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Supplemental Information

An *Acinetobacter baumannii*, Zinc-Regulated Peptidase Maintains Cell Wall Integrity during Immune-Mediated Nutrient Sequestration

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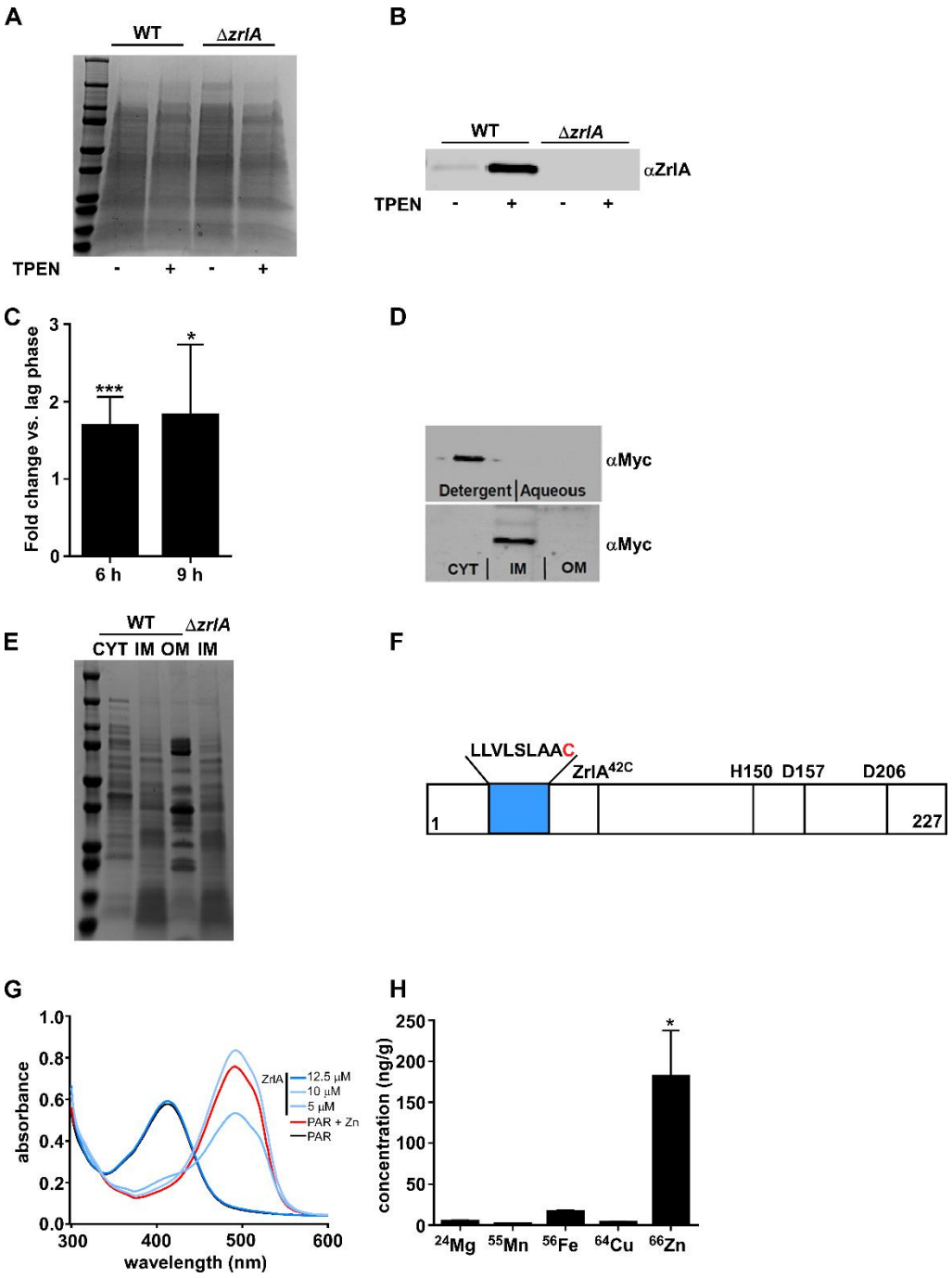

