Supplementary Material

A role for the periplasmic adaptor protein AcrA in vetting substrate access to the RND efflux transporter AcrB

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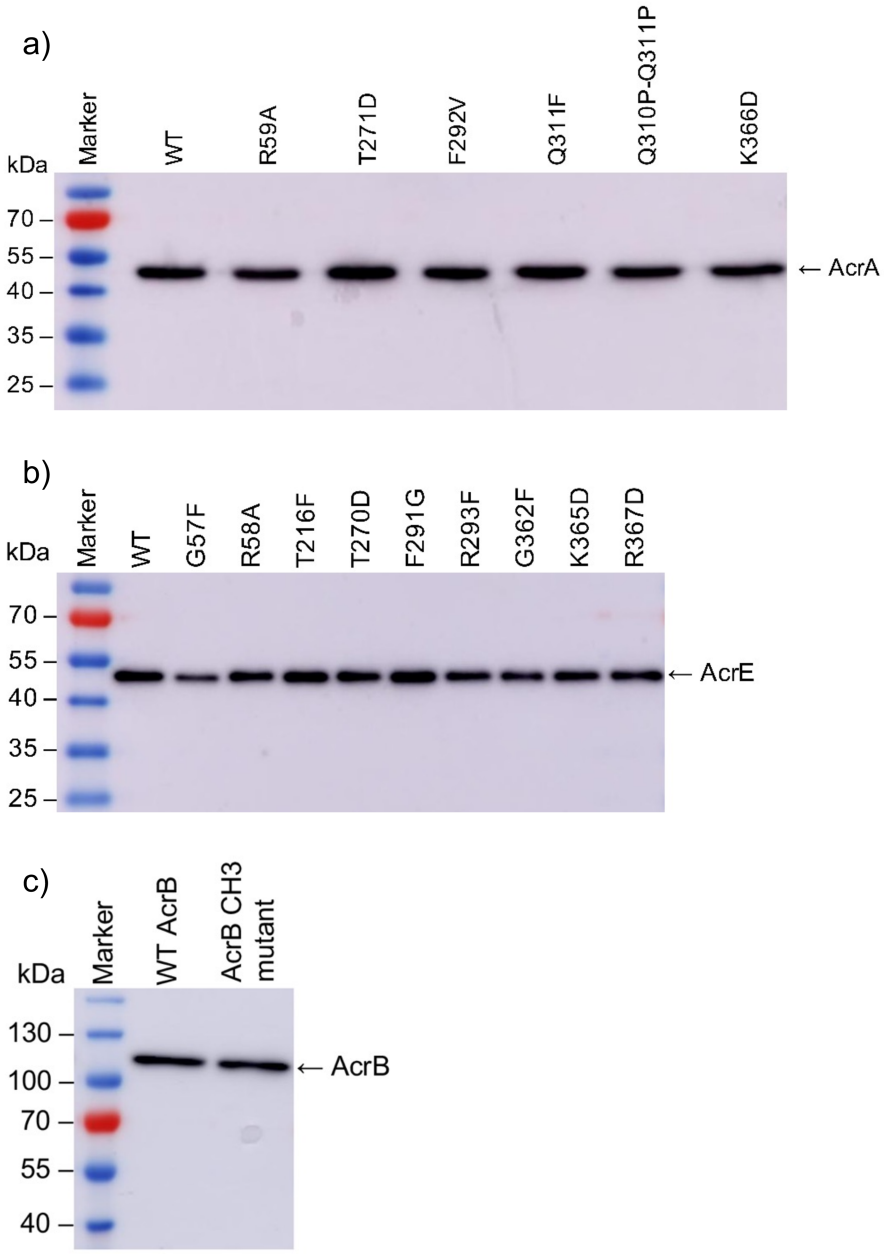

