

Supplementary information

S-nitrosylation-mediated activation of a histidine kinase represses the type 3 secretion system and promotes virulence of an enteric pathogen

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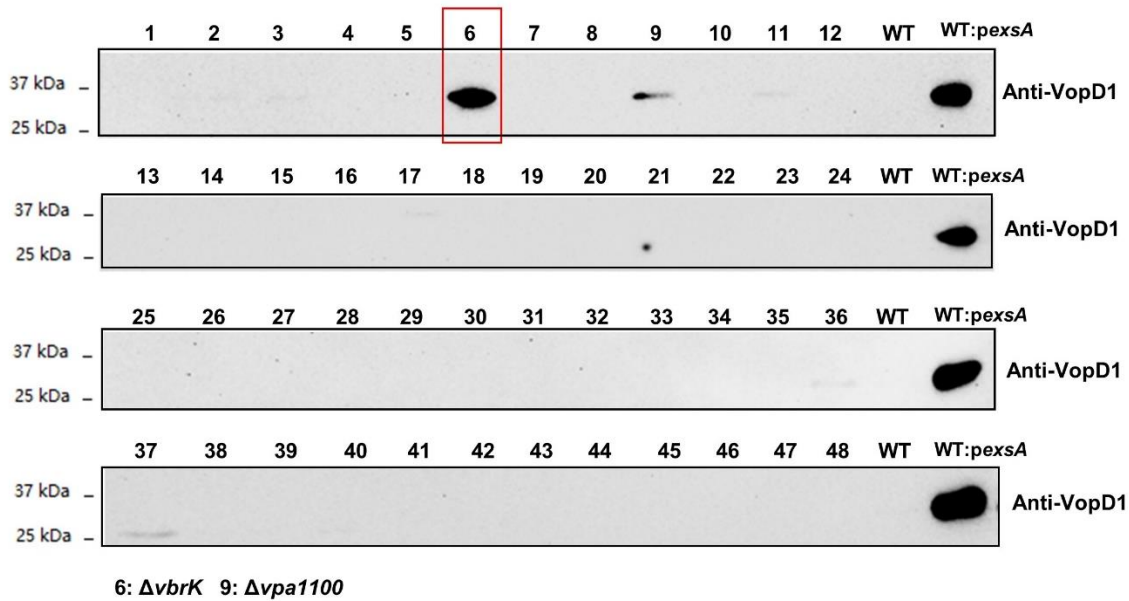
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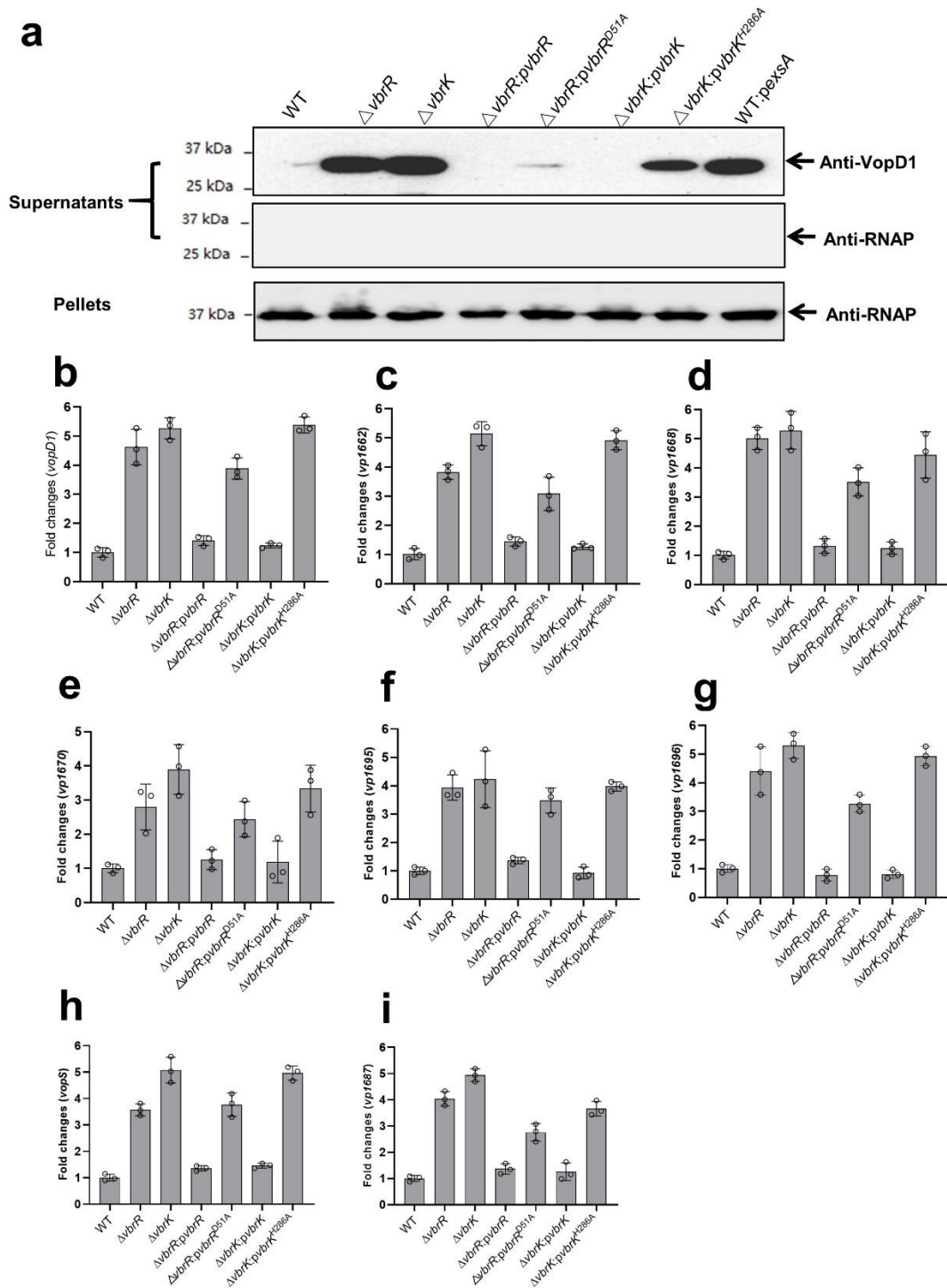
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Supplementary Table 1 Bacterial strains and plasmids used in this study

