Supplemental information for:

The Bacterial Septal Ring Protein RlpA is a Lytic Transglycosylase that Contributes to Rod Shape and Daughter Cell Separation in *Pseudomonas aeruginosa*

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Running Title: RlpA is a Peptidoglycan Hydrolase

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Supplemental Materials and Methods

Construction of plasmids

(i)Plasmid for rescue of Δ*rlpA*. pDSW1398 (P_{BAD}::*rlpA*) was constructed by amplifying *rlpA* from PA14 chromosomal DNA with primers P1603 and P1604. The 1205 bp product was cut with EcoRI and XbaI and ligated to the same sites of pJN105 to create the desired P_{BAD}::*rlpA* construct. Expression of *rlpA* from pDSW1398 did not require arabinose induction.

(ii) Plasmids for gene knockouts. In-frame deletions were constructed essentially as previously described using the pEXG2 vector (Schweizer, 1992, Rietsch *et al.*, 2005). pDSW1385 (pEXG2::'*sltb1*-Δ*rlpA*-*dacC*') was constructed by amplifying ~1 Kb of upstream sequence plus the first 8 codons of *rlpA* with primers P1507 and P1474. Similarly, the last 8 codons and ~1 Kb of sequence downstream of *rlpA* were amplified with primers P1475 and P1508. The 975 and 994 bp products, respectively, were cut with XbaI and ligated to each other to make a 1955 bp product, which was further amplified using primers P1507 and P1508. The 1955 bp product was cut with HindIII and MfeI and ligated to pEXG2 cut with HindIII and EcoRI. Similar procedures were used to construct pDSW1490 (pEXG2::'*rodA*-Δ*sltb1*-*rlpA*') and pDSW1516 (pEXG2::'*PA14_57740*-Δ*mltb1*-*cysD*') using the following primers: P1702-P1705 (pDSW1490) and P1713-1716 (pDSW1516).

(iii) Plasmids for localization of RlpA. To construct an RlpA-mCherry fusion, primers P1599 and P1600 were used to amplify *rlpA* from PA14 chromosomal DNA. The 1047 bp product was cut with EcoRI and XbaI and ligated to the same sites of pDSW913 (P_{206}::MCS-*mCherry*) to create pDSW1399. Similarly, to construct a SPOR

deletion mutant of RlpA for localization studies, primers P1599 and P1708 were used to amplify *rlpA*(Δ269-341) from PA14 chromosomal DNA. The 828 bp product was cut with XbaI and EcoRI and ligated to the same site of pDSW913 to produce pDSW1497.

For localization studies in *P. aeruginosa*, *rlpA-mCherry* was recombined onto the chromosome using procedures similar to make gene deletions. pDSW1489 (pEXG2::'*rlpA-mCherry-dacC*') was constructed by amplifying *rlpA-mCherry* from pDSW1399 using primers P1680 and P1681. In a subsequent reaction, ~1 Kb of sequence downstream of *rlpA* was amplified with P1682 and P1683. The 1761 bp and 1018 bp products, respectively, were cut with MfeI, ligated to each, and further amplified using primers P1680 and P1683. The 2.8 Kb product was digested with HindIII and KpnI and ligated to the same sites of pEXG2 to make pDSW1489. A similar procedure was used to generate a pEXG2 derivative for recombining the *rlpA* SPOR deletion fusion [*rlpA*(Δ269-341)-*mCherry*] onto the chromosome of *P. aeruginosa*. pDSW1504 (pEXG2::'*rlpA*(Δ269-341)-*mCherry-dacC*') was constructed by amplifying *rlpA*(Δ269-341)-*mCherry* from pDSW1497 using primers P1680 and P1681. The 1542 bp product was cut with HindIII and MfeI and ligated to the same sites of pDSW1489 to produce pDSW1504.

(iv) Plasmids for localization of RlpA proteins with amino acid substitutions in the DPBB domain. To generate amino acid substitutions in the DPBB domain of *rlpA*, *rlpA-mCherry* was amplified from pDSW1399 with primers P1599 and P1727. The 1764 bp product was cut with AatII and SacI, then ligated to the same sites of pDSW1398 to produce the vector pDSW1518. Amino acid substitutions in the DPBB domain of *rlpA* were then introduced by megapriming. For example, a D157N substitution in the DPBB

of *rlpA* was constructed by amplifying *rlpA* from pDSW1518 with primers P1754 and P1781. P1781 has a sequence change at the codon for D157. The 315 bp product was isolated by PCR column purification (Qiagen) and used in a subsequent reaction with primer P1755 to produce full length *rlpA* (with the D157N substitution) from pDSW1518. The 870 bp product was cut with AatII and XbaI and ligated to the same sites of pDSW1518 to produce pDSW1545. Similar procedures were used to introduce substitutions at other residues using the following primers in place of P1781: P1756 (E120A) to make pDSW1519, P1758 (D123A) to make pDSW1520 and P1760 (H131A) to make pDSW1537.

For functional studies in *P. aeruginosa*, pEXG2 derivatives containing *rlpA* variants with amino acid substitutions in the DPBB domain were generated for recombination onto the chromosome of *P. aeruginosa*. pDSW1614 (pEXG2::'*sltb1-rlpA-mCherry-dacC*') was constructed by amplifying ~1 Kb of sequence upstream of *rlpA* from PA14 chromosomal DNA using primers P1821 and P1822. The 1208 bp product was cut with BamHI and HindIII, and ligated to the same sites of pDSW1489 to make pDSW1614. pDSW1614 was then used as a destination vector for mutants of *rlpA* with substitutions in the DPBB domain. pDSW1615 (pEXG2::'*sltb1-rlpA*(E120A)-*mCherry-dacC*') was constructed by amplifying *rlpA* with an E120A substitution from pDSW1519 using primers P1823 and P1824. The 556 bp product was cut with BamHI and NotI and ligated to the same sites of pDSW1614. Similar procedures were used to introduce other *rlpA* variants with amino acid substitutions in the DPBB domain using the following plasmids as template: pDSW1520 (D123A) to make pDSW1616, pDSW1537 (H131A) to make pDSW1617 and pDSW1545 (D157N) to make pDSW1619.