Figure S1. Western blotting of mutant proteins. a) Wild type and mutant AcrA were in expressed in the *Salmonella* Typhimurium SL1344 Δ4PAP strain from p*acrA* plasmids. **b)** Wild type and mutant AcrE were in expressed in the *Salmonella* Typhimurium SL1344 Δ4PAP Δ*acrF* strain from p*acrE* plasmids. **c)** Wild type and the AcrB channel 3 (CH3) mutant (A33W T37W N298W) were in expressed in the *Salmonella* Typhimurium SL1344 Δ4PAP Δ*acrB* strain from p*acrAB* plasmids. Membrane fractions were harvested, separated on a 12% SDS-PAGE gel for AcrA and AcrE or 8% SDS-PAGE gel for AcrB, and transferred to a PVDF membrane. The His-tagged proteins were blotted using anti 6x-His tag HRP-conjugated monoclonal antibody and detected using ECL substrate. PageRuler Prestained Protein Ladder (Thermo Scientific, USA) was used as a molecular weight marker.

Table S1. List of primers used for site-directed mutagenesis reactions.

Primer name	Primer sequence
AcrA_R59A_F	CTTCCGGGTGCTACCGTTGCTTACCGTATC
AcrA_R59A_R	GCAACGGTAGCACCCGGAAGTTCAGTTGTG
AcrA_T270A_F	GTTGACCAAGCCACCGGGTCTATTACTTTG
AcrA_T270A_R	CCCGGTGGCTTGGTCAACGGTCACGTCGG
AcrA_T270D_F	GACCGTTGACCAAGACACCGGGTCTATTAC
AcrA_T270D_R	GACCCGGTGTCTTGGTCAACGGTCACGTCG
AcrA_T271A_F	GTTGACCAAACCGCCGGGTCTATTACTTTGC
AcrA_T271A_R	GTAATAGACCCGGCGGTTTGGTCAACGGTC
AcrA_T271D_F	GTTGACCAAACCGACGGGTCTATTACTTTGC
AcrA_T271D_R	GTAATAGACCCGTCGGTTTGGTCAACGGTC
AcrA_G272A_S273A_F	CAAACCACCGCGGCTATTACTTTGCGCGCC
AcrA_G272A_S273A_R	GTAATAGCCGCGGTGGTTTGGTCAACGGTCAC
AcrA_F292V_F	CCAGGAATGGTCGTTCGCGCACGTCTGC
AcrA_F292V_R	CGAACGACCATTCCTGGCAATAAGGTGTG
AcrA_Q310P_Q311P_F	CTGGTTCCACCACCGGGCGTTACCCGTACTC
AcrA_Q310P_Q311P_R	GTAACGCCCGGTGGTGGAACCAGTAATGCCG
AcrA_Q311F_F	GTTCCACAATTCGGCGTTACCCGTACTCC
AcrA_Q311F_R	GTAACGCCGAATTGTGGAACCAGTAATGC
AcrA_P317G_F	GTTACCCGTACTGGACGCGGCGATGCCAC
AcrA_P317G_R	TCGCCGCGTCCAGTACGGGTAACGCCCT
AcrA_P317F_F	GTTACCCGTACTTTCCGCGGCGATGCCACG
AcrA_P317F_R	CGCCGCGGAAAGTACGGGTAACGCCCTGTTG
AcrA_R318F_F	CGTACTCCATTCGGCGATGCCACGGTGCTG
AcrA_R318F_R	CATCGCCGAATGGAGTACGGGTAACGCCCTG
AcrA_I343F_G344F_F	GCCAGGCGTTCTTCGATAAGTGGCTGGTGAC
AcrA_I343F_G344F_R	CACTTATCGAAGAACGCCTGGCTTGCGACG
AcrA_Q365F_F	GCGGGCTGTTCAAAGTACGTCCTGGCGCAC
AcrA_Q365F_R	GACGTACTTTGAACAGCCCGCTGACGACTAC
AcrA_K346A_F	AGCCAGGCGATCGGCGATGCGTGGCTGGTG
AcrA_K346A_R	CACCAGCCACGCATCGCCGATCGCCTGGCT
AcrA_K366D_F	GGCTGCAAGATGTACGTCCTGGCGCACAGG
AcrA_K366D_R	GGACGTACATCTTGCAGCCCGCTGACGAC
AcrA_R368A_F	CAGCGGGCTGCAAAAAGTAGCTCCTGGCGCA
AcrA_R368A_R	TGCGCCAGGAGCTACTTTTTGCAGCCCGCTG
AcrA_R368F_F	GCAAAAAGTATTTCCTGGCGCACAGGTTAAAG
AcrA_R368F_R	CGCCAGGAAATACTTTTTGCAGCCCGCTGAC
AcrE_G57F_F	GTAACGACCGAACTTCCCTTCCGTACGTCCGCATTTCGC
AcrE_G57F_R	GCGAAATGCGGACGTACGGAAGGGAAGTTCGGTCGTTAC

