Supplementary Materials

Gain-of-function mutations in acid stress response (*evgS*) protect *Escherichia coli* from killing by gallium nitrate, an antimicrobial candidate

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Supplementary References

Table S1. Bacterial strains, plasmids, and MIC of gallium nitrate (GaNt) used in this study

Strains	Relevant genotype	Source and description	GaNt MIC, mM with LB medium
1	Wildtype	*E. coli* BW25113	18.4 (12.2)[a]
596	*ΔevgA*	This study, by kanamycin marker excision	18.4
597	*evgS* E701G	This study, constructed using CRISPR-cas9	18.4 (12.2)[a]
598	*evgS* S582P	This study, constructed using CRISPR-cas9	18.4
603	*arpA* E334A	This study, constructed using CRISPR-cas9	18.4
608	*ΔevgS*	This study, by kanamycin marker excision	18.4
610	GaNt-3	This study, GaNt-tolerant mutant 3	18.4
611	GaNt-4	This study, GaNt-tolerant mutant 4	18.4
612	GaNt-5	This study, GaNt-tolerant mutant 5	18.4
613	GaNt-6	This study, GaNt-tolerant mutant 6	18.4
636	*kdpD* P885T- *arpA* E334A	This study, constructed using CRISPR-cas9	18.4
638	*kdpD* P885T	This study, constructed using CRISPR-cas9	18.4
1044	*evgS* G658A	This study, constructed using CRISPR-cas9	18.4
1045	*evgS* S584F	This study, constructed using CRISPR-cas9	18.4
1046	*evgS* N573K	This study, constructed using CRISPR-cas9	18.4
1047	*evgS* S600I	This study, constructed using CRISPR-cas9	18.4
1048	*ΔgadE::kan,* Kan^R	CGSC# 11776, Keio collection JW3480	18.4
1049	*ΔydeO*	This study, constructed using CRISPR-cas9	18.4
1050	*evgS* E701G-*ΔydeO*	This study, constructed using CRISPR-cas9	18.4
1051	*evgS* E701G-*ΔgadE*	This study, constructed using CRISPR-cas9	18.4
1052	*ΔrpoS*	This study, constructed using CRISPR-cas9	18.4
1053	*evgS* E701G-*ΔrpoS*	This study, constructed using CRISPR-cas9	18.4
1054	*ΔsafA*	This study, constructed using CRISPR-cas9	18.4
1055	*evgS* E701G-*ΔsafA*	This study, constructed using CRISPR-cas9	18.4
1056	*evgA* D52A	This study, constructed using CRISPR-cas9	18.4
1057	*evgS* E701G-*evgA* D52A	This study, constructed using CRISPR-cas9	18.4

Plasmids

pCas		Kan^R, *repA*101(temperature-sensitive replicon), *lac*Iq-Ptrc fragment, sg-RNA-pMBI,λ-Red recombinase gene (*exo bet gam paraB*) (1).	
pTargetF		*aadA* (spec^R), pMB1, pj23119 (synthetic promoter) N20-sgRNA (1).	
pTargetF-sgRNA-FRT		spec^R, sgRNA with an N20 sequence targeting the *FRT* region of Keio collection mutants after antibiotic marker excision.	

pTargetF-sgRNA-*ydeO*	spec[R], sgRNA with an N20 sequence targeting the *ydeO* gene of the wildtype strain, also containing homologous template for *ydeO* knockout
pTargetF-sgRNA-*gadE*	spec[R], sgRNA with an N20 sequence targeting *gadE* gene of the wildtype strain, alsp containing homologous template for *gadE* knockout.
pTargetF-sgRNA-*safA*	spec[R], sgRNA with an N20 sequence targeting *safA* gene of the wildtype strain, alsp containing homologous template for *safA* knockout.
pTargetF-sgRNA-*rpoS*	spec[R], sgRNA with an N20 sequence targeting *rpoS* gene of the wildtype strain, alsp containing homologous template for *ropS* knockout.
pCP-20	plasmid containing yeast FLp recombinase gene, ts-*rep* (30°C), *recA*[-], Amp[R] (100 μg/ml), Cm[R] (25 μg/ml). pCP20 was used for excision of antibiotic marker from in-frame gene knockout mutant (2, 3).