(v) Plasmid for localization of AmiB. To construct an AmiB-mCherry fusion, primers P1805 and P1806 were used to amplify *amiB* from PA14 chromosomal DNA. The 1483 bp product was cut with EcoRI and XbaI and ligated to the same sites of pDSW1518 to produce pDSW1635.

(vi) Plasmids for purification of His_6-RlpA proteins. To overproduce RlpA with an N-terminal His-tag, *rlpA* was amplified from PA14 chromosomal DNA with primers P1787 and P1711. The 962 bp product was cut with BclI and HindIII and ligated to pQE-80L cut with BamHI and HindIII to make pDSW1557. Similar procedures were used to clone *rlpA* variants with amino acid substitutions [pDSW1600 (D157N), pDSW1601 (E120A), pDSW1604 (D123A) and pDSW1606 (H131A) using plasmids pDSW1545, pDSW1519, pDSW1520 and pDSW1537, respectively, as template]. Purification constructs contain amino acids 28-341 of *rlpA* and the sequence MRGSHHHHHHGS at the N-terminus.

Construction of strains for *in vivo* studies. pEXG2 derivatives were conjugated from donor *E. coli* strain SM10 to recipient *P. aeruginosa* strain UCBPP-PA14 on LB plates. Following incubation, the conjugation mixture was plated on LB plates containing Gent and Irg (to counter select against the *E. coli* donor strain) at 37°C overnight. Resolution of the co-integrant was selected for on LB0N plates containing 5% sucrose at 37°C overnight. Gene knockouts were made in the wild type (MJ1) background. Gene knock-ins were made in the Δ*rlpA* (MJ24) background.

Table S1. Functionality of various RlpA-mCherry fusion proteins.

Genotype[a]	Avg length µm, (SD)[b]	% Cells with indicated no. of constrictions:			
		0	1	3	>3
rlpA::*mCherry*	3.1 (0.7)	74	26	0	0
Δ*rlpA*	5.8 (2.0)	0	10	60	30
rlpA(ΔSPOR)-*mCherry*	3.1 (0.7)	71	29	0	0
rlpA(E120A)-*mCherry*	3.5 (0.9)	55	42	3	0
rlpA(D123A)-*mCherry*	3.1 (0.7)	73	27	0	0
rlpA(H131A)-*mCherry*	3.1 (0.7)	71	29	0	0
rlpA(D157N)-*mCherry*	5.6 (1.9)	0	14	58	28

[a]Strains shown are (in the order listed) MJ36, MJ24, MJ42, MJ81, MJ83, MJ85 and MJ89. At least 300 cells were scored for each.

[b]End-to-end length regardless of whether constrictions were observed.

Table S2. Amino acid and amino sugar analysis of muropeptides.

		nmoles				
Muropeptide	**Structure[a]**	**Glu**	**NAM**	**NAG+NAMol[b]**	**Ala**	**Dpm**
4	DS-Tri	2.4	0.0	4.3	2.5	1.8
5	TS	0.4	1.3	2.8	0.1	0.1
7	DS-Tetra	7.7	0.0	14.6	17.4	7.0
8	DS-Penta	0.6	0.0	1.3	0.5	0.3
9	HS	0.0	0.8	1.2	0.0	0.0
11	DS-Tetra-Tetra	0.4	0.0	0.6	0.6	0.1
13	TS-Tetra	0.2	0.2	0.6	0.4	0.1
17	DS-Tetra-DS-Tetra	8.2	0.0	12.1	19.7	7.9
20	DS-Tetra-DS-Tetra anhydro	1.3	0.5	1.7	2.9	1.0

[a]Abbreviations: DS, disaccharide (NAG-NAMol); TS, tetrasaccharide (NAG-NAM-NAG-NAMol); HS, hexasaccharide (NAG-NAM-NAG-NAM-NAG-NAMol). Tri, tripeptide (L-Ala-D-iGlu-m-Dpm); Tetra, tetrapeptide (L-Ala-D-iGlu-m-Dpm-D-Ala); Pent, pentapeptide (L-Ala-D-iGlu-m-Dpm-D-Ala-Gly). Anhydro, 1,6-anhydroNAM. The terminal NAM is in the alcohol form due to borohydride reduction except in the case of anhydro-NAM.

[b]During analysis, NAMol and NAG co-elute, and NAMol standards produce approximately 3-fold more absorbance than NAG standards, precluding a precise quantification of each. Nanomole values in this table are calculated assuming a one-to-one ratio of NAG and NAMol, which is true for disaccharides but not true for larger muropeptides.

Table S3. Tandem mass spectrometry analysis of muropeptides P5, P9 and P13.