AcrE_R58A_F	ACCGAACTTCCCGGAGCTACGTCCGCATTTCG
AcrE_R58A_R	CGAAATGCGGACGTAGCTCCGGGAAGTTCGGT
AcrE_T216F_F	CGATCCGATTTATGTCGACGTGTTCCAATCAAGCAACGACTTTATGC
AcrE_T216F_F	GCATAAAGTCGTTGCTTGATTGGAACACGTCGACATAAATCGGATCG
AcrE_T270D_F	GTTACCGTAGATGAAAGCGACGGCTCTATCACGCTCAG
AcrE_T270D_R	CTGAGCGTGATAGAGCCGTCGCTTTCATCTACGGTAAC
AcrE_F291G_F	CTGCTTCCCGGTATGGGTGTTCGCGCCCGCAT
AcrE_F291G_R	ATGCGGGCGCGAACACCCATACCGGGAAGCAG
AcrE_R293F_F	GTCTGCTTCCCGGTATGTTTGTTTTCGCCCGCATTGA
AcrE_R293F_R	TCAATGCGGGCGAAAACAAACATACCGGGAAGCAGAC
AcrE_G362F_F	CGATAAGGTCATCGTCAGCTTCTTACAAAAAGCGCGACCG
AcrE_G362F_R	CGGTCGCGCTTTTTGTAAGAAGCTGACGATGACCTTATCG
AcrE_K365D_F	CATCGTCAGCGGCTTACAAGATGCGCGACCGG
AcrE_K365D_R	CCGGTCGCGCATCTTGTAAGCCGCTGACGATG
AcrE_R367D_F	CGGCTTACAAAAAGCGGATCCGGGCGTCCAGGTG
AcrE_R367D_R	CACCTGGACGCCCGGATCCGCTTTTTGTAAGCCG
AcrB_A33W_F	GCGATCCTCAAATTGCCGGTATGGCAATATCCGACGAT
AcrB_A33W_R	ATCGTCGGATATTGCCATACCGGCAATTTGAGGATCGC
AcrB_T37W_F	GGTATGGCAATATCCGTGGATTGCGCCACCAGCA
AcrB_T37W_R	TGCTGGTGGCGCAATCCACGGATATTGCCATACC
AcrB_N298W_F	TGGCTACCGGCGCCTGGGCGCTGGATACCGC
AcrB_N298W_R	GCGGTATCCAGCGCCCAGGCGCCGGTAGCCA

Table S2. Antimicrobial susceptibility of the *Salmonella* Typhimurium SL1344 Δ4PAP strain complemented with mutated versions of AcrA.