Figure S1. ZrlA is a zinc-binding inner membrane lipoprotein. Related to Figure 1. A) SDS-PAGE analysis to assess total protein levels corresponding to the immunoblot in Fig 1A. B) Immunoblot on protein lysates from WT or Δ*zrlA* cells grown for 6 hours ± 40 μM TPEN (25 μg protein/lane). C) Relative expression of *zrlA* as determined by qRT-PCR on WT *A. baumannii* at 6 h and 9 h of growth compared to 2 h. * $p < 0.05$, *** $p < 0.001$ as determined by Student's *t* test from three independent experiments, mean ± SD. D) Immunoblot of membrane fractionations (15 μg protein/lane) reveals $ZrlA^{myc}$ localizes to the detergent phase and localizes to the inner membrane. E) SDS-PAGE analysis to assess total protein levels corresponding to the immunoblot in Fig 1D. F) Schematic of ZrlA, with the predicted lipobox denoted as a blue square and the conserved lipid-anchoring cysteine in red. The approximate N-terminus of the $ZrlA^{42C}$ variant is indicated (residues 42-227). H150, D157, and D206 are the predicted metal-coordinating residues. G) Incubation of 20 μM 4-(2-pyridylazo)-resorcinol (PAR) with 10 μM Zn results in an absorbance shift of PAR from 410 nm to 500 nm; the addition of increasing concentrations of recombinant ZrlA from 5 μM to 12.5 μM results in return in absorbance to 410 nm. H) Recombinant ZrlA was analyzed by ICP-MS for associated Mg, Mn, Fe, Cu, and Zn concentrations. * $p < 0.05$ as determined by one-way ANOVA with Tukey multiple comparisons test from three independent experiments, mean ± SD.