Abbreviations: Cm[R], chloramphenicol resistance; Tc, tetracycline resistance; *aadA* (spec), spectinomycin resistance; Amp[R]: ampicillin resistance; Kan[R]: kanamycin resistance

[a]Gallium nitrate MIC for MH medium is shown in parentheses; MIC was determined after correction for water content of gallium nitrate powder; MIC values were 4.7 mg/ml and 3.1 mg/ml with LB and MH media, respectively.

Table S2. Primers used in the study

Primer name	Purpose	Nucleotide sequence of primers (5'-3')
evgA (D52A)-P1	Construction of mutant *evgA* D52A using CRISPR system; amplification of upstream homologous sequence by PCR from wild-type strain	AATAGGCATAGGCACCTGAAAACGC
evgA (D52A)-P2		GTTAACTCCGGGGATATCGACAGCAATGAT GACGATATCAGGCTT
evgA (D52A)-P3	Construction of mutant *evgA* D52A using CRISPR system; amplification of downstream homologous sequence by PCR from wild-type strain	AAGCCTGATATCGTCATCATTGCTGTCGAT ATCCCCGGAGTT
evgA (D52A)-P4		TCGTCTGGGGGGTAATTTGCTA
kdpD-P1	Construction of mutant *kdpD* P885T using CRISPR system; amplification of homologous *kdpD* template by PCR from GaNt-3	TGACCACCGTACTGGGGCAATG
kdpD-P2		ATTGTTGAGCAGCACCGCCAGC
kdpD-check-P1	Amplification and sequencing of *kdpD* for mutation confirmation	TGACCACCGTACTGGGGCAATG
kdpD-check-P2		GCAGATGTCCCATATAAATACGCA
arpA-P1	Construction of mutant *arpA* Q334G using CRISPR system; amplification of homologous *arpA* template by PCR from GaNt-3	GCTCTAGACGCAAAGGGTTACATGCCTA
arpA-P2		AACTGCAGATGTCTGCCGCTCACGTTC
arpA-check-P1	Amplification and sequencing of *arpA* for mutation confirmation	GCCTGCTAACCACGATGCAA
arpA-check-P2		AGCTACCGCGAATATCTCGTT
FRT-sgRNA-5'	Construction of sgRNA targeting the FRT sequence of Keio mutants for backcrossing mutant alleles into the corresponding Keio mutant strain	TCCTAGGTATAAT**ACTAGT**TCCTATTCTCTA GAAAGTATGTTTTAGAGCTAGAAATAGC (SpeI)
FRT-sgRNA-3'		AA**CTGCAG**TTCAAAAAAAGCACCGACTC GG (PstI)
evgA D52A-*evgS* E701G-check 1	Amplification (with *evgA* D52A-*evgS* E701G-P2 primer) and sequencing of *evgA* D52A-*evgS* E701G for mutation confirmation	TTGATGACCATCCTCTTGCTA
evgA D52A-*evgS* E701G-P1	Construction of *evgA* D52A mutation in *evgS* E701G background using CRISPR system; amplification of upstream homologous fragment with *evgA* D52A-*evgS* E701G-P1 plus *evgA*	TTGCTGTCGATATCCCCGGAGTT
evgA D52A-*evgS* E701G-P2		TCTCTTGCGGATCTATGCGGCGGTG