		MIC (μg mL^{-1})									
Strain	**Box no.**	ACR	CLI	CV	DOX	EtBr	ERY	FA	MB	NOV	R6G
WT		256	256	64	512	>1024	128	1024	>1024	512	>1024
Δ4PAP		16	2	2	2	16	2	4	8	1	8
WT complement		64	128	16	64	128	64	256	128	128	128
R59A	**1**	**16**	**2**	**2**	**2**	**32**	**2**	**4**	**16**	**2**	**16**
T270A	4	64	128	16	64	128	64	256	128	128	128
T270D	4	64	128	16	64	128	64	256	128	128	128
T271A	4	64	16	8	32	64	**8**	**64**	**32**	**8**	**32**
T271D	**4**	**16**	**2**	**2**	**2**	**16**	**2**	**4**	**8**	**1**	**8**
G272A-S273A	4	64	128	16	64	128	64	256	128	128	128
F292V	**5**	**16**	**2**	**2**	**2**	**16**	**2**	**4**	**8**	**1**	**8**
Q310P-Q311P	**pre-6**	**16**	**2**	**2**	**2**	**16**	**2**	**4**	**8**	**1**	**8**
Q311F	pre-6	64	128	16	64	64	64	128	128	64	128
P317G	6	64	128	16	64	64	64	128	128	128	128
P317F	6	64	128	16	64	128	64	256	128	128	128
R318F	6	64	128	16	64	128	64	256	128	128	128
I343F-G344F	8	64	128	16	64	128	64	256	128	128	128
K346A	8	64	128	16	64	128	64	256	128	128	128
Q365F	9	64	128	16	64	128	64	256	128	128	64
K366D	9	64	**16**	16	**16**	64	**16**	**16**	128	**16**	64
R368F	9	64	128	16	64	128	64	256	128	128	128

Underlined values highlight values for the Δ4PAP strain complemented with wild type AcrA (WT complement). Bold values are at least two-fold or more different than the parent strain. ACR, acriflavine; CLI, clindamycin; CV, crystal violet; DOX, doxorubicin; EtBr, ethidium bromide; ERY, erythromycin; FA, fusidic acid; MB, methylene blue; NOV, novobiocin; R6G, rhodamine 6G. Box no. indicates the mapping of the mutation to its binding box.

Table S3. Antimicrobial susceptibility of the *Salmonella* Typhimurium SL1344 Δ4PAP Δ*acrF* strain complemented with mutated versions of AcrE.

		MIC (μg mL^{-1})												
Strain	**Box no.**	ACR	BZK	CHL	CLI	CV	DOX	EtBr	ERY	FA	MB	MIN	NOV	R6G
WT		256	64	4	512	64	1024	>1024	64	1024	1024	1	512	>1024
Δ4PAP Δ*acrF*		16	4	0.5	1	2	2	16	4	4	8	0.25	1	8
WT complement		256	64	4	128	32	512	>1024	64	512	1024	1	512	>1024
G57F	**1**	**16**	**4**	**0.5**	**4**	**1**	**2**	**16**	**4**	**4**	**8**	**0.25**	**2**	**8**
R58A	**1**	**16**	**4**	**0.5**	**4**	**1**	**2**	**16**	**4**	**4**	**8**	**0.25**	**2**	**8**
T216F	2	256	64	4	128	32	512	1024	64	1024	1024	1	512	1024
T270D	**4**	**16**	**4**	**0.5**	**4**	**1**	**2**	**16**	**4**	**8**	**8**	**0.25**	**1**	**8**
F291G	**5**	**16**	**4**	**0.5**	**4**	**2**	**2**	**16**	**4**	**8**	**8**	**0.25**	**1**	**8**
R293F	**5**	**16**	**8**	**0.5**	**8**	**2**	**8**	**64**	**4**	**32**	**64**	**0.25**	**16**	**64**
G362F	**9**	**32**	**8**	**0.5**	**16**	**2**	**8**	**64**	**4**	**32**	**128**	**0.25**	**16**	**64**
K365D	9	128	64	4	128	16	512	1024	64	256	1024	1	256	1024
R367D	9	256	64	4	128	32	512	1024	64	512	1024	1	256	1024

Underlined values highlight values for the Δ4PAP Δ*acrF* strain complemented with wild type AcrE (WT complement). Bold values are at least two-fold or more different than the parent strain. ACR, acriflavine; BZK, benzalkonium chloride; CHL, chloramphenicol; CLI, clindamycin; CV, crystal violet; DOX, doxorubicin; EtBr, ethidium bromide; ERY, erythromycin; FA, fusidic acid; MB, methylene blue; MIN, minocycline; NOV, novobiocin; R6G, rhodamine 6G. Box no. indicates the mapping of the mutation to its binding box.

