Supplemental data for 'Peptidoglycan DD-peptidases have distinct activities that impact fitness of *Acinetobacter baumannii*'

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Supplementary Figures

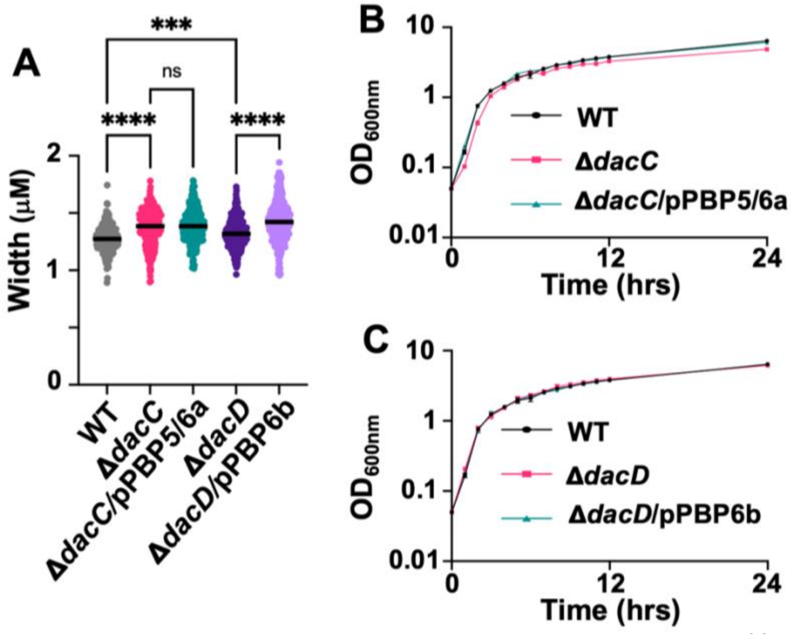

Figure S1: Analysis of Δ*dacC* and Δ*dacD* mutations in *A. baumannii* strain ATCC 17978. (A) Quantifications of cell width in wild-type (WT), Δ*dacC*, Δ*dacC*/pPBP5/6a, Δ*dacD*, and Δ*dacD*/pPBP6b (*n* ≥300), measured using ImageJ with the MicrobeJ plugin. Each dot represents a single cell. Error bars indicate standard deviation. Statistical significance was determined using one-way ANOVA (*** P < 0.001, **** P < 0.0001, ns = not significant). **(B)** Growth curves of the Δ*dacC* mutant. **(C)** Growth curves of the Δ*dacD* mutant.