	(D52A)-P2 and downstream homologous fragment with *evgA* (D52A)-P3 plus *evgA* D52A-*evgS* E701G-P2 by PCR from *evgS* E701G mutant template. The *evgA* D52A-*evgS* E701G-P1 and *evgA* D52A-*evgS* E701G-P2 primer pair was used to amplify full-length homologous template after annealing the upstream and downstream homologous fragments.	
evgS-P1	Shared upstream primer for pairing with various *evgS* P2 primers to amplify upstream *evgS* fragment for construction of various *evgS* point mutations using CRISPR system	TAGCAGAGTTGACTGAAGGCG
evgS-P4	Shared downstream primer for pairing with various *evgS* P3 primers to amplify downstream *evgS* fragment for construction of various *evgS* point mutations using CRISPR system	GCCGATTACGATTTGTTGTGG
evgS S584F-P2	Primers paired with *evgS*-P1 for amplification of upstream *evgS* S584F, evgS N573K, and *evgS* S600I fragments used for subsequent full-length *evgS* mutant template construction needed for CRISPR-based allelic exchange	GGATTCGGTAAGAAATCCGAGAGTGC
evgS N573K-P2		CGGAATGATATTTGTTTTTCTAAATCACCC
evgS S600I-P2		GCACTATTATGAATAATGACATTACC
evgS S584F-P3	Primers paired with *evgS*-P4 for amplification of downstream *evgS* S584F, *evgS* N573K, and *evgS* S600I fragments used for subsequent full-length *evgS* mutant template construction needed for CRISPR-based allelic exchange	GCACTCTCGGATTTCTTACCGAATCC
evgS N573K-P3		GGGTGATTTAGAAAAACAAATATCATTCCG
evgS S600I-P3		GGTAATGTCATTATTCATAATAGTGC
gadE-sgRNA-5'	Amplification of *gadE*-sgRNA for CRISPER-based mutant construction	GG**ACTAGT**GATTTTTGATACTTTCTTTGGT TTTAGAGCTAGAAATAGC (SpeI)

gadE-sgRNA-3'		CTCAAAAAAAGCACCGACTCGG
gadE-P1	Primer sequence for PCR amplification of homologous template upstream of *gadE* for CRISPR-based *gadE* mutant construction	CGAGTCGGTGCTTTTTTTGAGCTGAAGAC ATGAATGCG
gadE-P2		AGTTGCTTATGTCCTGACTACATAACTTGC TCCTTAGCCG
gadE-P3	Primer sequence for PCR amplification of homologous template downstream of *gadE* for CRISPR-based *gadE* mutant construction	CGGCTAAGGAGCAAGTTATGTAGTCAGGA CATAAGCAACTG
gadE-P4		AA**CTGCAG**GTTTTCCGCCGATTTGTCAT (pstI)
gadE-CRISPR-check-P1	Primer (paired with *gadE*-P4) for PCR confirmation of *gadE* deletion	GGGTTCATTTTTTTGCAACTGGCG
evgS-PAS-check-P1	Primer pair for amplification and sequencing confirmation of various *evgS* PAS domain for mutations	GCTGAAGCCTTCATAACAACG
evgS-PAS-check-P2		TAACAGCGTGGTTGTCATCA
ydeO-SgRNA-P1	Amplification of *ydeO*-sgRNA for CRISPER-based mutant construction	GG**ACTAGT**TGTAATGAAACCTTTGCATGG TTTTAGAGCTAGAAATAGC
ydeO-SgRNA-P2		CTCAAAAAAAGCACCGACTCGG
ydeO-P1	Primer sequence for PCR amplification of homologous template upstream of *ydeO* for CRISPR-based *yedO* mutant construction	CCGAGTCGGTGCTTTTTTTGAGCGTTATCC GCTAAATAAACA
ydeO-P2		TACTCGTTAGCAAATAATCACATTTTATCTC CTTAAAACA
ydeO-P3	Primer sequence for PCR amplification of homologous template downstream of *ydeO* for CRISPR-based *ydeO* mutant construction	TGTTTTAAGGAGATAAAATGTGATTATTTG CTAACGAGTA
ydeO-P4		AA**CTGCAG**GTCCATAAAGCGTTGACATT (pstI)
ydeO-CRISPR- check-P1	Primer (paired with *ydeO*-P4) for PCR confirmation of *ydeO* deletion	ACACTTACGTTGATAGCCAT
safA-sgRNA-P1	Amplification of *safA*-sgRNA for CRISPER-based mutant construction	GG**ACTAGT**CTGATCAATTGCCGAAAAAAG TTTTAGAGCTAGAAATAGC (SpeI)
safA-sgRNA-P2		CTCAAAAAAAGCACCGACTCGG
safA-P1	Primer sequence for PCR amplification of homologous template upstream of *safA* for CRISPR-based *safA* mutant construction	CCGAGTCGGTGCTTTTTTTGAGCTATTTTC CAGAATCGAAT
safA-P2		GCTGTTTGTTTTCAGCCGGTCGCATGCATT TCAAATATG
safA-P3	Primer sequence for PCR amplification of homologous template	GAAATGCATGCGACCGGCTGAAAACAAAC AGCAAA