Muropeptide	Structure	Expected *m/z* $[M+Na]^+$	Observed *m/z* $[M+Na]^+$	Formula
P5, Tetrasaccharide	NAG-NAM-NAG-NAMol[a]	999.4	999.5	$C_{38}H_{64}N_4O_{25}$
	NAG-NAM-NAG-NAMol*[b]	927.4	928.0	$C_{35}H_{60}N_4O_{23}$
	NAM-NAG-NAMol	796.3	796.9	$C_{30}H_{51}N_3O_{20}$
	NAG-NAM-NAG^{+c}	704.3	704.8	$C_{27}H_{44}N_3O_{17}$
	NAG-NAMol	521.2	521.6	$C_{19}H_{34}N_2O_{13}$
	NAG-NAM$^+$	501.2	501.6	$C_{19}H_{31}N_2O_{12}$
P9, Hexasaccharide	NAG-NAM-NAG-NAM-NAG-NAMol	1477.6	1477.6	$C_{57}H_{94}N_6O_{37}$
	NAG-NAM-NAG-NAM-NAG-NAMol*	1405.5	1406.0	$C_{54}H_{90}N_6O_{35}$
	NAM-NAG-NAM-NAG-NAMol	1274.5	1275.0	$C_{49}H_{81}N_5O_{32}$
	NAG-NAM-NAG-NAM-NAG$^+$	1183.4	1182.9	$C_{46}H_{74}N_5O_{29}$
	NAG-NAM-NAG-NAMol	999.4	999.7	$C_{38}H_{64}N_4O_{25}$
	NAG-NAM-NAG-NAM	980.4	979.7	$C_{38}H_{61}N_4O_{24}$
	NAM-NAG-NAMol	796.3	796.6	$C_{30}H_{51}N_3O_{20}$
	NAG-NAM-NAG$^+$	704.3	704.5	$C_{27}H_{44}N_3O_{17}$
	NAG-NAMol	521.2	521.4	$C_{19}H_{34}N_2O_{13}$
	NAG-NAM$^+$	501.2	501.4	$C_{19}H_{31}N_2O_{12}$
P13, Tetrasaccharide-tetrapeptide	NAG-NAM-NAG-NAMol-Ala-Glu-Dpm-Ala	1442.6	1442.9	$C_{56}H_{93}N_9O_{33}$
	NAG-NAM-NAG-NAMol-Ala-Glu-Dpm-Ala*	1371.5	1371.1	$C_{53}H_{88}N_8O_{32}$
	NAG-NAM-NAG-NAMol-Ala-Glu-Dpm	1354.5	1354.1	$C_{53}H_{87}N_8O_{31}$
	NAM-NAG-NAMol-Ala-Glu-Dpm-Ala	1239.5	1240.1	$C_{48}H_{80}N_8O_{28}$
	NAG-NAM-NAG-NAMol-Ala-Glu	1182.5	1182.0	$C_{46}H_{76}N_6O_{28}$
	NAG-NAM-NAG-NAMol-Ala	1070.4	1070.9	$C_{41}H_{69}N_5O_{26}$
	NAG-NAMol-Ala-Glu-Dpm-Ala	964.4	964.8	$C_{37}H_{63}N_7O_{21}$
	NAMol-Ala-Glu-Dpm-Ala	761.3	761.6	$C_{29}H_{50}N_6O_{16}$
	NAG-NAM-NAG$^+$	704.3	704.6	$C_{27}H_{44}N_3O_{17}$
	NAG-NAM$^+$	501.2	501.4	$C_{19}H_{31}N_2O_{12}$

[a]The terminal NAM is in the alcohol form due to borohydride reduction.

[b]The asterisk indicates loss of a pyruval group from one of the NAM residues.

[c]The + symbol (e.g., NAG-NAM$^+$ or NAG-NAM-NAG$^+$) indicates that a water molecule has been lost during fragmentation.

Table S4. Tandem mass spectrometry analysis of muropeptides Pa and Pb.

Muropeptide	Structure	Expected *m/z* $[M+Na]^+$	Observed *m/z* $[M+Na]^+$	Formula
Pa, Tetrasaccharide anhydro	NAG-NAM-NAG-NAM anhydro	979.3	979.3	$C_{38}N_4O_{24}H_{60}$
	NAG-NAM-NAG-NAM anhydro*[a]	907.3	907.2	$C_{35}N_4O_{22}H_{56}$
	NAM-NAG-NAM anhydro	776.3	776.2	$C_{30}N_3O_{19}H_{47}$
	NAG-NAM-NAG^{+}[b]	704.3	704.2	$C_{27}N_3O_{17}H_{44}$
	NAG-NAM anhydro	501.2	501.1	$C_{19}N_2O_{12}H_{30}$
Pb, Hexasaccharide anhydro	NAG-NAM-NAG-NAM-NAG-NAM anhydro	1457.5	1457.8	$C_{57}N_6O_{36}H_{90}$
	NAG-NAM-NAG-NAM-NAG-NAM anhydro*[a]	1385.5	1385.2	$C_{54}N_6O_{34}H_{86}$
	NAM-NAG-NAM-NAG-NAM anhydro	1254.5	1254.3	$C_{49}N_5O_{31}H_{77}$
	NAG-NAM-NAG-NAM-NAG^{+}	1182.4	1182.3	$C_{46}N_5O_{29}H_{74}$
	NAG-NAM-NAG-NAM anhydro	979.3	979.3	$C_{38}N_4O_{24}H_{60}$
	NAG-NAM-NAG-NAM anhydro*[a]	907.3	907.3	$C_{35}N_4O_{22}H_{56}$
	NAM-NAG-NAM anhydro	776.3	776.2	$C_{30}N_3O_{19}H_{47}$
	NAG-NAM-NAG^{+}	704.3	704.2	$C_{27}N_3O_{17}H_{44}$
	NAG-NAM anhydro	501.2	501.1	$C_{19}N_2O_{12}H_{30}$

[a]The asterisk indicates loss of a pyruval group from one of the NAM residues.

[b]The + symbol (e.g., NAG-NAM^{+} or NAG-NAM-NAG^{+}) indicates that a water molecule has been lost during fragmentation. This explains why NAG-NAM^{+} and NAG-NAManhydro have the same expected *m*/*z* ratio.

Table S5. Strains used in this study

Strain	Relevant features	Source or reference
E. coli		
BL21	*dcm ompT hsdS*(r_B^- m_B^-) *gal* [*malB*$^+$]$_{K-12}$(λ^S)	Lab collection
EC251	K-12 wild type MG1655	Lab collection
EC2292	BL21(λDE3)/pET28a-AmiD	Tom Bernhardt
EC3087	BL21/pDSW1557	This study
EC3204	BL21/pDSW1600	This study
EC3220	BL21/pDSW1601	This study
EC3223	BL21/pDSW1604	This study
EC3225	BL21/pDSW1606	This study
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Kan^R	(Simon, 1983)
P. aeruginosa		
MJ1	UCBPP-PA14 pathogenic isolate wild type	Lab collection
MJ7	PA14 *rlpA*::*MAR2xT7*	(Liberati *et al.*, 2006)
MJ18	PA14 *dacC*::*MAR2xT7*	(Liberati *et al.*, 2006)
MJ24	MJ1 Δ*rlpA*	This study
MJ26	MJ1 Δ*rlpA*/pJN105	This study
MJ27	MJ1 Δ*rlpA*/pDSW1398	This study
MJ34	MJ1 Δ*sltb1*	This study