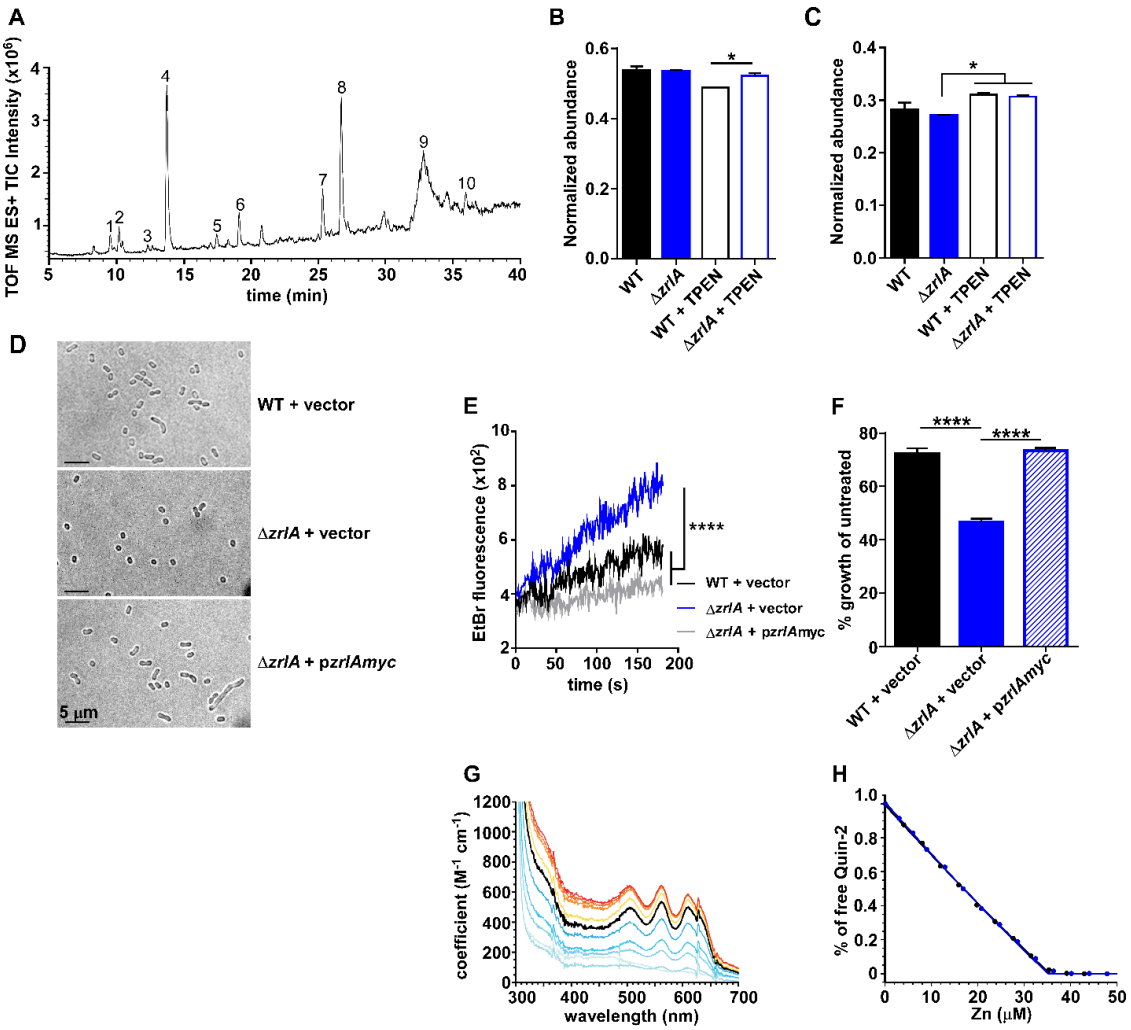

Figure S2. ZrlA contributes to PG homeostasis and cellular morphology. Related to Figure 2. A) Representative total ion chromatogram (TIC) and accompanying UPLC-MS analysis of a muropeptide profile of *A. baumannii* ATCC 17978 Δ*zrlA* grown with 40 μM TPEN treatment. Other TICs were obtained for the WT strain grown with 40 μM TPEN. See **Table S1** for corresponding peak descriptions of peaks labeled 1-10 including molecular composition, retention time, theoretical *m/z*, experimental *m/z* and difference in *m/z*. Note that peaks 1-2 (monomeric trimers) and 3-4 (monomeric tetramers include muropeptides that harbor a disaccharide or monosaccharaide (–GlcNAc) sugar moiety (see Table S1 for details). B) Normalized abundance of muropeptide tetrapeptide monomers [sum M4,M4(–GlcNAc)] (Figure S2, Table S1) in WT or Δ*zrlA* ± 40 μM TPEN. C) Normalized abundance of muropeptide dimeric tetrapeptides crosslinks (sum D44, M44; Figure S2, Table S1) in WT or Δ*zrlA* ± 40 μM TPEN. * $p < 0.05$ as determined by one-way ANOVA with Tukey multiple comparisons test on biological duplicates, mean ± SD. D) Live-cell imaging of WT + vector, Δ*zrlA* + vector, or Δ*zrlA* + p*zrlAmyc* following