Table S4. Antimicrobial susceptibility of the *Salmonella* Typhimurium SL1344 Δ4PAP Δ*acrB* strain complemented with K366D AcrA and the AcrB channel 3 mutation.

	MIC (μg mL^{-1})									
	HMMD				PAC					
Strain	ERY	DOX	FA	NOV	ACR	BZK	CV	EtBr	MB	R6G
WT	128	1024	1024	512	256	64	64	>1024	1024	>1024
Δ4PAP Δ*acrB*	4	2	4	1	8	2	2	8	8	8
WT AcrAB complement	64	64	128	64	128	32	16	256	256	128
WT AcrA + AcrB CH3 mutation	16	32	32	16	32	8	8	32	32	32
K366D AcrA + WT AcrB	16	16	16	16	128	32	16	128	128	64
K366D AcrA + AcrB CH3 mutation	**4**	**2**	**4**	**1**	**8**	**2**	**2**	**8**	**8**	**8**

Underlined values highlight values for the Δ4PAP Δ*acrB* strain complemented with WT AcrAB. Bold values highlight the MIC values of the Δ4PAP Δ*acrB* strain complemented with K366D AcrA and the AcrB CH3 (A33W T37W N298W AcrB) mutation compared to its single mutation parent strains. HMMD, high-molecular-mass drugs; ERY, erythromycin; DOX, doxorubicin; FA, fusidic acid; NOV, novobiocin; PAC, planar aromatic cation; ACR, acriflavine; BZK, benzalkonium chloride; CV, crystal violet; EtBr, ethidium bromide; MB, methylene blue; R6G, rhodamine 6G.

