measured in a time dependent manner. (1) Purpurogallin-4-carboxylic acid and (4) Sanguinarine sulfate were excluded from further study because of growth inhibitory effects. The remaining 10 antivirulence hits were considered secondary hits and evaluated further using qRT-PCR.

Figure S4.

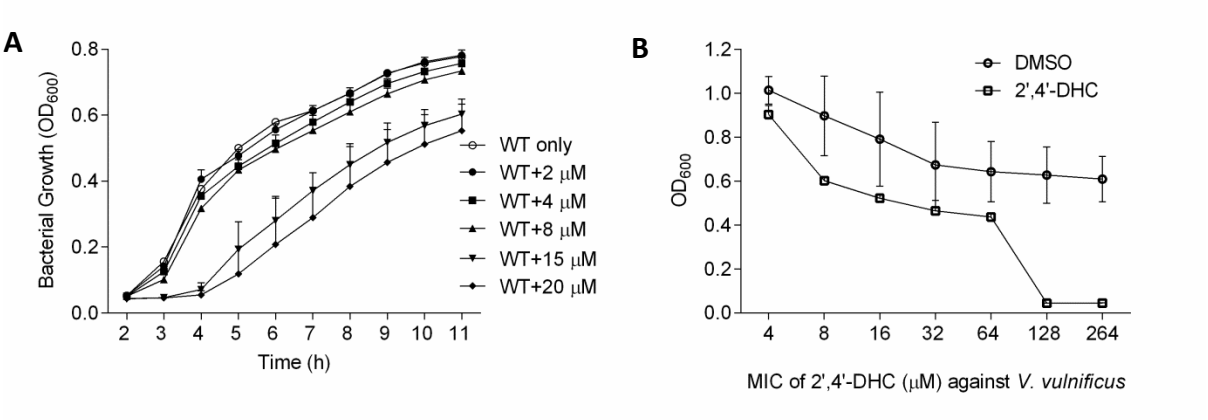


Figure S4. Effect of 2′,4′- DHC on the growth of *V. vulnificus.* (**A**) Growth pattern of *V. vulnificus* with different concentrations of 2′,4′- DHC. Freshly grown culture of WT *V. vulnificus* was supplemented with 2, 4, 8, 15, and 20 μM of 2′,4′- DHC and the growth response was recorded in 48-well plates by measuring OD_{600} over time in LBS medium. (B) Minimum inhibitory concentration (MIC) of 2′,4′- DHC against *V. vulnificus.* MIC was determined with an initial inoculum of 8×10^5 CFU/mL in MH broth and the observation was recorded after 18 h of incubation according to the CLSI protocol [3].

Figure S5.