growth in LB Carb75 (100X). Scale bar is 5 μm. E) Ethidium bromide uptake assessed over time in WT + vector, Δ*zrlA* + vector, or Δ*zrlA* + p*zrlAmyc* following growth in LB Carb75. **** $p < 0.0001$ as determined by Student's *t* test on mean line slope from three independent experiments. F) WT + vector, Δ*zrlA* + vector, and Δ*zrlA* + p*zrlAmyc* were grown in 0.01 % SDS. Graph depicts percent growth relative to untreated strains at eight hours. *** $p < 0.001$ as determined by one-way ANOVA with Tukey multiple comparisons test from three indepdenent experiments, mean ± SD. G) Co(II) titration of apo A1S_1248^{56C} with the spectra colored as a function of the Co(II):protein ratio as in Fig 2E, main text: *cyan*, less than 1 mol•equivalent Co(II); *black*, 1:1 mol ratio; *tan to orange*, greater than 1:1 mol ratio. H) Replicate normalized titrations (*black*, *blue* filled symbols) of Zn(II) into a solution of the quin-2 and A1S_1248^{56C}, with the continuous lines individual fits to a 1:1 binding model. 12.4 μM A1S_1248^{56C} with 22.4 μM quin-2 (black filled circles) and 15.0 μM A1S_1248^{56C} with 20.2 μM quin-2 (blue filled circles). The average K_{Zn} of 2.2 (±0.1) x10^{11} M^{-1}.