MJ36	MJ1 *rlpA*::*mCherry*	This study
MJ42	MJ1 *rlpA*(Δ269-341)::*mCherry*	This study
MJ47	MJ1 Δ*mltb1*	This study
MJ49	MJ1 Δ*sltb1*Δ*mltb1*	This study
MJ81	MJ1 *rlpA*(E120A)::*mCherry*	This study
MJ83	MJ1 *rlpA*(D123A)::*mCherry*	This study
MJ85	MJ1 *rlpA*(H131A)::*mCherry*	This study
MJ89	MJ1 *rlpA*(D157N)::*mCherry*	This study
MJ117	MJ1 Δ*rlpA*/pDSW1635	This study
MJ119	MJ1/pDSW1635	This study
MJ137	MJ24/pMRP9-1	This study

Table S6: Plasmids used in this study

Plasmid	Relevant features	Source or reference
pDSW913	*rfp* fusion vector; Amp^R	(Arends *et al.*, 2010)
pDSW1385	pEXG2::'*sltb1*-Δ*rlpA*-*dacC*'	This study
pDSW1398	pJN105::*rlpA*	This study
pDSW1399	pDSW913::*rlpA*	This study
pDSW1489	pEXG2::'*rlpA*-*mCherry*-*dacC*'	This study
pDSW1490	pEXG2::'*rodA*-Δ*sltb1*-*rlpA*'	This study
pDSW1497	pDSW913::*rlpA*(Δ269-341)	This study
pDSW1504	pEXG2::'*rlpA*(Δ269-341)-*mCherry*-*dacC*'	This study
pDSW1516	pEXG2::'*PA14_57740*-Δ*mltb1*-*cysD*'	This study
pDSW1518	pJN105::*rlpA*-*mCherry*	This study
pDSW1519	pJN105::*rlpA*(E120A)-*mCherry*	This study
pDSW1520	pJN105::*rlpA*(D123A)-*mCherry*	This study
pDSW1537	pJN105::*rlpA*(H131A)-*mCherry*	This study
pDSW1545	pJN105::*rlpA*(D157N)-*mCherry*	This study
pDSW1557	pQE-80L::*rlpA* (28-341)	This study
pDSW1600	pQE-80L::*rlpA*(D157N) (28-341)[a]	This study
pDSW1601	pQE-80L::*rlpA*(E120A) (28-341) [a]	This study
pDSW1604	pQE-80L::*rlpA*(D123A) (28-341) [a]	This study

pDSW1606	pQE-80L::*rlpA*(H131A) (28-341) [a]	This study
pDSW1614	pEXG2::'*sltb1-rlpA-mCherry-dacC*'	This study
pDSW1615	pEXG2::'*sltb1-rlpA*(E120A)-*mCherry-dacC'*	This study
pDSW1616	pEXG2::'*sltb1-rlpA*(D123A)-*mCherry-dacC*'	This study
pDSW1617	pEXG2::'*sltb1-rlpA*(H131A)-*mCherry-dacC*'	This study
pDSW1619	pEXG2::'*sltb1-rlpA*(D157N)-*mCherry-dacC*'	This study
pDSW1635	pJN105::*amiB-mCherry*	This study
pEXG2	Suicide vector; ColEI ori *mob sacB* $Gent^R$	(Rietsch *et al.*, 2005)
pET28a-AmiD	*his6-amiD*	(Uehara & Park, 2007)
pJN105	Arabinose regulation (P_{BAD}); pBBR ori $Gent^R$	(Newman & Fuqua, 1999)
pMRP9-1	Constitutive expression of *gfp* in *P. aeruginosa*; $Carb^R$	(Davies *et al.*, 1998)
pQE-80L	P_{T5} containing *lac* operators; $lacI^q$ ColE1 ori Amp^R	Qiagen

[a]The numbers 28-341 refer to the residues of RlpA included in the construct; the first 27 a.a. of RlpA encode the signal sequence and were omitted.

Table S7: Primers used in this study

Primer	Sequence[a]
P1474	AAAATCTAGAGGAGGAGCGGACACGCTTGCTC
P1475	AAAATCTAGACCGACGCTGGTACGCCCCGACTG
P1507	AAAAAAGCTTCGGCCCAGGCGGGGGACTAC
P1508	AAAACAATTGCTTCCAGACCAGGCCCTTGG
P1599	GCCGAATTCAGCAAGCGTGTCCGCTCCTCC
P1600	CTGTCTAGAGTTGTTGTTGTCGGGGCGTACCAGCGTCGG
P1603	GCAGAATTCGACCAGAAGGTCACGGCGATG
P1604	CAATCTAGATCAGTCGGGGCGTACC
P1680	GCAAAGCTTAAGCGTGTCCGCTCCTCCCTG
P1681	GCCCAATTGTTACTTGTACAGCTCGTCCAT
P1682	GCACAATTGGCGCCTACTCACGCAGGGAAT
P1683	GGCGGTACCGTCATGGTCAGGTCTTCGGCG
P1702	CAGAAGCTTCATGCTGATGAAGCAGGCCAC
P1703	CTGCTCGAGCAGTACTTGCATTGCGTTCTT
P1704	CAGCTCGAGCGCGCGCGAGGTGCCCATTGA
P1705	CTGGAATTCTGCTGGTTGCGTACGACCGAG
P1708	CTGTCTAGAGTTGTTGTTGAGATACAGGCCATCGGCTGG
P1711	CTGAAGCTTCAGTCGGGGCGTACCAGCGTC

P1713 CAGAAGCTTGAAGGCAGCGTCGAAACCGTAC

P1714 CTGCTCGAGCAGGGCGAGGGCGGTACGGCG

P1715 CAGCTCGAGTCCGTCGTCAGGCAGGATTAG

P1716 CTGGGTACCCTGAGCACCCTGGTCGAAGAG

P1727 CTGGAGCTCTTACTTGTACAGCTCGTCCATG

P1754 TGGGACGTCGACGTGTCGCGGATC

P1755 CATTCTAGAGTTGTTGTTGTCGGG

P1756 TAGAGGTCGTAGGTCGCGCCGTTGGCGGTGG

P1758 GTCATGCCGTAGAGGGCGTAGGTCTCGCCGT

P1760 AACGGCAGGGTCTTGGCCGCGGCGGTCATGCC

P1781 ATAGAACGGGCCGCGGTTGTTGACGCGGACGATC

P1787 CATTGATCATCCAGCAAGGCGCCCCAGCAG

P1805 CAGGAATTCCCACCCTGACCATGGGAGCATG

P1806 CTGTCTAGAGTTGTTGTTCTGGGCCGCCAGGGCGGTGCT

P1821 CAGAAGCTTTACTGCGTACATGGGCGGCCAG

P1822 CGGGGATCCGCGACACGTCGACGTC

P1823 CGCGGATCCCCGATGCGGTGCCGA

P1824 ACGGCGGCCGCGTGCTGCGCCGGC

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[a]All primer sequences are written 5' to 3'. Restriction sites are underlined.