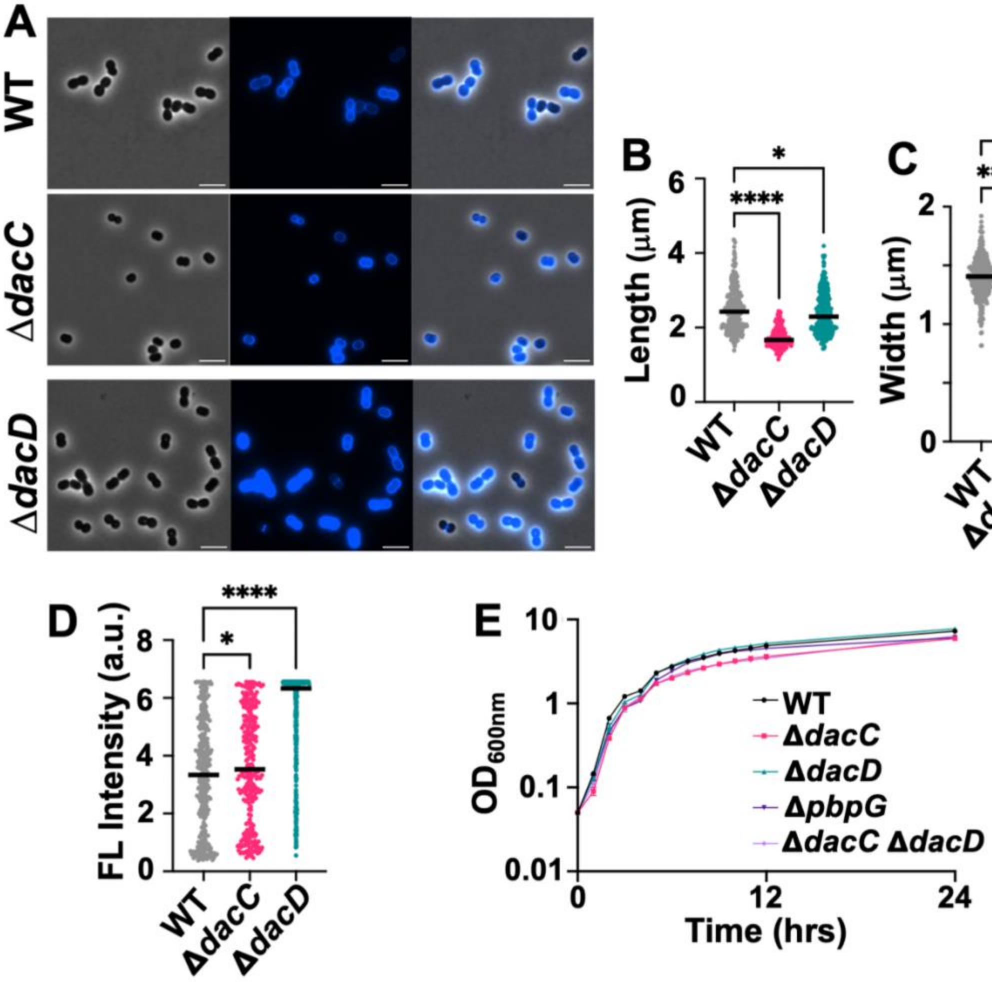

Figure S2 : Analysis of *A. baumannii* strain AB5075 mutants. (A) Phase-contrast (left) fluorescence (middle) and merged (right) images of wild-type (WT), Δ*dacC*, Δ*dacD* cells. Scale bar: 5 µm. (**B)** Quantification of cell length (pole to pole) for each strain ($n \geq 300$) was measured using ImageJ with the MicrobeJ plugin. Each dot represents a single cell. Error bars indicate standard deviation. Statistical significance was determined using one-way ANOVA ($^{*}P < 0.05$, $^{****}P < 0.0001$, ns = not significant). **(C)** Quantification of cell width. **(D)** Quantification of fluorescence intensity. **(E)** Growth curves of Δ*dacC,* Δ*dacD,* and Δ*pbpG* mutants. Each experiment was independently replicated three times; one representative data set is shown.