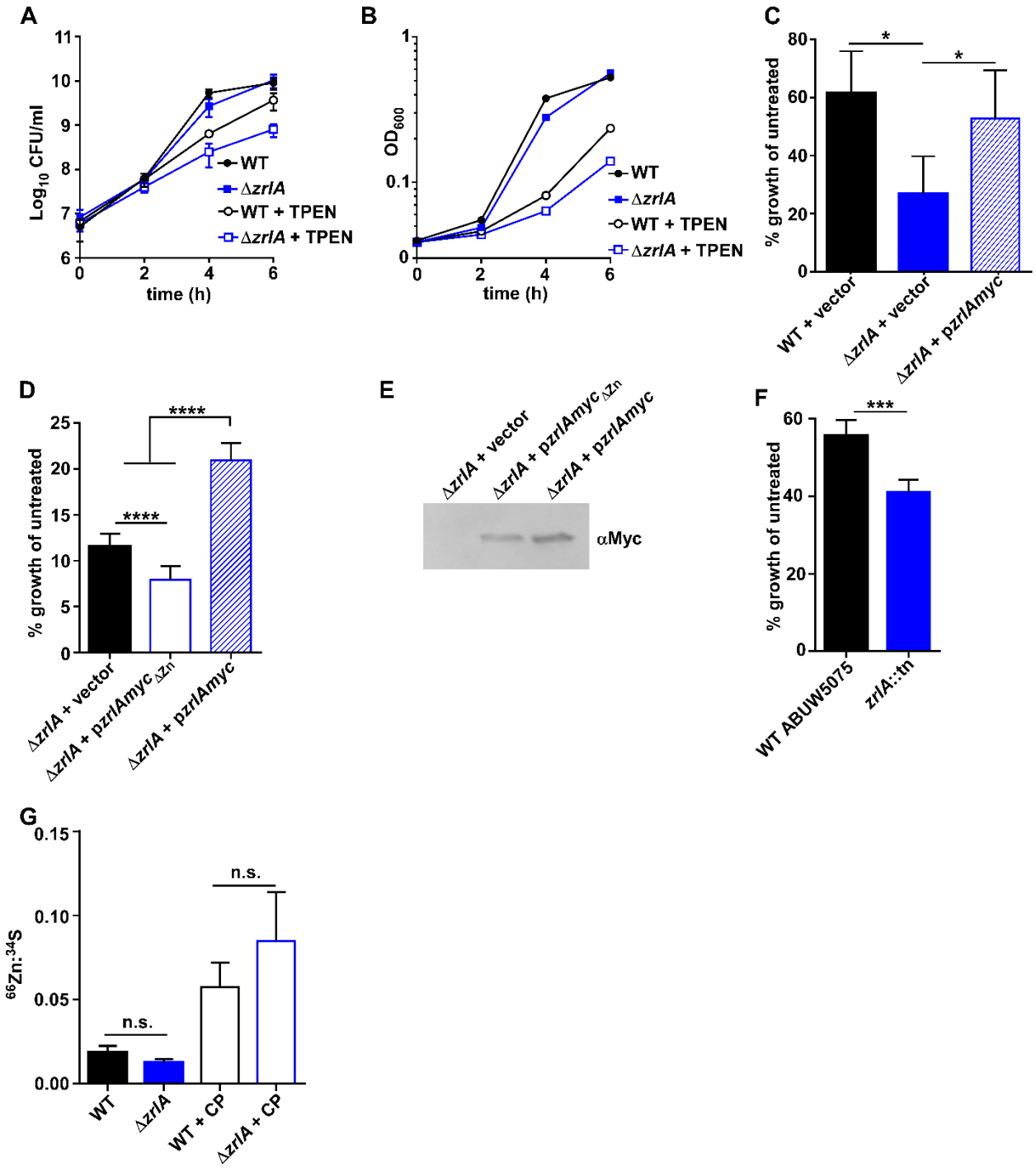

Figure S3. ZrlA is critical for full growth in low Zn. Related to Figure 3. A) Colony forming units (CFU) were monitored over time for WT or Δ*zrlA* ± 40 μM TPEN. B) WT or Δ*zrlA* growth over time ± 40 μM TPEN as determined by OD_{600}. C) Growth of WT + vector, Δ*zrlA* + vector, or Δ*zrlA* + p*zrlAmyc* was monitored in the presence of 250 μg/ml calprotectin. Graph depicts percent growth at eight hours compared to untreated strains. * $p < 0.05$ as determined by Student's *t* test from three independent experiments, mean ± SD. D) Δ*zrlA* + vector, Δ*zrlA* + p*zrlAmyc*$_{\Delta Zn}$, or Δ*zrlA* + p*zrlAmyc* was monitored over time ± 40 μM TPEN. Graph depicts percent growth at eight hours compared to untreated strains. **** $p < 0.0001$ as determined by Student's *t* test from three independent experiments, mean ± SD. E) Immunoblot on protein lysates from Δ*zrlA* + vector, Δ*zrlA* + p*zrlAmyc*$_{\Delta Zn}$, or Δ*zrlA* + p*zrlAmyc* (20 μg protein/lane). F) *A. baumannii* strain ABUW5075 or a transposon mutant with an insertion in *zrlA* was grown ± 20 μM TPEN. Graph depicts percent growth at eight hours compared to untreated strains. *** $p < 0.001$ as determined by Student's *t* test from three independent experiments, mean ± SD. G) Total cellular ^{66}Zn was quantified in WT or Δ*zrlA* ± 250 μg/ml calprotectin by ICP-MS. n.s. = no significant difference as determined by one-way ANOVA with Tukey multiple comparisons test from three independent experiments, mean ± SD.