safA-P4	downstream of *safA* for CRISPR-based *safA* mutant construction	AA**CTGCAG**TCGCAGGAACAGGCA (PstI)
safA-CRISPR-check-P1	Primer (paired with *safA*-P4) for PCR confirmation of *safA* deletion	AGCTCACGCCGCATGGATAGATT
rpoS-sgRNA-P1	Amplification of *rpoS*-sgRNA for CRISPER-based mutant construction	GG**ACTAGT**GCAAACGAATAGTACGGGTTG TTTTAGAGCTAGAAATAGC (SpeI)
rpoS-sgRNA-P2		CTCAAAAAAAGCACCGACTCGG
rpoS-P1	Primer sequence for PCR amplification of homologous template upstream of *rpoS* for CRISPR-based *rpoS* mutant construction	GTCGGTGCTTTTTTTGAGCGCAGATGGCC GCGTTGTTTATGC
rpoS-P2		GACAGATGCTTACTTACATAAGGTGGCTCC TAC
rpoS-P3	Primer sequence for PCR amplification of homologous template downstream of *rpoS* for CRISPR-based *rpoS* mutant construction	CACGGGTAGGAGCCACCTTATGTAAGTAA GCATCTGTCAGA
rpoS-P4		AA**CTGCAG**ACTCTCAGAAGCGGTGATGG GA (PstI)
rpoS-CRISPR-check-P1	Primer (paired with *rpoS*-P4) for PCR confirmation of *rpoS* deletion	CAAGGGGATTGATATCGCAGGCA

Table S3. Primers used for RT-PCR

Primer[a]	Nucleotide sequence of primers (5'-3')
ahpC-F	TTCTACCCGGCTGACTTTACTT
ahpC-R	CTTCATCTTCACGCATGTTGTC
ahpF-F	ACATCGAACTGGCCGGTATTT
ahpF-R	GGTGCGAATCAGGTAGTCAAA
katE-F	ATGCCAAACTGCTCTACTCCC
katE-R	CAGCGCAATCGGTTTAAGGT
oxyR-F	ACCTGCTACCGCATATTATCCCT
oxyR-R	CTCATCAAACAACGGCACTTCA
soxR-F	TATCCGTAACAGCGGCAATCAG
soxR-R	CCACAACCAATACATCCGTCCAGT
soxS-F	TATCAAAAATCGGACGCTCGGTGGT
soxS-R	CAGGATCTTATCGCATGGATTGACG
acnA-F	CACGCATAAAGATCGCCAGC
acnA-R	CTTGAGGCCCAGAGTTACGG
acnB-F	GTTCTTCGCGTAAATCCGCC
acnB-R	CAATCACGTCGCCCATGTTC
icd-F	CTATCAGGGCACTCCAAGCC
icd-R	ACAATGTTCCGGGAAGCGAA
sucA-F	ATGGGACGAAGAGTACCCGA
sucA-R	GTAAGCGAGGTTTTCCGCAC
sucC-F	AGCCGCTTCAAAAATCGGTG
sucC-R	GGTTAACCGGTTGGCCATTG
sdhA-F	GCTGTTTCTGCCAACTTCCG