Supplementary Table 2 Primers used in this study



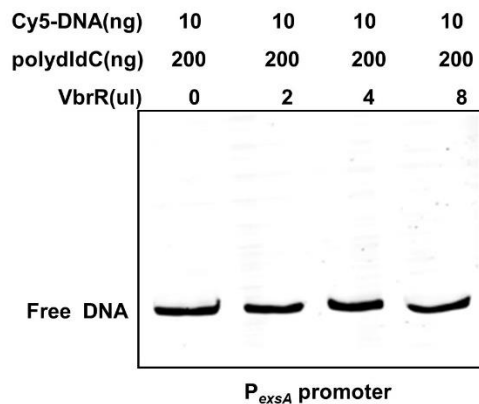
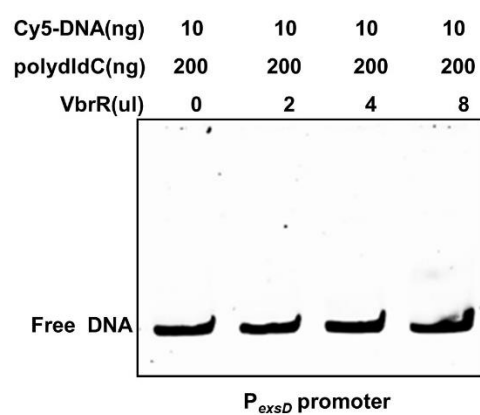
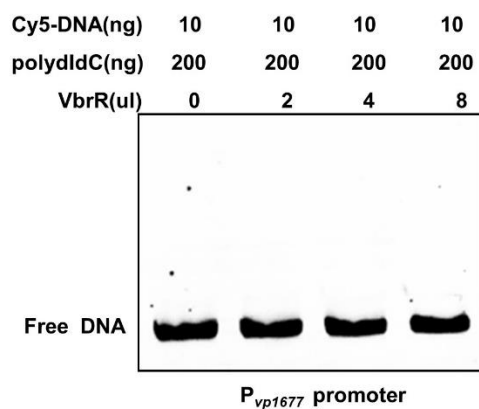
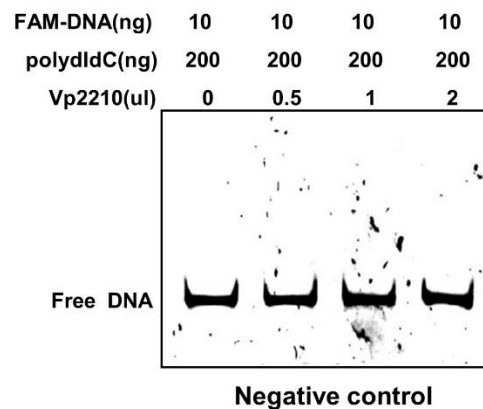
Supplementary Fig. 1 Identification of histidine kinase responsible for T3SS1 repression in the LB culture medium. Genes encoding predicted histidine kinases were deleted individually from the genome of WT *V. parahaemolyticus* and each mutant was grown in LB medium in the presence of carbenicillin (except for $\Delta vbrK$) for 3 hours. Culture supernatant was collected and western blot was performed using anti-VopD1 antibody. #1 to #48 on top of the blot indicate mutants lacking *vpa0009*, *vpa0149*, *vpa0710*, *vpa0736*, *vpa0763*, *vpa0920* (*vbrK*), *vpa0965*, *vpa1075*, *vpa1100*, *vpa1130*, *vpa1277*, *vpa1433*, *vpa1515*, *vpa1664*, *vp0362*, *vp0487*, *vp1069*, *vp1201*, *vp1212*, *vp1375*, *vp1503*, *vp1712*, *vp1735*, *vp1908*, *vp1968*, *vp1984*, *vp2010*, *vp2478*, *vp2567*, *vp2859*, *vp2874*, *vpa0020*, *vpa0182*, *vpa0826*, *vpa1196*, *vpa1731*, *vpa1220*, *vpa1229*, *vpa0675*, *vp0155*, *vp0539*, *vp0570*, *vp0915*, *vp1245*, *vp2252*, *vp1755*, *vp1876*, and *vp2229*, respectively. WT:pexsA was used as a positive control. Red box indicates the secretion of VopD1 by $\Delta vpa0920$ ($\Delta vbrK$). Representative image of three independent experiments is shown.



Supplementary Fig. 2 VbrK/VbrR regulates T3SS1 gene expression.