Supplemental Figure Legends

Figure S1. Phenotypes associated with *rlpA*. (A) The cytoplasm is compartmentalized between cells in chains of a Δ*rlpA* mutant as demonstrated by fluorescence loss in photobleaching (FLIP). The figure shows an overlay of DIC and fluorescence images of strain MJ137, a Δ*rlpA* mutant harboring a plasmid that produces high levels of cytoplasmic GFP. The cell to be bleached is indicated with an arrow. The cell was bleached by iterative exposure to a beam of light from an argon laser. Cells were photographed immediately before, immediately after, and 30 sec after bleaching. Note that the neighboring cell did not lose fluorescence, indicating that septation had gone to completion. A total of 21 cells from 16 different chains were analyzed by FLIP; those on the end of a chain have only one septum but those internal to the chain have two septa, so 35 septa were tested in total. Of these, 30 were closed (86%) while 5 were open (14%). (B) Rescue of Δ*rlpA* by osmolytes. Tenfold serial dilutions of WT and Δ*rlpA* cells were spotted onto LB0N plates containing the indicated concentrations of NaCl, proline, or sucrose. Plates were photographed after incubation overnight at 37°C. Strains shown are MJ1 (WT) and MJ24 (Δ*rlpA*). (C) Two examples showing that the Δ*rlpA* mutant lyses on LB0N. Numbers in the lower right refer to the time in minutes between images. Strain MJ24 (Δ*rlpA*) in LB0N was spotted on an agarose pad and photographed under phase contrast over a period of five hours. About 10% of the cells lysed during the period of observation. In photographs taken the next morning, this had increased to 50%. The remaining cells were phase-dark but did not grow after the first few hours. For the cells that lysed, we observed a general disintegration of the wall and

rounding-up, not specific lysis at constrictions. Note that the cells shown here were maintained at room temperature, whereas plates and growth curves shown elsewhere in this paper were incubated at 37°C. Although the Δ*rlpA* mutant does not form colonies on LB0N plates at room temperature, we do not know if the proportion of lysing cells is different at different temperatures. (D) A field of cells showing representative results for localization of wild-type *rlpA-mCherry*. Filled arrows point to septal localization in cells at different stages of the constriction process. Filled triangles point to examples of polar localization. Open arrows point to foci along the lateral wall. The strain shown is MJ36 (*rlpA-mCherry*).

Figure S2. RlpA is not upregulated by low osmolarity. Cells producing RlpA-mCherry (strain MJ36) were grown to an OD_{600} ~0.5 in LB or LB0N before harvest. Whole-cell extracts were diluted as indicated and subjected to Western blotting with anti-mCherry sera. Molecular mass standards are shown at the left. The expected molecular mass of RlpA-mCherry is 61 kDa after removal of the signal sequence.

Figure S3. RlpA digests PG sacculi from a Δ*rlpA* mutant. Unlabeled PG sacculi isolated from MJ1 (WT) or MJ24 (Δ*rlpA*) after growth in LB0N were incubated with His_6-RlpA. Reaction mixtures were centrifuged to separate residual insoluble PG from soluble fragments released into the supernatant. Both fractions were subjected to muropeptide analysis. Peaks are numbered and were characterized as described in the legends to Figures 6 and 8.

Figure S4. RlpA does not cleave isolated tetrasaccharide. A portion of the P5 product (NAG-NAM-NAG-NAMol) that had been isolated by RP-HPLC for mass spectrometry analysis was divided into three aliquots and incubated with buffer (negative control), 4 μM His_6-RlpA or 4 μM His_6-RlpA(D157N) (another negative control). Reaction mixtures were analyzed by RP-HPLC.

Figure S5. RlpA activity is potentiated by AmiD. This is a companion to Figure 8 and shows additional controls. Dye-labeled sacculi from a wild-type *E. coli* strain were incubated overnight with buffer ("untreated"), 1 μM His_6-AmiD or 1 μM His_6-RlpA, as indicated. These sacculi preparations were then incubated with 4 μM His_6-RlpA, His_6-RlpA(D157N), His_6-AmiD or buffer. Dye-release was read after 480 min of incubation.

Figure S6. Sequence analysis of RlpA. (A) Identification of conserved residues in the RlpA-like DPBB domain targeted for mutagenesis. Mutagenized residues are bolded and highlighted in grey. Conserved amino acids are indicated below the sequence with * (invariant), : (highly conserved) and . (moderately conserved). Sequences were aligned using Clustal Omega (Sievers *et al.*, 2011) with default parameters. Sequences of RlpA were obtained from: *P. aeruginosa* UCBPP-PA14 protein PA14_12090 residues 100-194, *Vibrio parahaemolyticus* RIMD 2210633 protein VP0720 residues 84-178, *Yersinia pestis* Z176003 protein YPZ3_2296 residues 65-171, *Klebsiella pneumoniae* 342 protein KPK_3908 residues 79-170, *E. coli* K-12 MG1655 RlpA residues 79-171, and *Caulobacter crescentus* ATCC 19089 protein CC_1825 residues 67-161. (B) Sequence of the active site from RlpA and MltA aligned with other

suspected lytic transglycosylases. The catalytically important D157 of RlpA (this study) is bold and highlighted in red. The catalytic D308 of MltA (van Straaten *et al.*, 2007) is bold and highlighted in blue. Conserved amino acids are indicated below the alignment as in (A). To produce this alignment, we used Phyre to model the DPBB domain of *P. aeruginosa* RlpA onto the structures of PA4485, MltA and YoaJ. The relevant portions of these sequences, together with the corresponding region from *E. coli* RlpA, were then aligned using Clustal Omega (Sievers *et al.*, 2011) with default parameters. The Clustal alignment conformed to the Phyre models. The sequences shown are: *P. aeruginosa* UCBPP-PA14 protein PA14_12090 residues 152-174; *E. coli* K12 MG1655 RlpA residues 131-153; *P. aeruginosa* PAO1 PAO1-UW protein PA4485 residues 84-106; *E. coli* K12 MG1655 MltA residues 292-314 (numbering is for the mature protein, after removal of the signal sequence); and *B. subtilis* 168 YoaJ residues 90-112.