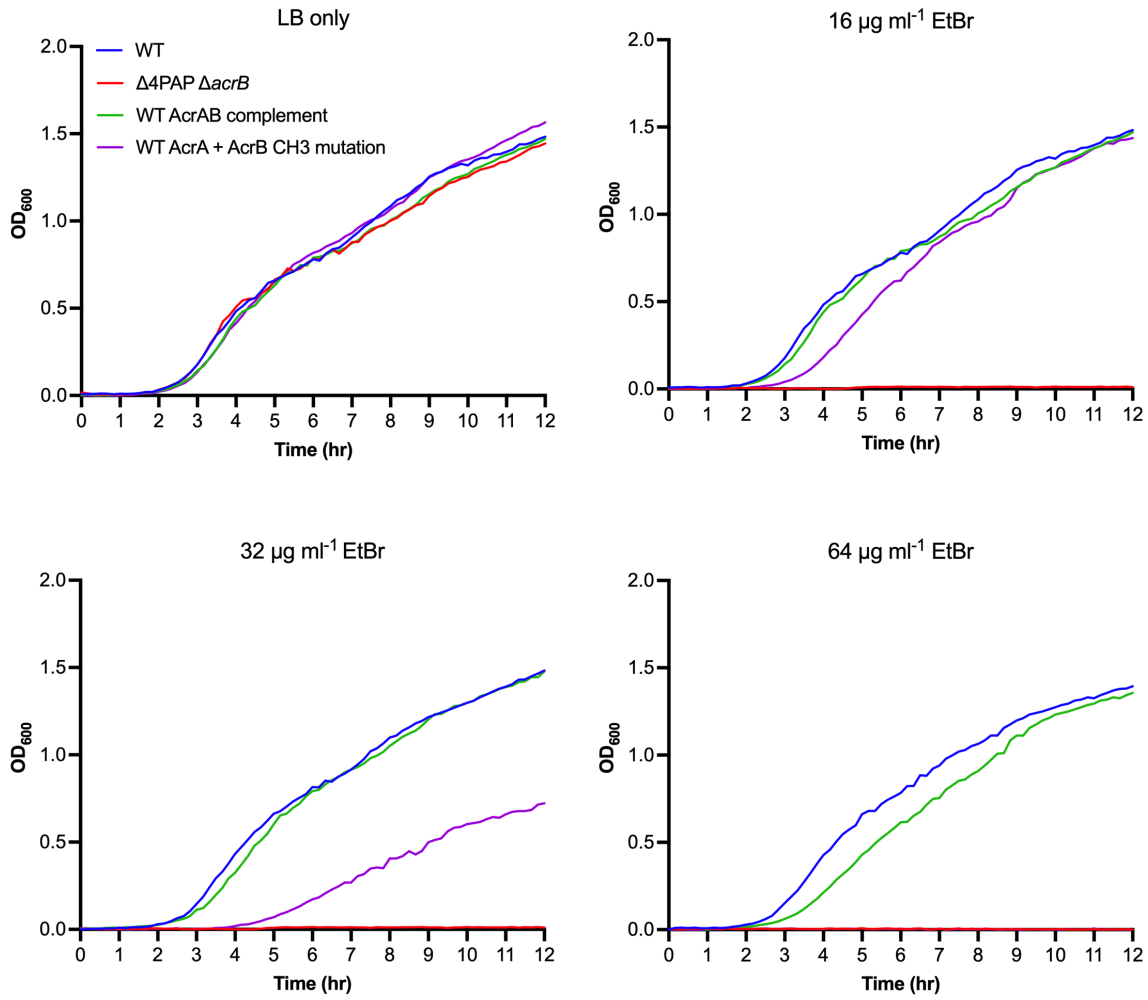

Figure S2. Growth kinetics of the *Salmonella* Typhimurium SL1344 Δ4PAP Δ*acrB* strain complemented with wild type AcrA and the AcrB channel 3 (CH3) mutation in various concentrations of ethidium bromide (EtBr). Data shown are the mean OD_{600} values of three biological replicates. The AcrB CH3 mutation refers to A33W T37W N298W AcrB.