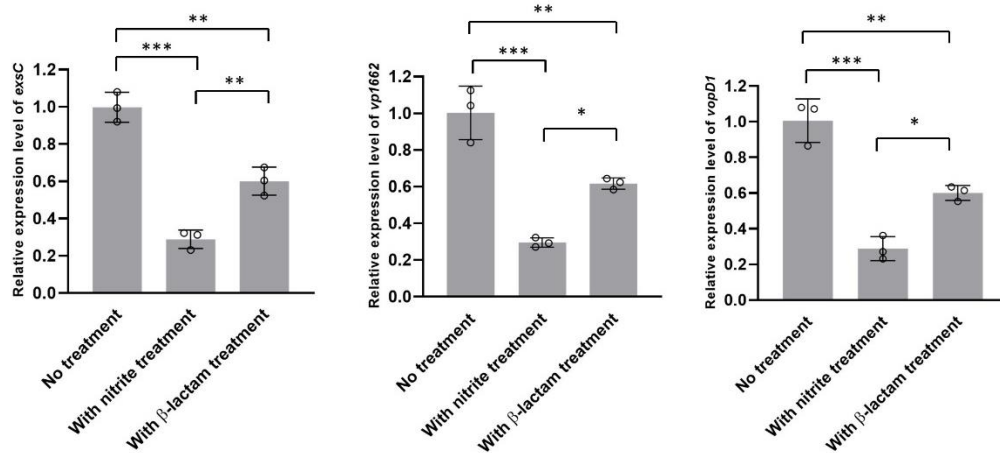
(a) Indicated strains of *V. parahaemolyticus* were grown in the LB medium and supernatant was processed for western blot using anti-VopD1 and anti-RNAP antibodies. Pellet samples were also processed for western blot using anti-RNAP antibody to ensure that the supernatant was prepared from equal amount of bacteria cells across the samples.

(b-i) RNA was isolated from indicated *V. parahaemolyticus* strains cultured in LB medium and qRT-PCR analysis of *vopD1* (b), *vp1662* (c), *vp1668* (d), *vp1670* (e), *vp1695* (f), *vp1696* (g), *vopS* (h) and *vp1687* (i). Bars with standard deviation indicate average fold changes relative to WT. All error bars represent mean $\pm$ standard deviation (n=3 biologically independent experiments).

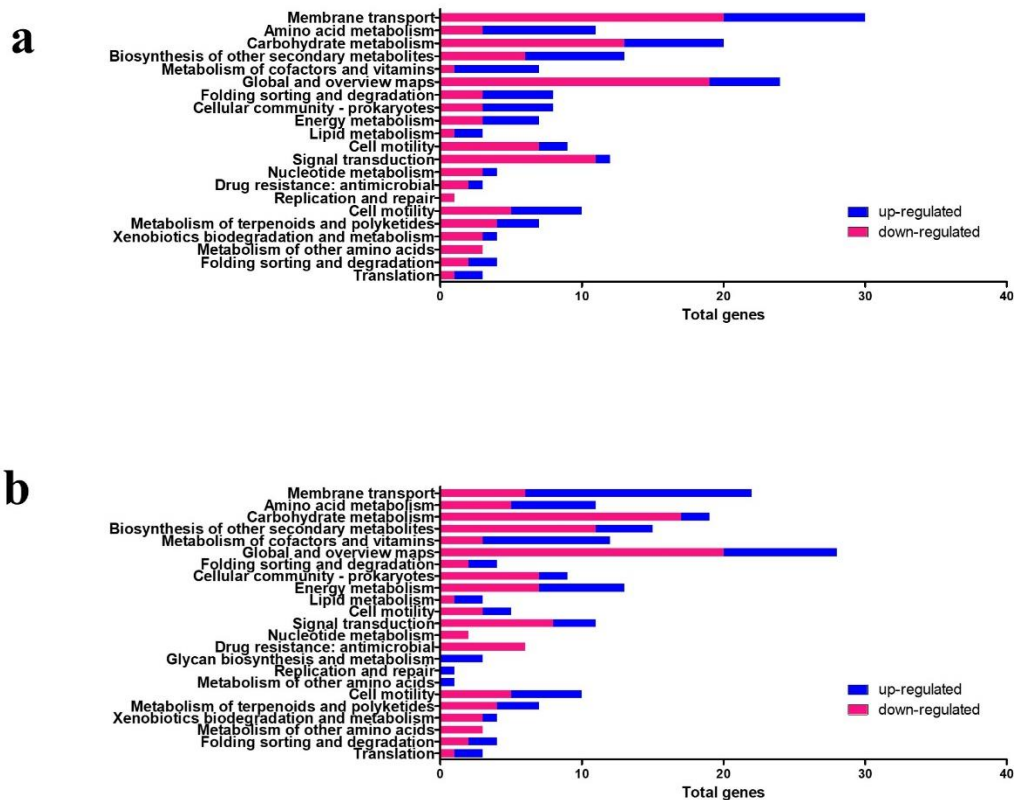
a**b****c****d**

Supplementary Fig. 3 EMSA analysis for the binding of VbrR or Vp2210 with promoters.