Figure S7. Other PG hydrolases: SltB1, MltB1 and AmiB. (A) Division phenotypes of lytic transglycosylase mutants do not mimic Δ*rlpA*. Cells grown at 37°C in LB or LB0N to an OD_{600} ~0.5 were fixed, stained with the membrane dye FM4-64 and photographed under fluorescence. The white bar represents 2 µm. Strains shown are MJ1 (WT), MJ24 (Δ*rlpA*), MJ34 (Δ*sltb1*), MJ47 (Δ*mltb1*), and MJ49 (Δ*sltb1*Δ*mltb1*). (B) Septal localization of AmiB-mCherry does not require *rlpA*. Cells of MJ119 (WT/pJN105::*amiB-mCherry*) and MJ117 (Δ*rlpA*/ pJN105::*amiB-mCherry*) were grown in LB0N to OD_{600} ~0.5 and photographed under phase (above) and fluorescence (below). Filled triangles point to blebs where cells are lysing at division sites, perhaps

provoked by the AmiB-mCherry fusion. The fluorescence micrographs were inverted to better visualize localization and blebbing.

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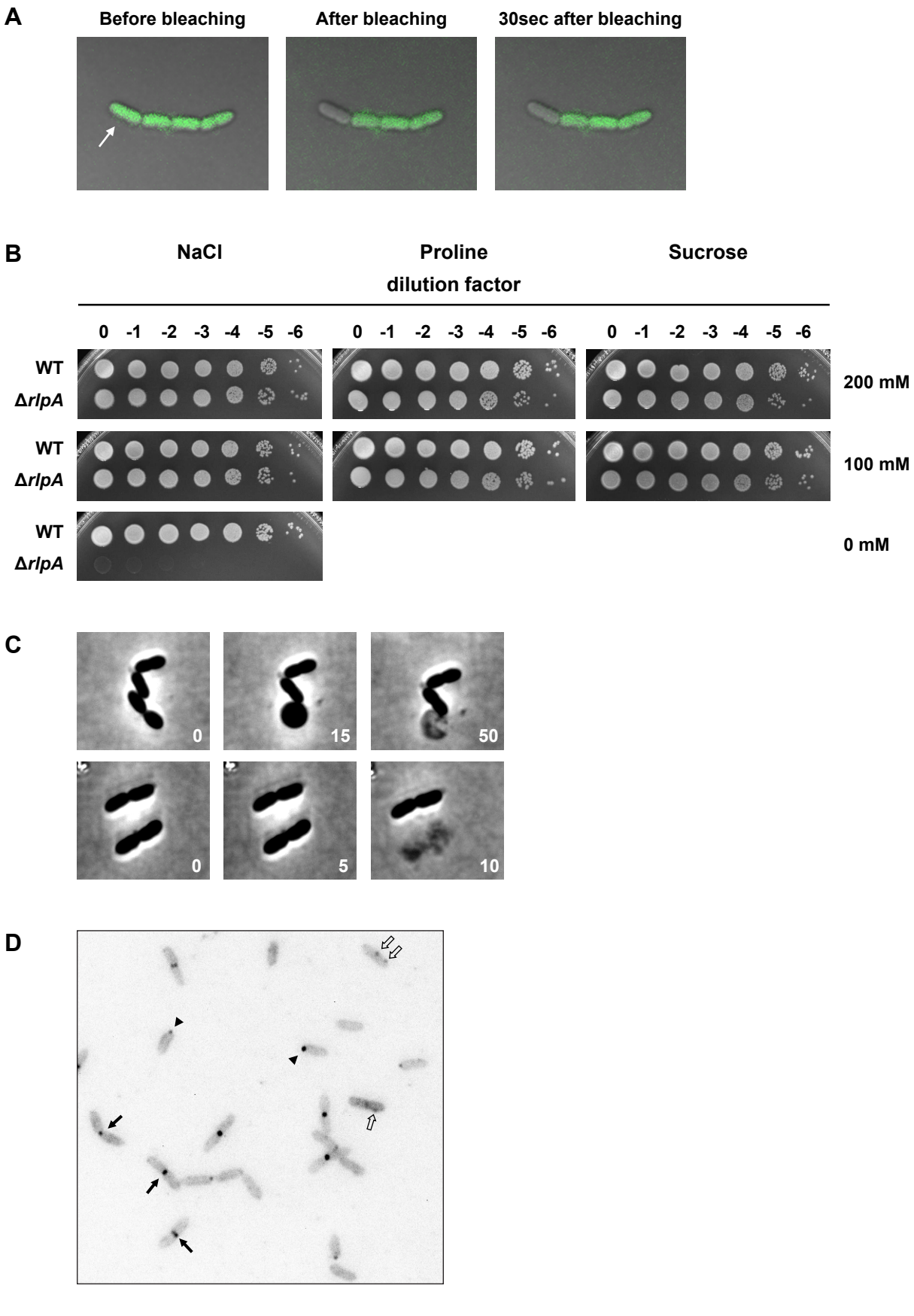
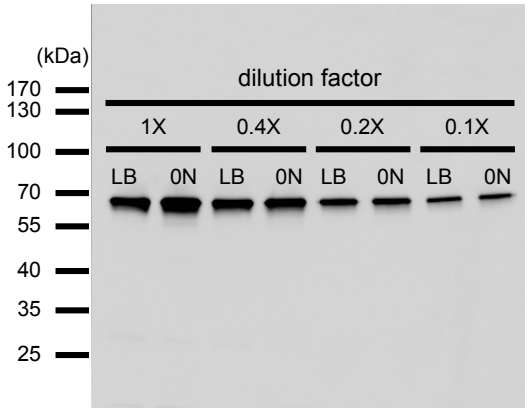