sdhA-R	AAGTACGAATCTTCGGCGGG
frdA-F	TCCACGCGACAAAGTCTCTC
frdA-R	TCTTTAACCGGATCGACGCC
frdD-F	CAAAGCGTTCTGACGAACCG
frdD-R	TACGCGACCAATGAAGCTCT
fumA-F	CGAAGATCAACCGTCAGGGG
fumA-R	GCCGTTAAGCGATAAGCGTG
fumB-F	GCGGTGATGAAGAAACGCTG
fumB-R	CCTTTCGCAACGCAAAGGAA
fumC-F	TTTCCGCATTTCGACGGAGA
fumC-R	GATAGCCAGCGGGAATTCGT
mdh-F	CCGGTTAACACCACAGTTGC
mdh-R	CACCAGAGTGACCGCCAATA
mqo-F	GAACTGAACTACACCCCGCA
mqo-R	ATTGACGTTATCCTCGCCCC
aceA-F	AGTGCCAACTCAGGAAGCTATT
aceA-R	TAAATTCGCTGTCATACGGGTC
aceB-F	TTCCGAGCAAAGATGAAGAGC
aceB-R	CGAGAATGTCGTTGAATACCG
16S-qPCR-F[b]	CTTACGACCAGGGCTACACAC
16S-qPCR-R[b]	CGGACTACGACGCACTTTATG

[a]F and R indicate forward and reverse, respectively

[b]16S ribosomal RNA

Table S4. Gallium nitrate concentration-dependent acidification of citrate-buffered LB medium

[Gallium nitrate] (mM)	pH (before/after)[a]
0	7.16/8.37
9.2	6.14/5.91
18.4[b]	5.60/5.55
36.8	4.88/4.86
55.2	4.25/4.19
73.6	3.20/3.16

[a]Indicating pH values measured before and after incubation of bacterial inoculum for MIC determination.

[b]Indicating MIC value determined with citrate (54.3 mM)-buffered LB medium.

Table S5. Gallium nitrate concentration-dependent acidification of citrate-buffered MH medium

[Gallium nitrate] (mM)	pH (before/after)[a]
0	7.25/8.29
6.1	6.45/6.40
12.2[b]	6.00/5.98
18.4	5.72/5.73
24.4	5.42/5.43
36.8	5.16/5.12
48.8	4.26/4.20
55.1	3.99/3.91

[a]Indicating pH values measured before and after incubation of bacterial inoculum for MIC determination.