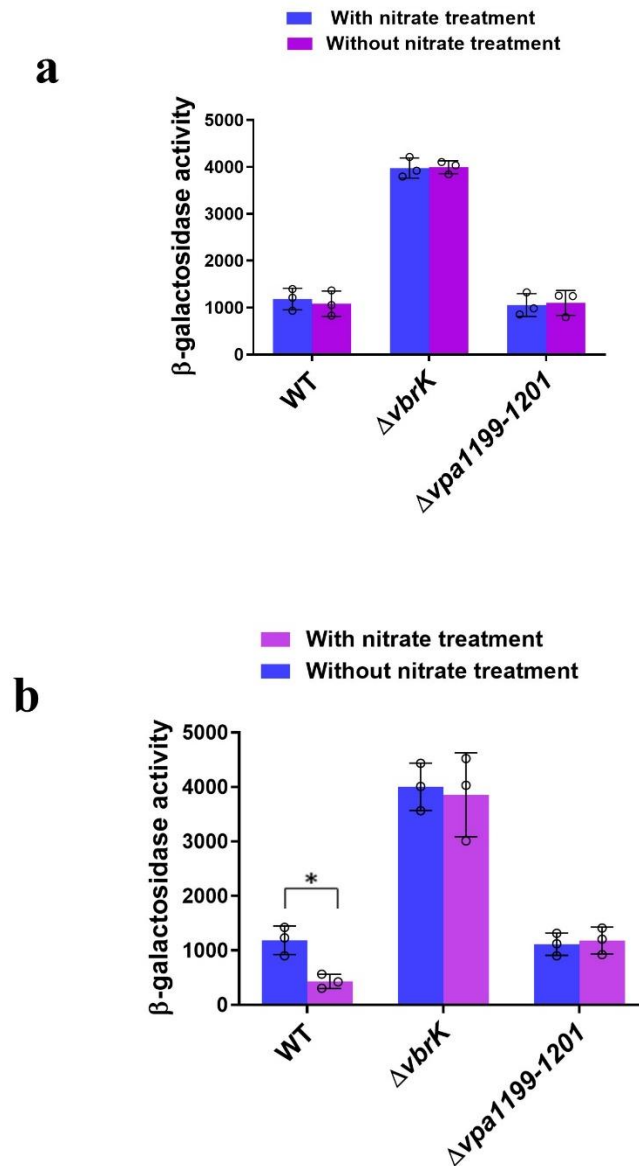
(a-c) Purified VbrR protein does not bind promoter of *exsA* (a), *exsD* (b) and *vp1677* (c). (d) Purified Vp2210 protein does not bind negative control promoter of *gyrB*. Representative image of three independent experiments is shown.



Supplementary Fig. 4 Effect of nitrite and β -lactam on the expression of T3SS1 genes. RNA was isolated from WT *V. parahaemolyticus* cultured in LB medium with or without treatment and qRT-PCR analysis of *exsC* (left panel), *vp1662* (middle panel) and *vopD1* (right panel). Bars with standard deviation (n=3 biologically independent experiments) indicate average fold changes relative to the no treatment condition. Statistical significance was calculated using one-way ANOVA with Bonferroni correction. Asterisks indicate p values * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$.



Supplementary Fig. 5 KEGG analysis of pathways regulated by nitrite (a) or nitrate (b). Red color shows the genes that have reduced (by three-fold) transcription level in nitrite- or nitrate-treated samples compared to untreated sample. Blue color shows the genes that have elevated (by three-fold) transcription level in nitrite- or nitrate-treated samples compared to untreated sample.

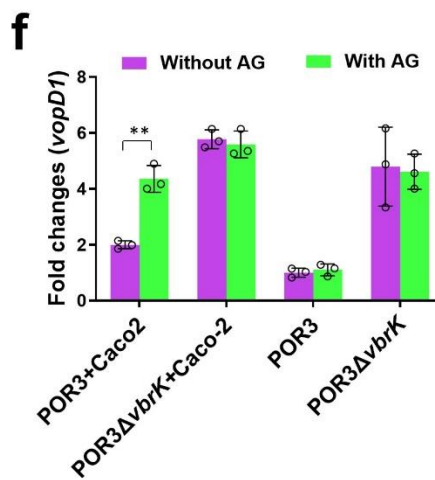
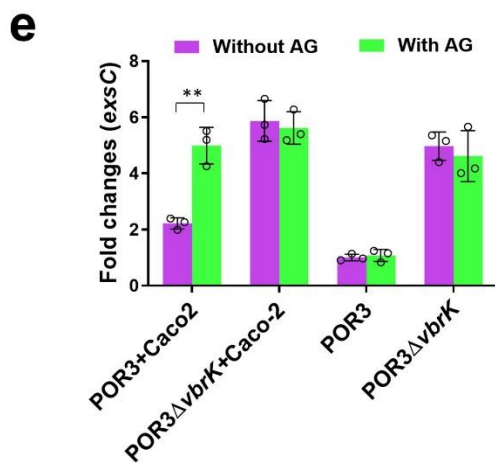
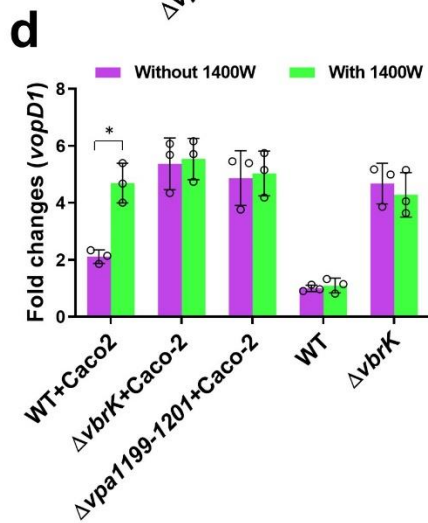
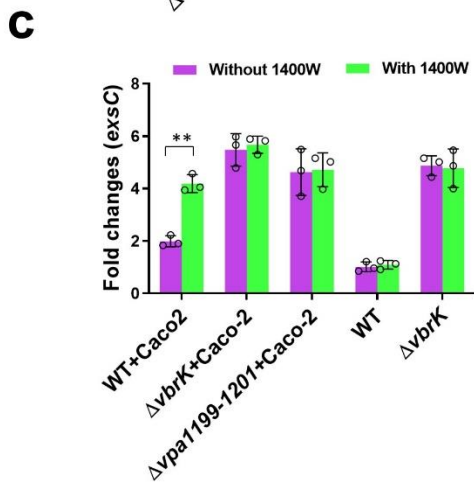
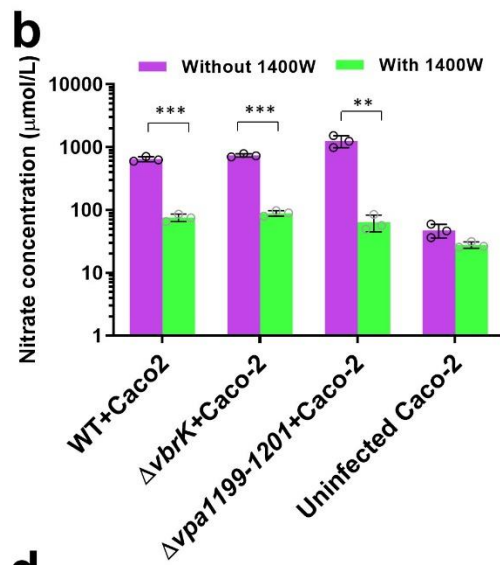
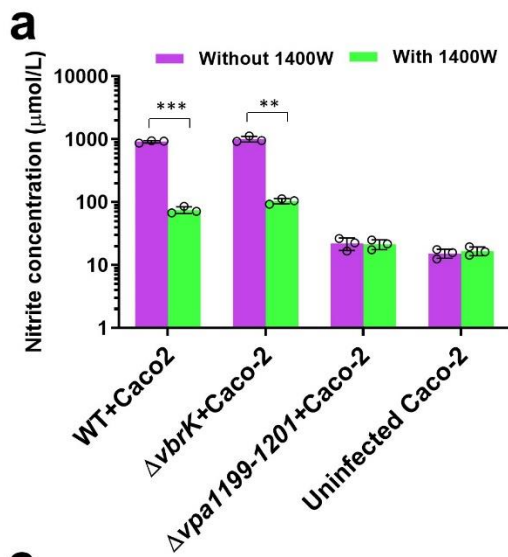