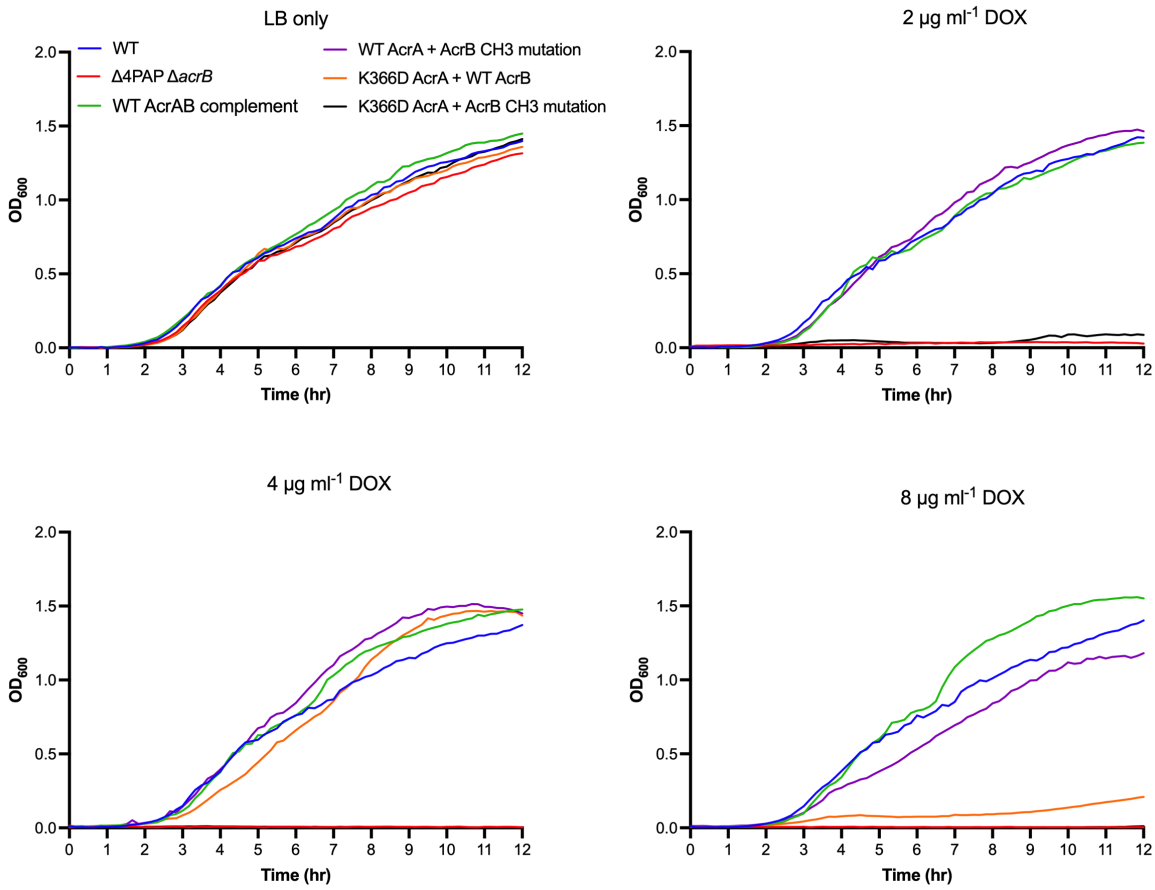

Figure S3. Growth kinetics of the *Salmonella* Typhimurium SL1344 Δ4PAP Δ*acrB* strain complemented with K366D AcrA and the AcrB channel 3 (CH3) mutation in various concentrations of doxorubicin (DOX). Data shown are the mean OD_{600} values of three biological replicates. The AcrB CH3 mutation refers to A33W T37W N298W AcrB.

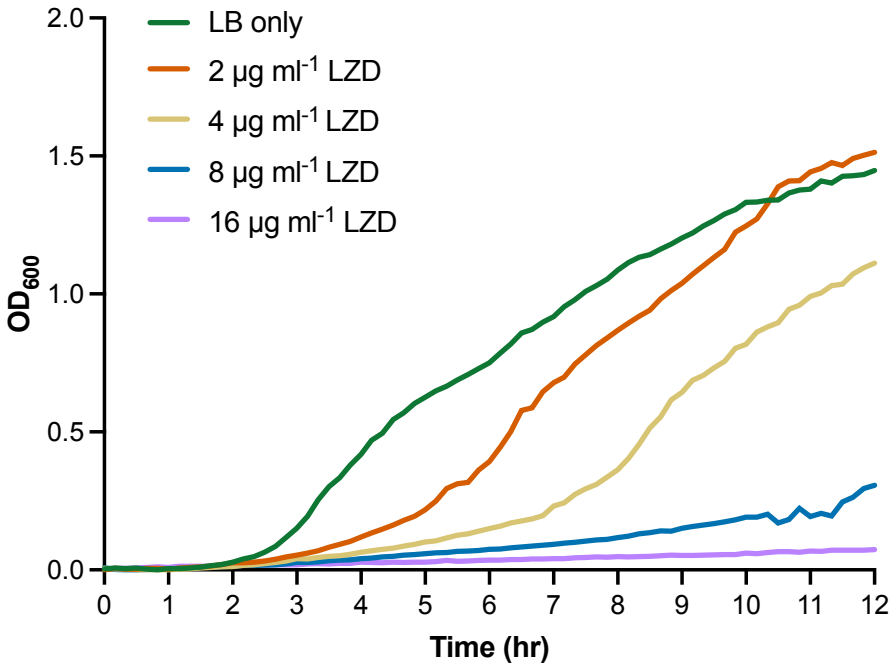

Figure S4. Growth kinetics of the *Salmonella* Typhimurium SL1344 Δ4PAP strain complemented with the K366D AcrA mutation in various concentrations of linezolid (LZD). Data shown are the mean OD_{600} values of three biological replicates.