[b]Indicating MIC value determined with citrate (54.3 mM)-buffered MH medium.
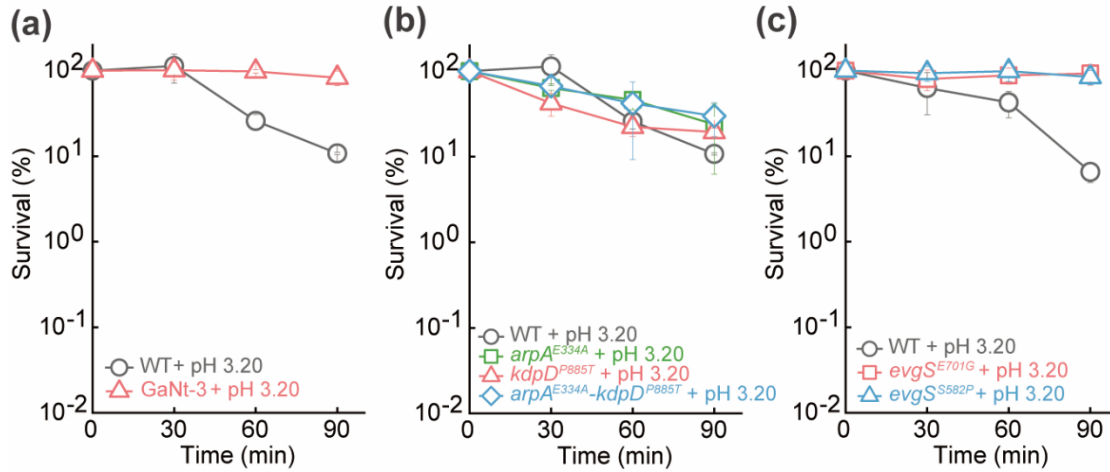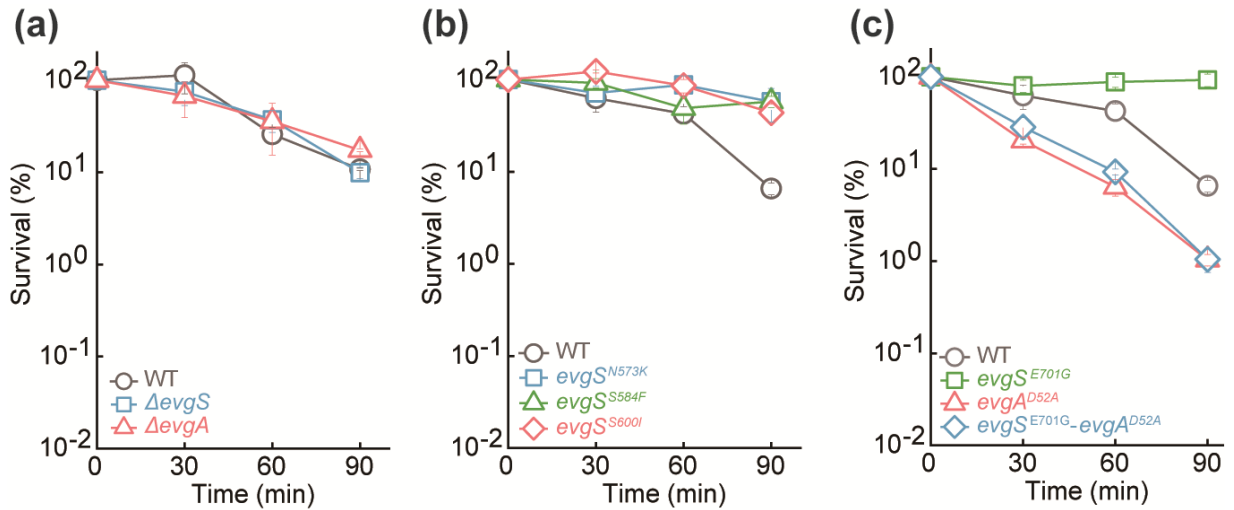
Figure S1. Killing of wild-type, GaNt-3, *arpA*, *kdpD*, *arpA-kdpD*, and *evgS* mutants by hydrochloric-acid-acidified (pH 3.20) medium. Exponentially growing cultures of *E. coli* were treated by adjusting the pH of the LB medium (supplemented with 54.3 mM sodium citrate) to 3.20 with hydrochloric acid and then incubated for the indicated times followed by determination of survival as colony forming units (CFU). Percent survival was determined relative to CFU at the time of acid addition. Panel *a*. Protection from acid-mediated killing by mutant GaNt-3. Panel *b*. Lack of protection from killing by mutations in *arpA* and *kdpD*. Panel *c*. Protection from acid-mediated killing by two *evgS* mutants. All experiments were performed in triplicate; error bars indicate standard error of the mean.
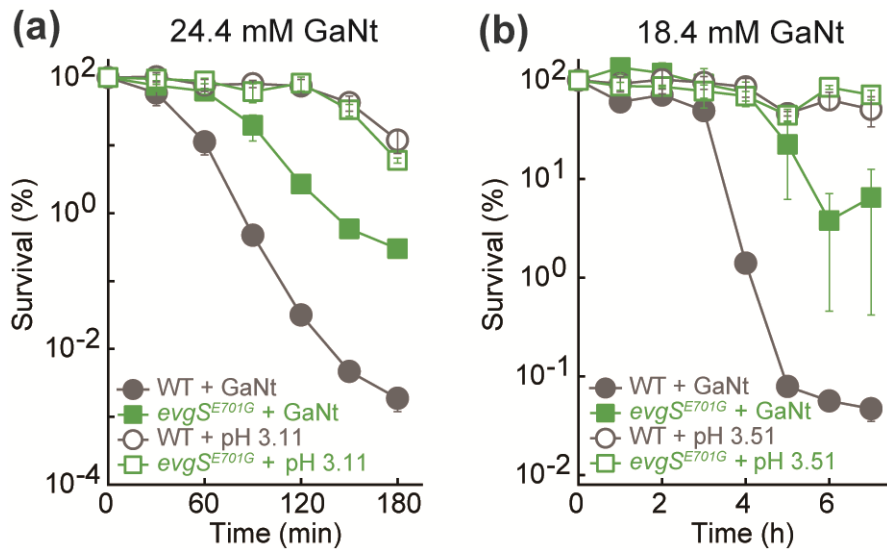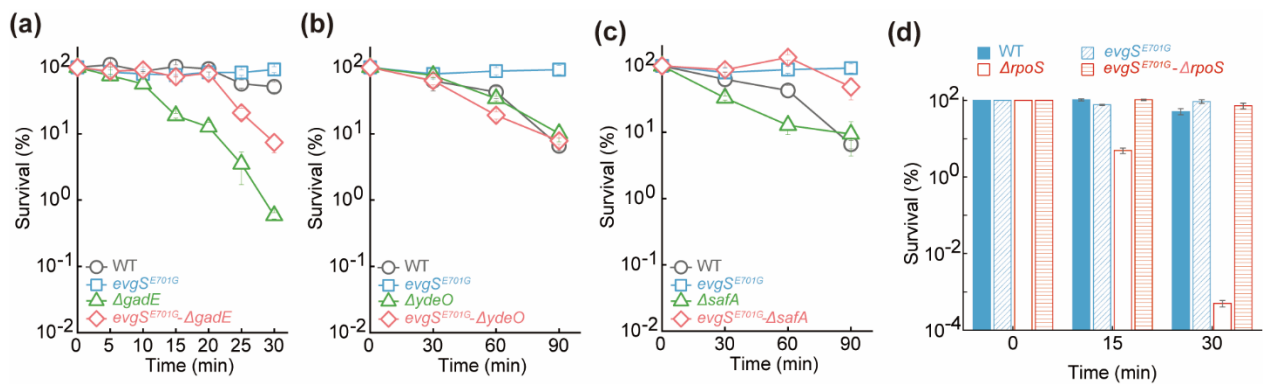