Supplementary Fig. 6 Nitrate affects *exsC* expression under anaerobic condition.

(a) Indicated strains of *V. parahaemolyticus* carrying P_{exsC} -lacZ construct were cultured under aerobic condition in the presence or absence of nitrate. Bars indicate average lacZ activity.

(b) Indicated strains of *V. parahaemolyticus* carrying P_{exsC} -lacZ construct were cultured under anaerobic condition in the presence or absence of nitrate and bars indicate average lacZ activity.

(a, b) All error bars represent mean $\pm$ standard deviation (n=3 biologically independent experiments). Statistical significance was calculated using two-tailed multiple *t* test with Bonferroni correction. Asterisks indicate p values **P* < 0.05, ***P* < 0.005, and ****P* < 0.0005.



Supplementary Fig. 7 Host-derived nitrite represses T3SS1 gene expression during infection.

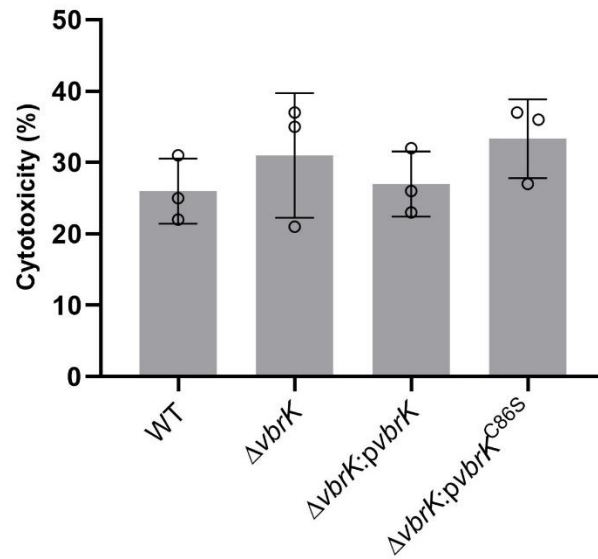
(a) Nitrite concentration measured in the culture medium of Caco-2 cells infected with the indicated strains in the presence or absence of 1400W. All error bars represent mean $\pm$ standard deviation (n=3 biologically independent experiments).

(b) Nitrate concentration measured in the culture medium of Caco-2 cells infected with the indicated strains in the presence or absence of 1400W. All error bars represent mean $\pm$ standard deviation (n=3 biologically independent experiments).

(c, d) Quantitative RT-PCR analysis of T3SS1 gene expression in *V. parahaemolyticus* during infection. Caco-2 cells were infected with WT (WT+Caco-2), $\Delta vbrK$ ($\Delta vbrK$ +Caco-2) or $\Delta vpa1199-1201$ ($\Delta vpa1199-1201$ +Caco-2) and RNA was collected from the infected samples for qRT-PCR analysis of *exsC* (C) and *vopD1* (D). Transcripts of these two genes in WT and $\Delta vbrK$ in the LB medium were also included as controls. Bars indicate average fold changes relative to WT under LB growth condition without 1400W treatment. All error bars represent mean $\pm$ standard deviation (n=3 biologically independent experiments).