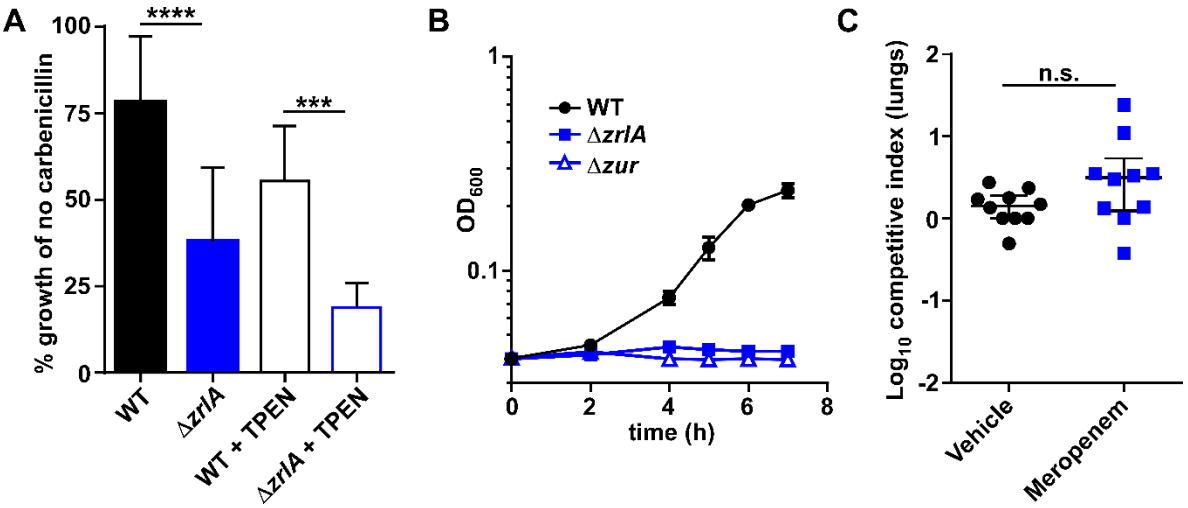

Figure S4. *zrlA* is required for overcoming antibiotic exposure. Related to Figure 4. A) WT or Δ*zrlA* was grown with 3.125 μg/ml carbenicillin ± 20 μM TPEN with OD_{600} monitored over time. Data depict growth at eight hours relative to no carbenicillin treatment. *** $p < 0.001$, **** $p < 0.0001$ as determined by one-way ANOVA with Tukey multiple comparisons test from three independent experiments, mean ± SD. B) WT, Δ*zur*, or Δ*zrlA* was grown in the presence of 6.25 μg/ml carbenicillin with OD_{600} monitored over time. C) Mice were intranasally infected with a 1:1 mixture of an unmarked and marked Δ*zrlA* strain and intraperitoneally administered meropenem (12.5 mg/kg) or vehicle at 12 hour intervals post-infection (t = 0, 12, 24 hpi). Data depict the competitive index of unmarked *zrlA* mutant: marked *zrlA* mutant in the lungs at 36 hpi. n.s. = no significant difference as determined by Student's *t* test ($n = 10$).

Supplemental Tables

Table S1. Descriptions for muropeptide peak labeling depicted in Figure S2, and related to Figure 2. The theoretical masses were reported previously (Kuhner et al., 2014; Brown et al., 2012). The first letter indicates whether the muropeptide is a monomer (M), or crosslinked dimer (D) or crosslinked trimer (T). The numbers indicate the number of amino acids of the peptide side chains from the donor to acceptor; G indicates glycine; (-GlcNAc) and (-Acetyl) indicates loss of GlcNAc or acetyl group. The difference column denotes the difference in experimental mass relative to the theoretical mass.

UPLC peak	Muropeptide structure	Retention time in TIC (min)	Theoretical molecular mass	Theoretical m/z [M+H]+	Theoretical m/z [M+2H]2+	Theoretical m/z [M+3H]3+	Experimental m/z [M+xH]x+	Difference
1	M3	9.54	870.36	871.37			871.370	0.000
1	M3(-GlcNAc)	9.54	667.29	668.30			668.299	0.000
2	M3G	10.18	927.38	928.39			928.395	0.005
2	M3G(-GlcNAc)	10.20	724.31	725.32			725.316	-0.005
3	M4G(-GlcNAc)	12.34	795.35	796.36			796.349	-0.009
3	M4G	12.39	998.43	999.44			999.414	-0.023
4	M4	13.75	941.39	942.40			942.397	-0.003
4	M4(-GlcNAc)	13.76	738.33	739.34			739.335	-0.001
5	M43	17.45	1313.56	1314.57	657.79		657.791	0.002
6	M44	19.12	1384.59	1385.60	693.30		693.306	0.002
7	D34D and D43	25.34	1793.75	1794.76	897.88		897.877	-0.007
8	D44	26.72	1864.79	1865.80	933.40		933.401	-0.003
9	T444	32.8	2788.19	2789.20		930.41	930.390	-0.015
10	anhydroD44	35.97	1844.78	1845.79	923.40		923.388	-0.009

Table S2. Oligonucleotides used in this study. Related to Figures 1 and 2 and STAR Methods.