Figure S2. Killing of wild-type and *evgS* (E701G) mutants by gallium nitrate is more rapid and extensive than by hydrochloric-acid-acidified LB medium. Exponentially growing cultures of *E. coli* were treated either with 24.4 mM (panel *a*) or 18.4 mM (panel *b*) gallium nitrate or by adjusting the pH of the LB medium to 3.11 or 3.51 with hydrochloric acid as medium acidification controls for 24.4 mM and 18.4 mM gallium nitrate treatment, respectively. Samples were then incubated for the indicated times, followed by determination of survival as in Figure S1. All experiments were performed in triplicate; error bars indicate standard error of the mean.
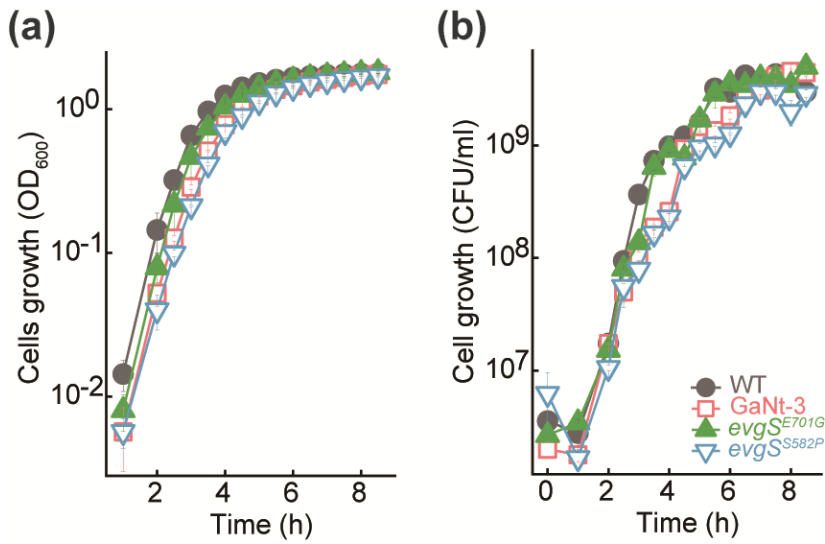
Figure S3. Bacterial growth rate is affected little by the GaNt-3, *evgS* (E701G), and *evgS* (S582P) mutations. Overnight cultures of *E. coli* were diluted by 2000-fold into fresh LB medium supplemented with 54.3 mM of sodium citrate. Cultures were incubated at 37 °C with rotary shaking at 250 rpm for 9 h. At the indicated times, aliquots were taken for determination of OD_{600} and colony forming units (CFU). Log of OD_{600} (*a*) or CFU (*b*) was plotted as a function of incubation time. All experiments were performed in triplicate; error bars indicate standard error of the mean.