(e, f) Caco-2 cells were infected with POR3 (POR3+Caco-2) and POR3 $\Delta vbrK$ (POR3 $\Delta vbrK$ +Caco-2) and RNA was collected from the infected samples for qRT-PCR analysis of *exsC* (E) and *vopD1* (F). Bars indicate average fold changes relative to POR under LB growth condition without 1400W treatment. All error bars represent mean $\pm$ standard deviation (n=3 biologically independent experiments).

(a-f) Statistical significance was calculated using two-tailed multiple *t* test with Bonferroni correction. Asterisks indicate p values **P* <0.05, ***P* <0.005, and ****P* < 0.0005.



Supplementary Fig. 8. Cytotoxicity of indicated *V. parahaemolyticus* strains to Caco-2 cells under anaerobic condition. Caco-2 cells were infected for 4 hours and LDH assay was performed to measure the cytotoxicity. All error bars represent mean $\pm$ standard deviation (n=3 biologically independent experiments).

Supplementary Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
<i>E. coli</i>		
DH5α <i>λpir</i>	For plasmid propagation	Laboratory collection
SM10 <i>λpir</i>	For plasmid conjugation	Laboratory collection
BL21(DE3)	Host strain for protein expression	Laboratory collection
pET28a:vp2210/BL21	BL21 carrying pET28a that expresses <i>vp2210</i>	This study
<i>V. parahaemolyticus</i>		
RIMD 2210633	WT	Laboratory collection
POR3	WT with deletion of T3SS2 and TDH	Laboratory collection
WT:pexsA	WT expressing the <i>exsA</i> gene	Laboratory collection
Δ <i>vbrK</i>	WT with in-frame deletion of <i>vbrK</i>	34
Δ <i>vbrR</i>	WT with in-frame deletion of <i>vbrR</i>	34
Δ <i>vbrR</i> /Δ <i>vp2210</i>	WT with in-frame deletion of <i>vbrR</i> and <i>vp2210</i>	This study
Δ <i>vpa1199-1201</i>	WT with in-frame deletion of <i>vpa1199</i> , <i>vpa1200</i> , and <i>vpa1201</i>	This study
Δ <i>vbrK</i> : <i>pvbrK</i> _6xHis	Δ <i>vbrK</i> harboring pMMB207 that expresses <i>vbrK</i> _6xHis gene	34
Δ <i>vbrR</i> : <i>pvbrR</i> _6xHis	Δ <i>vbrR</i> harboring pMMB207 that expresses <i>vbrR</i> _6xHis	34
Δ <i>vbrK</i> : <i>pvbrK</i> ^{H286A} _6xHis	Δ <i>vbrK</i> harboring pMMB207 that expresses <i>vbrK</i> ^{H286A} _6xHis	34
Δ <i>vbrR</i> : <i>PvbrR</i> ^{D51A} _6xHis	Δ <i>vbrR</i> harboring pMMB207 that expresses <i>vbrR</i> ^{D51A} _6xHis	34
Δ <i>vbrK</i> : <i>PvbrK</i> ^{C86S} _6xHis	Δ <i>vbrK</i> harboring pMMB207 that expresses <i>vbrK</i> ^{C86S} _6xHis	This study
Δ <i>vbrK</i> Δ <i>exsA</i>	WT with in-frame deletion in <i>vbrK</i> and <i>exsA</i>	This study
Δ <i>vbrK</i> Δ <i>exsC</i>	WT with in-frame deletion in <i>vbrK</i> and <i>exsC</i>	This study
Δ <i>vbrK</i> : <i>pexsD</i>	Δ <i>vbrK</i> harboring pMMB207 that expresses <i>exsD</i> _6xHis gene	This study
Δ <i>vbrR</i> : <i>pexsD</i>	Δ <i>vbrR</i> harboring pMMB207 that expresses <i>exsD</i> _6xHis gene	This study
WT/ <i>exsC</i> _6xHis	6xHis tag inserted at the C-terminus of <i>exsC</i> in the genome of WT	This study
Δ <i>vbrR</i> / <i>exsC</i> _6xHis	6xHis tag inserted at the C-terminus of <i>exsC</i> in the genome of Δ <i>vbrR</i>	This study