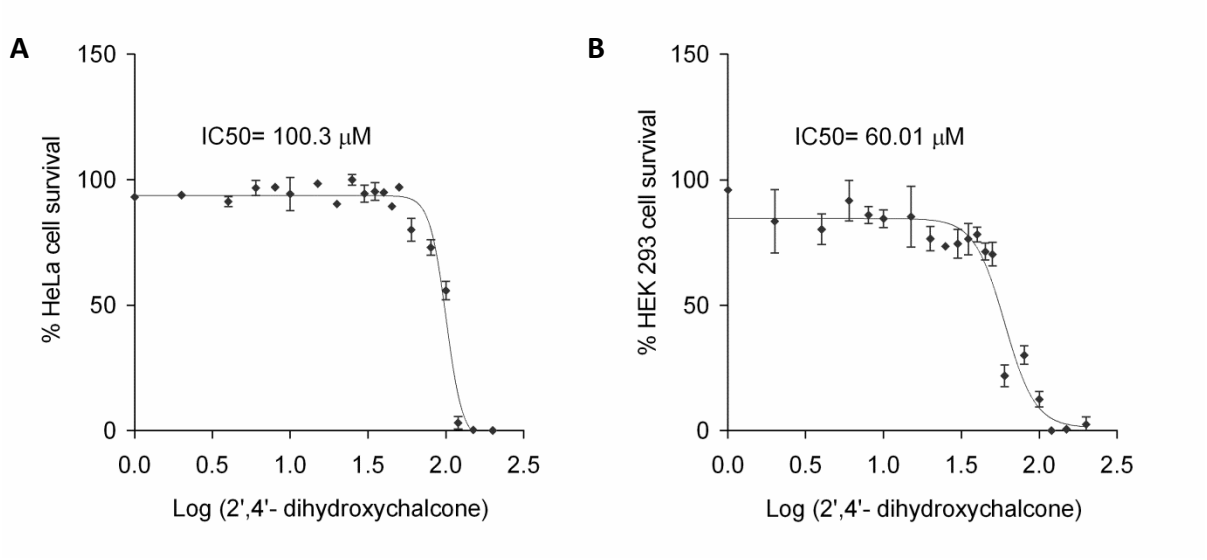


Figure S5. *In vitro* cytotoxicity of 2′,4′- DHC against cell lines. Toxicity of 2′,4′- DHC was tested with a EZ-Cytox kit for (**A**) HeLa cells and (**B**) HEK293 cells. The cytotoxicity was measured as a function of cellular viability. A total of 2×10^4 HeLa cells (in DMEM and 10 % FBS medium) were incubated with varying concentrations of 2′,4′- DHC (1–200 µM) for 48 h at 37°C with 5% CO_2. Absorbance (A_{450}) was measured using a multi-plate reader (Tecan Infinite M200), after adding the kit solution. The cell culture media was used as blank and the cells with DMSO treatment were considered as 100% viable. GraphPad Prism was used to determine the IC50 [1].

3. Tables

Table S1. Strains and plasmids

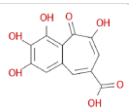
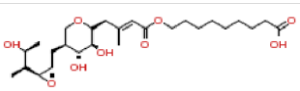
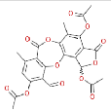
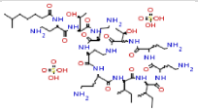
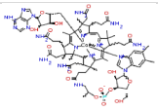
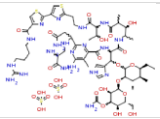
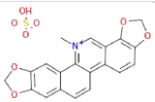
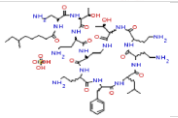
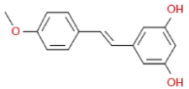
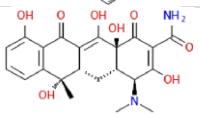
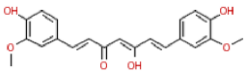
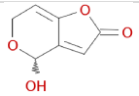
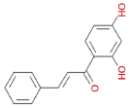
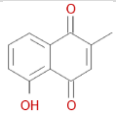
Plasmid/Strain	Description	Reference
Plasmids		
pMAL-c2X	Cytoplasmic expression plasmid for cytoplasm, *amp*^{r}	[2]
pMAL-c2X_*hlyU*	HlyU cloned at SalI and SacI site in pMAL-c2X plasmid (*amp*^{r})	This study
pMAL-c2X_*km*^{r}_*hlyU*	pMAL-c2X_*hlyU* resistance marker changed to kanamycin (*km*^{r} , *amp*^{s})	This study
pProEx-HTb	N-termial His_6 protein overexpression (*amp*^{r})	Lab stock
pProEx-HTb_*hlyU*	*hlyU* cloned at BamHI (F) and XhoI (R) site in pProEx-HTb (*amp*^{r})	This study
pProEx-HTb_*hlyU**	L91A/L17A point mutated *hlyU** (*amp*^{r})	This study
pBBRMCS2_P $_{rtxA1}$*::luxCDABE*	*E. coli* with pBBRMCS2_P$_{rtxA1}$*::luxCDABE (cm*^{r}*)*	[1]
pKD46	λ-red recombinase encoding plasmid under arabinose inducible promoter, temperature sensitive origin of replication (*amp*^{r})	[4]
Strains		
E. coli DH5α	F$^-$ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK$^-$, mK$^+$) *phoA supE44 λ– thi-1 gyrA96 relA1*	Lab stock
E. coli BL21(DE3)	*E. coli str*. B F– ompT gal dcm lon hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS)	Lab stock
E. coli K-12 MG1655	Wild-type (WT) *E. coli* strain	Lab stock
E. coli-P$_{rtxA1}$*::lux_cm*^{r}	Reporter cassette; chromosomal integration of ~8 kb P$_{rtxA1}$*::luxCDABE_cm*^{r}	[5]
E. coli-P$_{rtxA1}$*::lux_cm*^{r} reporter	*E. coli*-P$_{rtxA1}$*::lux_cm*^{r} with pMAL-c2X_*km*^{r}_*hlyU*	This study
WT *Vibrio vulnificus*	Wild type MO6-24/O clinical isolate	This study
Δ*hlyU*	*V. vulnificus hlyU* gene deletion mutant	[6]
		[7]