A
Before bleaching
After bleaching
30sec after bleaching
B
NaCl
Proline
Sucrose
dilution factor
0 -1 -2 -3 -4 -5 -6
WT
ΔrlpA
200 mM
100 mM
0 mM
C
0
15
50
0
5
10
D

Figure S1


(kDa)
170
130
100
70
55
40
35
25
dilution factor
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0.2X
0.1X
LB
0N
LB
0N
LB
0N
LB
0N

Figure S2

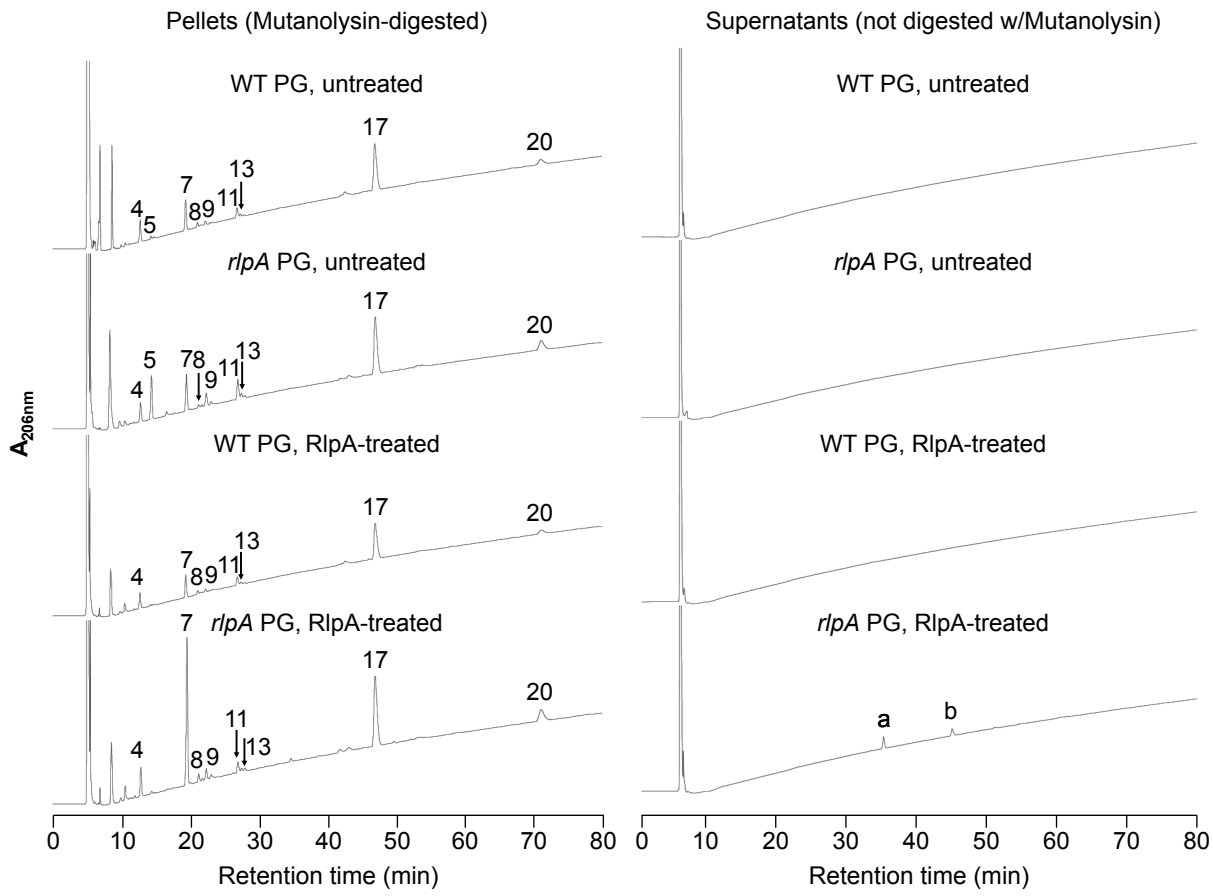

Pellets (Mutanolysin-digested)
Supernatants (not digested w/Mutanolysin)
WT PG, untreated
rlpA PG, untreated
WT PG, RlpA-treated
rlpA PG, RlpA-treated
A206nm
4
5
7
8
9
11
13
17
20
a
b
0
10
20
30
40
50
60
70
80
Retention time (min)

Figure S3

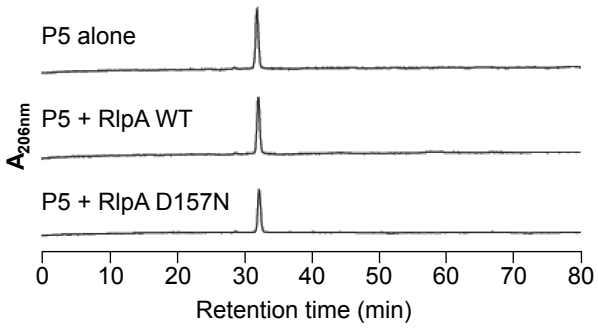
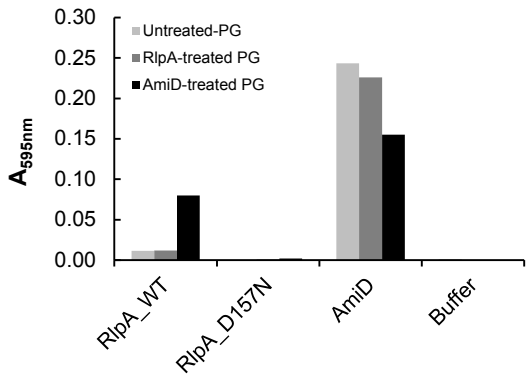

P5 alone
P5 + RlpA WT
P5 + RlpA D157N
A206nm
0
10
20
30
40
50
60
70
80
Retention time (min)