<i>ΔvbrK/exsC_6xHis</i>	6xHis tag inserted at the C-terminus of <i>exsC</i> in the genome of <i>ΔvbrK</i>	This study
VP0055-his/WT	WT harboring pMMB207 that expresses <i>vp0055</i> -6xHis gene	This study
VP0404-his/WT	WT harboring pMMB207 that expresses <i>vp0404</i> -6xHis gene	This study
VP2210-his/WT	WT harboring pMMB207 that expresses <i>vp2210</i> -6xHis gene	This study
VP2232-his/WT	WT harboring pMMB207 that expresses <i>vp2232</i> -6xHis gene	This study
VP-2358-his/WT	WT harboring pMMB207 that expresses <i>vp2358</i> -6xHis gene	This study
VP2553-his/WT	WT harboring pMMB207 that expresses <i>vp2553</i> -6xHis gene	This study
VP2578-his/WT	WT harboring pMMB207 that expresses <i>vp2578</i> -6xHis gene	This study
VP2670-his/WT	WT harboring pMMB207 that expresses <i>vp2670</i> -6xHis gene	This study
VP2953-his/WT	WT harboring pMMB207 that expresses <i>vp2953</i> -6xHis gene	This study
VPA1690-his/WT	WT harboring pMMB207 that expresses <i>vpa1690</i> -6xHis gene	This study
<i>P_{exsC}</i> -lacZ/WT	WT harboring pDM8 that contains the fusion of WT <i>exsC</i> promoter with promoterless <i>lacZ</i> gene	This study
<i>P_{exsC}</i> -lacZ/ <i>ΔvbrR</i>	<i>ΔvbrR</i> harboring pDM8 that contains the fusion of WT <i>exsC</i> promoter with promoterless <i>lacZ</i> gene	This study
<i>P_{exsC}</i> -lacZ/ <i>ΔvbrK</i>	<i>ΔvbrK</i> harboring pDM8 that contains the fusion of WT <i>exsC</i> promoter with promoterless <i>lacZ</i> gene	This study
<i>P_{exsCΔ50}</i> -lacZ/WT	WT harboring pDM8 that contains the fusion of mutant <i>exsC</i> promoter (<i>exsCΔ50</i>) with promoterless <i>lacZ</i> gene	This study
<i>P_{exsCΔ50}</i> -lacZ/ <i>ΔvbrR</i>	<i>ΔvbrR</i> harboring pDM8 that contains the fusion of mutant <i>exsC</i> promoter (<i>exsCΔ50</i>) with promoterless <i>lacZ</i> gene	This study
<i>P_{exsCΔ50}</i> -lacZ/ <i>ΔvbrK</i>	<i>ΔvbrK</i> harboring pDM8 that contains the fusion of mutant <i>exsC</i> promoter (<i>exsCΔ50</i>) with promoterless <i>lacZ</i> gene	This study
<i>P_{exsC}</i> -lacZ/ <i>ΔvbrK/Δvp2210</i>	<i>ΔvbrK/Δvp2210</i> harboring pDM8 that contains the fusion of WT <i>exsC</i> promoter with promoterless <i>lacZ</i> gene	This study
<i>P_{exsC}</i> -lacZ/ <i>Δvpa1199-1201</i>	<i>Δvpa1199-1201</i> harboring pDM8 that contains the fusion of WT <i>exsC</i> promoter with promoterless <i>lacZ</i> gene	
Plasmids		
pDM4	Suicide vector (<i>pir</i> dependent, R6K, <i>SacBR</i>) for making gene deletion mutant	Laboratory collection
pMMB207	Plasmid for complementation of the mutant	Laboratory collection
pDM8	Plasmid with promoterless <i>lacZ</i> gene	Laboratory collection

Supplementary Table 2 Primers used in this study

Primer name	Primer sequence (5' to 3')	Function
VPA1199-1201-UP-F:	GCTTATCGATACCGTCGACCCTCGATAGAACTGGGAAAAGTAGCACAACC	For deletion of vpa1199-1201
VPA1199-1201-UP-R	TTTGTCTCCTCACAAACGCACGTCTTGTCATTTTC	For deletion of vpa1199-1201
VPA1199-1201-DOWN-F	TGCGTTTGTGAGGAGACAAACCATGAGTACT	For deletion of vpa1199-1201
VPA1199-1201-DOWN-R	CACTAGTGACGCGTACTCGAGCAATGCAGAACACAAAGAAGG	For deletion of vpa1199-1201
EMSA-FAM	TGCCTGCAGGTCGACGAT	For EMSA
<i>exsA</i> EMSA-F	TGCCTGCAGGTCGACGATACTACTCATTGAGAAGCCTG	For EMSA
<i>exsA</i> EMSA-R	TTTCTACCCTTCATAATTTT	For EMSA
<i>exsC</i> EMSA-F	TGCCTGCAGGTCGACGATACGTTCCACGTACAACACGT	For EMSA
<i>exsC</i> EMSA-R	AGAAACAGTCCTTTTGAGAA	For EMSA
<i>exsD</i> -EMSA-F	TGCCTGCAGGTCGACGATACTCAAAGCTATCGACGTCG	For EMSA
<i>exsD</i> -EMSA-R	CCCTTTTCGAACACTCCAGAA	For EMSA
<i>vp1677</i> -EMSA-F	TGCCTGCAGGTCGACGATTGTAAAAAATATGCGCAATG	For EMSA
<i>vp1677</i> -EMSA-R	TCACTTGCACTCCTTTTCGTA	For EMSA
<i>gyrB</i> EMSA-F	TGCCTGCAGGTCGACGATGCGAAAGAAGTGGGTGCAAG	For EMSA
<i>gyrB</i> EMSA-R	TTTGATGTACGCGCCCTCTT	For EMSA
ExsC-his1	GCTTATCGATACCGTCGACCCTCGACACGATATCGAATATGTTTCCCGAT	For insertion of 6xHis to the C-terminus of <i>exsC</i> in the genome
ExsC-his2	AAGACACCTAGTGATGATGATGATGATGAACTCTCAGATCTAACTTTGAGGC	For insertion of 6xHis to the C-terminus of <i>exsC</i> in the genome
ExsC-his3	TCATCATCACTAGGTGTCTTATGTCTAATGACATC	For insertion of 6xHis to the C-terminus of <i>exsC</i> in the genome
ExsC-his4	CACTAGTGACGCGTACTCGATGCTGGATCACTACCAGAATACTAA	For insertion of 6xHis to the C-terminus of <i>exsC</i> in the genome
VP1662-RT-F	CACCAAAGCCAGCACTAGGA	For RT-PCR
VP1662-RT-R	TGTTTCCCACGAAGACTCGG	For RT-PCR
VP1668-RT-F	TCTTGCGGTTTCGCTCCATAG	For RT-PCR
VP1668-RT-R	CGCCAATCAGAGCAAGAACG	For RT-PCR
VP1670-RT-F	GAACTCGCGGCTGCTAAAAC	For RT-PCR