Name	Sequence (5' to 3')	Description
***zrlA* mutant construction and confirmation**		
3412FL1For	CCCGGGAGAGTAAGTGCAATTG	Cloning into pFLP2, 5' flank, forward
3412FL1Rev	CTCCTAGTTAGTCACATATGACGCTTCATAAATATATTC	Cloning into pFLP2, 5' flank, reverse
3412FL2For	AGGGAATAATGACATATGGGACTATAAAAGCAATTT	Cloning into pFLP2, 3' flank, forward
3412FL2Rev	CCCGGGTTGTGACCATGAATAAG	Cloning into pFLP2, 3' flank, reverse
3412KanFor	GAATATATTTATGAAGCGTCATATGTGACTAACTAGGAG	Cloning into pFLP2 for Km cassette from pUC18-k1, forward
3412KanRev	AAATTGCTTTTATAGTCCCATATGTCATTATTCCCT	Cloning into pFLP2 for Km cassette from pUC18-k1, reverse
3412_KO_F	cgtccttttttattttttaaaatttacaacagaaattaaaggatttaataattgttatattataacatttctgaaattaaatgattaaaggcttccagatgtttagggaagatagaatatatttGTG TAG GCT GGA GCT GCT TC	To generate unmarked *zrlA* mutant, foward
3412_KO_R	tggccagatcacaggcgtaatctatttctaaaccacaataatgttatgttataacataactattgatcaagtttttattctagttttatctaagccaaatcacttagtgtttgaaaaaattgcttCAT ATG AAT ATC CTC CTT AG	To generate unmarked *zrlA* mutant, foward
3412ExtR	GACCAGCTCTGCCAAACTTGC	External to *zrlA* for Tn insertion confirmation
Pgro-172	TGAGCTTTTTAGCTCGACTAATCCAT	T26 transposon-specific
***zrlA* complementation**		
3412mycFor	GGATTCATGGTGGCTTATCAATTTAAGCGCCG	Cloning into pWH1266, forward
3412mycRev	GGATTCTTATAGATCTTCTTCAGATATCAG TTTCTGTTCTAGTCCCTGACAAAT	Cloning into pWH1266, reverse with cMyc tag
qRT-PCR		
r01RTf	CTGTAGCGGGTCTGAGAGGAT	*A1S_r01* (16S), forward
r01RTr	CCATAAGGCCTTCTTCACAC	*A1S_r01* (16S), reverse
3412RTf	CGCAACTGCAGATCACAGAC	*zrlA*, forward
3412RTr	GGAACTGCGTAAGGCTCAAC	*zrlA*, reverse
A1S_2479_RTFor	CAATGCCGGATCATTACACGACAG	*A1S_2479*, forward
A1S_2479_RTRev	AACCTGCTGCTTTGGTATAGCCAG	*A1S_2479*, reverse
A1S_2435_RTFor	ATGAACGAATCTGCTTGGTGTAAAGGC	*A1S_2435*, forward
A1S_2435_RTRev	CCTTCATTCCCAGCAATATGTTCAGCC	*A1S_2435*, reverse
A1S_1248_RTFor	CCACCTAAAGTTGAACCTGCTTCCTAT	*A1S_1248*, forward
A1S_1248_qRTRev	TCTGCCAATCACGAGCGGATCTTAATA	*A1S_1248*, reverse
Protein purification		
3412-sspMAL_fwd	gcggccgcgatatcgtcgacAGTACTCAAACACCCCAGC	Cloning into pMAL-c5X, forward

3412-sspMAL_rev	cctgcagggaattcggatccTTATAGTCCCTGACAAATTGAG	Cloning into pMAL-c5X, reverse
ZrlA^{42C}-F	CAACGACCGAAAACCTGTATTTTCAGGGCGCCATGGA TAAACAACAGCCT GAAGATTAT	Cloning into pET-22b, forward
ZrlA^{42C}-R	CACTAGTTGAGCTCGTCGACGTAGGCC TTTGAATTCTT ATAGTCCCTGACAAATTGAGGT	Cloning into pET-22b, reverse
A1S_1248^{56C}-F	GCCGGCGATGGCCATGGATAAAGTTGA ACCTGCTTCCTAT	Cloning into pET-22b, forward
A1S_1248^{56C}-R	CGG CCG CAA GCT TGT CGA CGG AGC TCG AAT TCT TAG AAA TTA CAC ATT GAC GTA TTG C	Cloning into pET-22b, reverse