Figure S4. Killing of wild-type, *evgA,* and various *evgS* mutants by hydrochloric-acid-acidified (pH 3.20) medium. Exponentially growing cultures of *E. coli* were treated by adjusting the pH of the LB medium (supplemented with 54.3 mM sodium citrate) to 3.20 with hydrochloric acid and then incubated for the indicated times followed by determination of survival as in Figure S1. Panel *a*. Survival is unaffected by deletion of *evgA* or *evgS*. Panel *b*. Three *evgS* point mutations slightly protect from acid-mediated killing. Panel *c*. Point mutation in *evgA* is moderately hypersensitive to acid-mediated killing; when combined with a point mutation in *evgS* the double mutant is as sensitive as the *evgA* single mutant to acid treatment. All experiments were performed in triplicate; error bars indicate standard error of the mean.

Figure S5. Killing of wild-type and various *evgS* (E701G), *gadE*, *ydeO*, *safA*, and *rpoS* mutants by hydrochloric-acid-acidified (pH 3.20) medium. Exponentially growing cultures of *E. coli* were treated by adjusting the pH of the LB medium (supplemented with 54.3 mM sodium citrate) to 3.20 with hydrochloric acid and then incubated for the indicated times followed by determination of survival as in Figure S1. Panel *a*. Deletion of *gadE* renders cells hypersensitive to acid, a feature that is partially reversed by a point mutation (E701G) in *evgS*. Little killing occurs with wild-type and the *evgS* (E701G) mutant. Panel *b*. An *evgS* point mutation, which by itself is protective, has no protective effect for a *ydeO* deletion. Panel *c*. Slight hypersensitivity to acid by a *safA* deletion mutant is reversed by an additional point mutation in *evgS*. Panel *d*. Hypersensitivity to acid by an *rpoS* deletion is reversed by a point mutation in *evgS*.

Supplementary references

1. Jiang Y, Chen B, Duan C, Sun B, Yang J, Yang S. 2015. Multigene editing in the *Escherichia coli* genome via the CRISPR-Cas9 system. Appl Environ Microbiol 81:2506-14.
2. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:2006 0008.
3. Cherepanov PP, Wackernagel W. 1995. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158:9-14.