Figure S4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0.25
0.20
0.15
0.10
0.05
0.00
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Untreated-PG
RlpA-treated PG
AmiD-treated PG
RlpA_WT
RlpA_D157N
AmiD
Buffer

Figure S5

A

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P. aeruginosa        MVGTASWYGTKFHGQATANGETYDLYGMTAAHKTLPLPSYVRVTNLD
V. parahaemolyticus  EKGRASWYGKKFQGHLTSNGEIYDMYSMTAAHKTLPLPSYVKVTNTD
Y. pestis            QIGLASSYGEEARGNTTATGEIFDPNALTAAHPTLPIPSYVRVTNVS
K. pneumoniae        QAGFAAIYDAEPNSNLTASGETFDPTQLTAAHPTLPIPSYARITNLA
E. coli              QAGLAAIYDAEPGSNLTASGEAFDPTQLTAAHPTLPIPSYARITNLA
C. crescentus        VVGIGSWYGEQFHNRKTSNGEIFDMNLPSAAHKTLPLPSLVEVTNLD
                       * .: *  :   . *:.** :*    :*** ***:** ..:**

P. aeruginosa        NGKSVIVRVNDRGPFYSDRVIDLSFAAAKKLGYAETGTARVKVEGIDP
V. parahaemolyticus  NGKTTVVRVNDRGPFHDGRIIDLSYAAAHKLDVIKTGTANVEIEVISV
Y. pestis            NGRQIVVRVNDRGPYTPGRVIDLSRAAADRLNISN--NTKVKIDFINV
K. pneumoniae        NGRMIVVRINDRGPYGNDRVISLSRASADRLNTSN--NTKVRIDPIIV
E. coli              NGRMIVVRINDRGPYGNDRVISLSRAAADRLNTSN--NTKVRIDPIIV
C. crescentus        NGRKMILRVNDRGPFVGDRIIDLSKAAADELGYRRQGVARVRVKYVGP
                     **:  ::*:*****:   *:*.** *:*..*   .   ..:    :
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B

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RlpA_P. aeruginosa          IVRVNDRGPFYSDRVIDLSFAAA
RlpA_E. coli                VVRINDRGPYGNDRVISLSRAAA
PA4485_P. aeruginosa        VVRINDRGPFRRGRIIDVSRKAA
MltA_E. coli                LMVALDVGGAIKGQHFDIYQGIG
YoaJ_B. subtilis            TVYVTDLYPEGARGALDLSPNAF
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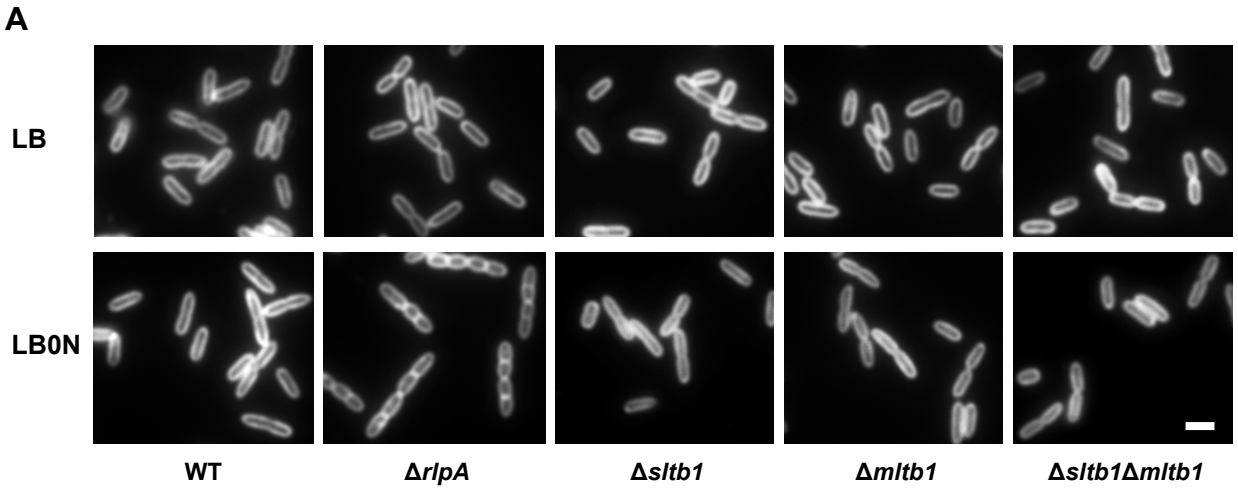
Figure S6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LB0N
WT
ΔrlpA
Δsltb1
Δmltb1
Δsltb1Δmltb1

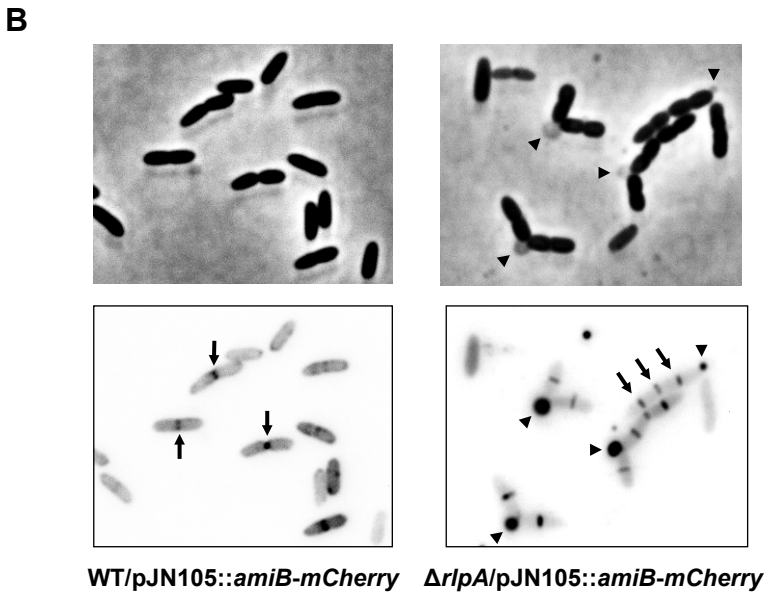
B
WT/pJN105::amiB-mCherry
ΔrlpA/pJN105::amiB-mCherry

Figure S7