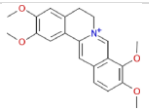
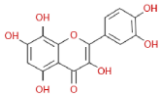
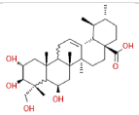
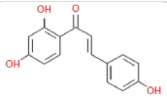
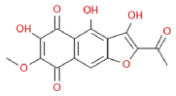
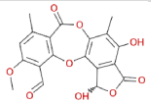
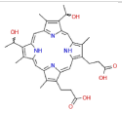
Table S2. List of primers

Purpose	Name	Sequence
Chromosomal Integration	*Int_F*	CGCGGGGAACTCTCGGTTCAGGCGTTGCAAACCTGGCTACTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGAGCTCGAATCAAATAAAATG
	Int_R	TAAACCGTTTGGATCGGGTCTGGAATTTCTGAGCGGTCGCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCATGATCGGCACGTAAGAGGTTC
	km^r_F	ACCATAAGTACTCAGCATCGCAGTGGGAACGATGCCC
	km^r_R	ACCATAAGTACTTGGCCGGGGGACTGTTGGGCGCCATC
Confirmation of Integration	P$_{rtxA1}$Lux_*cm*^{r}_Intconf_F	TGGGTTTGAAAATGGGAGCTGGGAGTTC
	P$_{rtxA1}$Lux_*cm*^{r}_Intconf_R	TTAGCTGATCTTTAATAATAAGGAAATG
HlyU cloning in pMAL-c2X-*km*^{r}	*hlyU*-F	ACGGCGGTCGACATGAACTTAAAAGATATGGAG (SalI)
	hlyU-R	ACGGCGGAGCTCTTATTCTTCGCAATAAAGACTG (SacI)
qRT-PCR	*rtxA1*-F	GATGGTTACAAAGCCGATAC
	rtxA1-R	TCTGGGTTATCAAGCAGAAT
	vvhA-F	AGACTATCGCATCAACAACC
	vvhA-R	AAACGTCATAGTTCGGTTTG
	hlyU-F	TTCTGCTAAAGCTGTCGTATT
	hlyU-R	AAACCGTTTGTGCTTCTTTA
	hns-F	GAACAAATTGCTAAAGATGGT
	hns-R	GATTTACCCGCATCTAATTG
	gyrB-F	TCAGTTTCTGTTAGCGATGA
	gyrB-R	ATCGTCAACAGCACTTTTTC
Site Directed Mutation of HlyU	*hlyU*L91A-F	TAAAAGCAATGATTAAACTGGCTCACAGTCTTTATTGCGAAGAA
	*hlyU*L91A-R	TTCTTCGCAATAAAGACTGTGAGCCAGTTTAATCATTGCTTTTA
	*hlyU*L17A-F	CTGCTAAAGCTGTCGTATTAGCTAAAGCCATGGCCAATGAAAG
	*hlyU*L17A-R	CTTTCATTGGCCATGGCTTTAGCTAATACGACAGCTTTAGCAG
EMSA	P$_{rtxA1}$ probe-F	TCAAATAAAATGGCGGGTG
	P$_{rtxA1}$probe-R	TCAAAAACGCTGCAATAAAC
HlyU-His$_6$ protein expression	His-HlyU-F	ACGGCGGGATCCAACTTAAAAGATATGGAG (BamHI)
	His-HlyU-R	ACGGCGCTCGAGCTATTCTTCGCAATAAAG (XhoI)

Restiction endonuclease shown in bracket.

Table S3. List of antivirulent and antimicrobial hits

Sr. No.	Antivirulent Hits		Antimicrobial Hits	
	Compounds	**Structure**	**Compounds**	**Structure**
1.	Purpurogallin-4-carboxylic acid		Mupirocin	
2.	Norstictic acid triacetate		Colistin sulfate	
3.	Coenzyme B12		Bleomycin	
4.	Sanguinarine sulfate		Polymyxin B sulfate	
5.	Resveratrol 4’-methyl ether		Tetracycline hyrochloride	
6.	Curcumin		Patulin	
7.	2’,4’-dihydroxychalcone		Plumbagin	

8.	Palmatine			
9.	Gossypetin			
10.	Madecassic acid			
11.	Isoliquiritigenin			
12.	Rhodocladonic acid			
13.	Stictic acid			
14.	Haematoporphyrin			

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