VP1670-RT-R	CAGGATGAGAGGCGAGTGAC	For RT-PCR
VP1695-RT-F	CAGTGCGGTTCTTTTCCAGC	For RT-PCR
VP1695-RT-R	GTGAATCGGGCATCGAGCTA	For RT-PCR
VP1696-RT-F	CCTAGGTACGGAATGTCGCC	For RT-PCR
VP1696-RT-R	GATCCCAGATGGTGGTGTGG	For RT-PCR
VP1687-RT-F	GCCAACAAAACCGACTTCCTC	For RT-PCR
VP1687-RT-R	TGATTGACCAAGACACGGCA	For RT-PCR
VP1686 (VopS)-RT-F	AAACTCGCAACGTTGGCTTC	For RT-PCR
VP1686 (VopS)-RT-R	ACTACCGCGAAAGTGCTGAA	For RT-PCR
VP1656 (VopD1)-RT-F	CTGAGCTTGTCCCTGTTCGT	For RT-PCR
VP1656 (VopD1)-RT-R	GGCAAACCTCAGCATTGGTGG	For RT-PCR
<i>gyrB</i> -RT-F	TGACAGCCGTTGTTTCGGTA	For RT-PCR
<i>gyrB</i> -RT-R	AGTCTGCAAGTTTGCCTGGT	For RT-PCR
exsCEMSA23-F	TGCCTGCAGGTCGACGATTTAACATAGTATTTAATTTTAAGA	For EMSA
exsCEMSA48-F	TGCCTGCAGGTCGACGATTAACACAATTCTAATTCTTCATCGC	For EMSA
exsCEMSA73-F	TGCCTGCAGGTCGACGATTTGTGATATAAACCAAAATCCAC	For EMSA
exsCEMSA98-F	TGCCTGCAGGTCGACGATCATAATAGAATTTAATAACCGTTAA	For EMSA
exsCEMSA123-F	TGCCTGCAGGTCGACGATTCTTTATATGCACCAGCACTCATTT	For EMSA
exsCEMSA148-F	TGCCTGCAGGTCGACGATGTAAGATTAGAAGCAAGGAGTCAAT	For EMSA
exsCEMSA173-F	TGCCTGCAGGTCGACGATGGTTTCCGGCCAAAATATAA	For EMSA
exsCEMSA198-F	TGCCTGCAGGTCGACGATATAACTAACAACCAAAATTG	For EMSA
exsCEMSA165-F	TGCCTGCAGGTCGACGATGAGTCAATTCTGGGTTTCCG	For EMSA
exsCEMSA111-R	TAAATTCTATTATGGTGGGA	For EMSA
Vp0055-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGGC AACGACTTCAGCCCTA	For vp0055 overexpression
Vp0055-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGTGC GTGTGCCATTCTTGATTG	For vp0055 overexpression
VP0404-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGGAT CAAAATCCGCAGTCAC	For vp0404 overexpression
VP0404-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGTTCG TCGAGGAAGCTGCGCAG	For vp0404 overexpression
VP2210-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGGAG GCTCCTGTGGCCATA	For vp2210 overexpression

VP2210-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGTCCA TTTTTTTGTCTCT	For vp2210 overexpression
VP2232-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAGTGAATA AAGCGATAACCTATG	For vp2232 overexpression
VP2232-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGGTCAT TTTGTGTCCACGC	For vp2232 overexpression
VP2358-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGATGAG TGATAGCCCACAA	For vp2358 overexpression
VP2358-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGTAGTTG CTCCGTATGCATTG	For vp2358 overexpression
VP2553-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGAGTATC AGCAACACAGTA	For vp2553 overexpression
VP2553-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGGGATTT CACGAAGTCGACG	For vp2553 overexpression
VP2578-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGAACGAG CAGCTGACCGATC	For vp2578 overexpression
VP2578-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGGCGCTGT AAAAGAGGTTTG	For vp2578 overexpression
VP2670-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGAAACCTT CATTACAACCTC	For vp2670 overexpression
VP2670-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGAAGTAGG CGTTTACGCTG	For vp2670 overexpression
VP2953-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGACAAAAG AAGCGTATCCG	For vp2953 overexpression
VP2953-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGGAATCAC CAACGGCCAGT	For vp2953 overexpression
VPA1690-his-F	AGCTCGGTACCCGGGGATCCTCTAGTAAGGAGGTAGGATAATAATGGAAAAACA AAAACGTTACG	For vpa1690 overexpression
VPA1690-his-R	TCTCATCCGCCAAAACAGCCAAGCTTTAGTGATGATGATGATGATGATGGTTTCC TTGCTGCGCG	For vpa1690 overexpression
PexsC-lacZ-F	ATTGCTGCAGGTCGACGGATCCGGGAATTATTCTACAGATGGACAGCAATAACG	For construction exsC promoter-LacZ fusion
PexsC-lacZ-R	GTCGACCTGCAGCCCAAGCTTATCGATTGAGAAACAGTCCTTTTGAGAATTTTA	For construction exsC promoter-LacZ fusion

PexsCΔ50-lacZ-2	ATTCTATTATGATCTTAAAAATTAAATACTATG	For construction <i>exsCΔ50</i> promoter-LacZ fusion
PexsCΔ50-lacZ-3	ATTTTAAAGATCATAATAGAATTAAATAACCGT	For construction <i>exsCΔ50</i> promoter-LacZ fusion