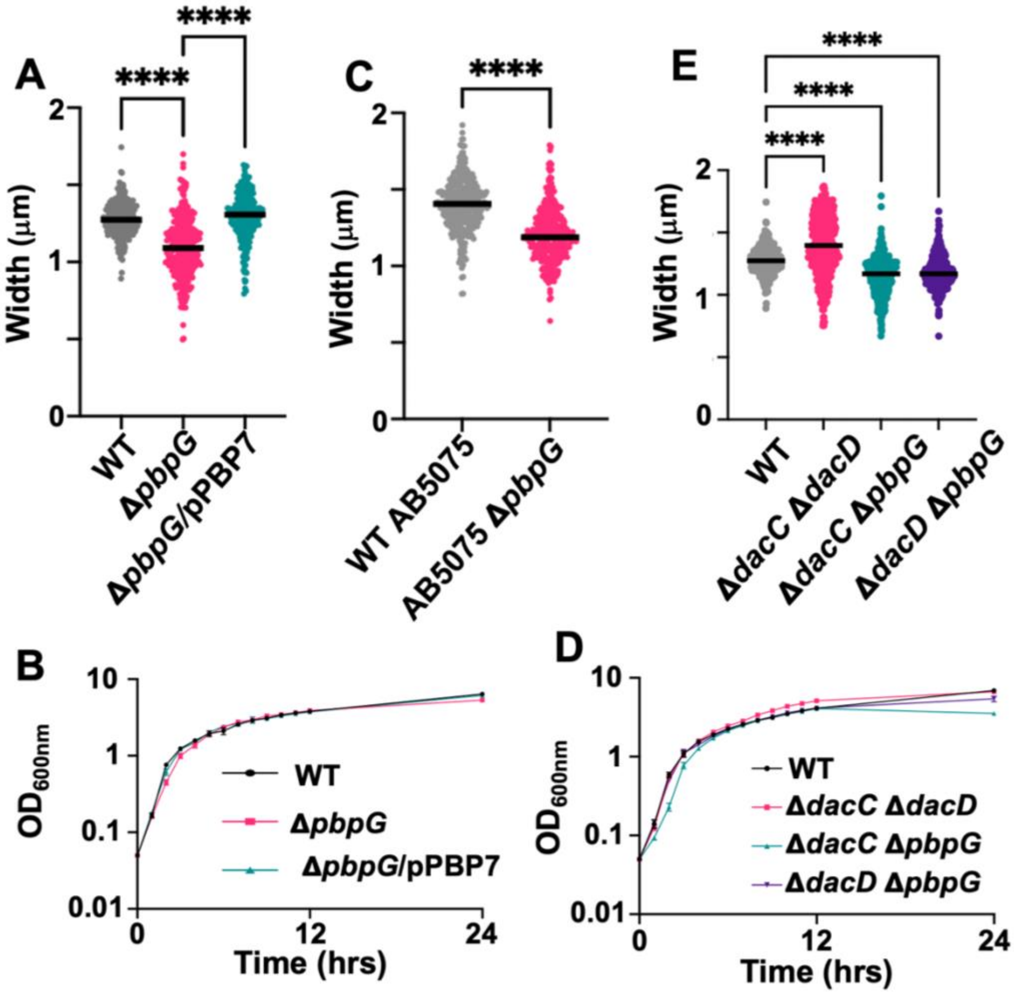

Figure S3: Cell morphology measurement and growth curves of Δ*pbpG* mutants and double mutants. (A) Quantifications of cell width in *A. baumannii* strain ATCC 17978 Δ*pbpG* mutants (*n* ≥300) , measured using ImageJ with the MicrobeJ plugin. Each dot represents a single cell. Error bars represent standard deviation. Statistical significance was determined using one-way ANOVA (**** $P < 0.0001$). **(B)** Growth curves of Δ*pbpG* mutants in strain ATCC 17978. **(C)** Same as described in (A), but for Δ*pbpG* mutants in *A. baumannii* strain AB5075. **(D)** Growth curves of double mutants in strain *A. baumannii* ATCC 17978. **(E)** Same as described in (A), but for double mutants in *A. baumannii* strain ATCC 17978.

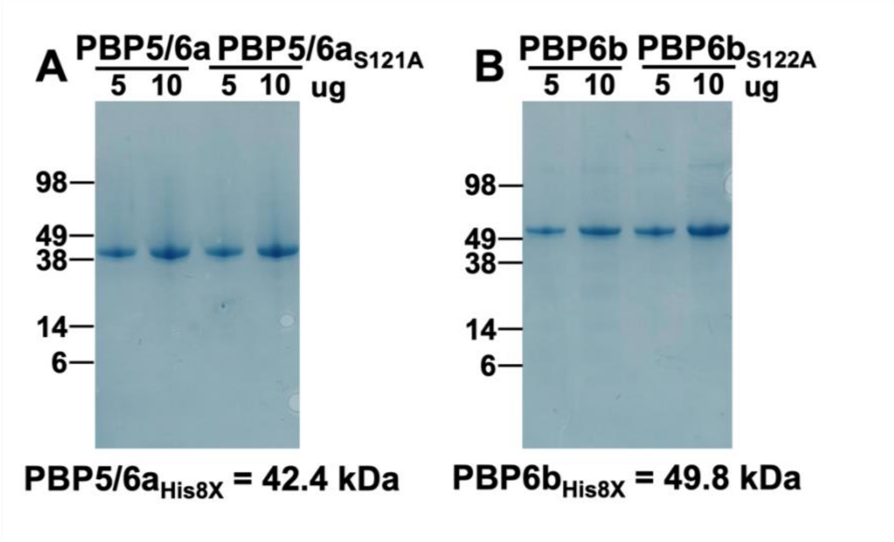

Figure S4: Purification of PBP5/6a and PBP6b enzymes. (A) Coomassie stained SDS-PAGE gel of PBP5/6a$_{His8X}$ and the catalytically inactive mutant PBP5/6a$_{S121A\ His8X}$. **(B)** Coomassie stained SDS-PAGE gel of PBP6b$_{His8X}$ and the catalytically inactive mutant PBP6b$_{S122A\ His8X}$.

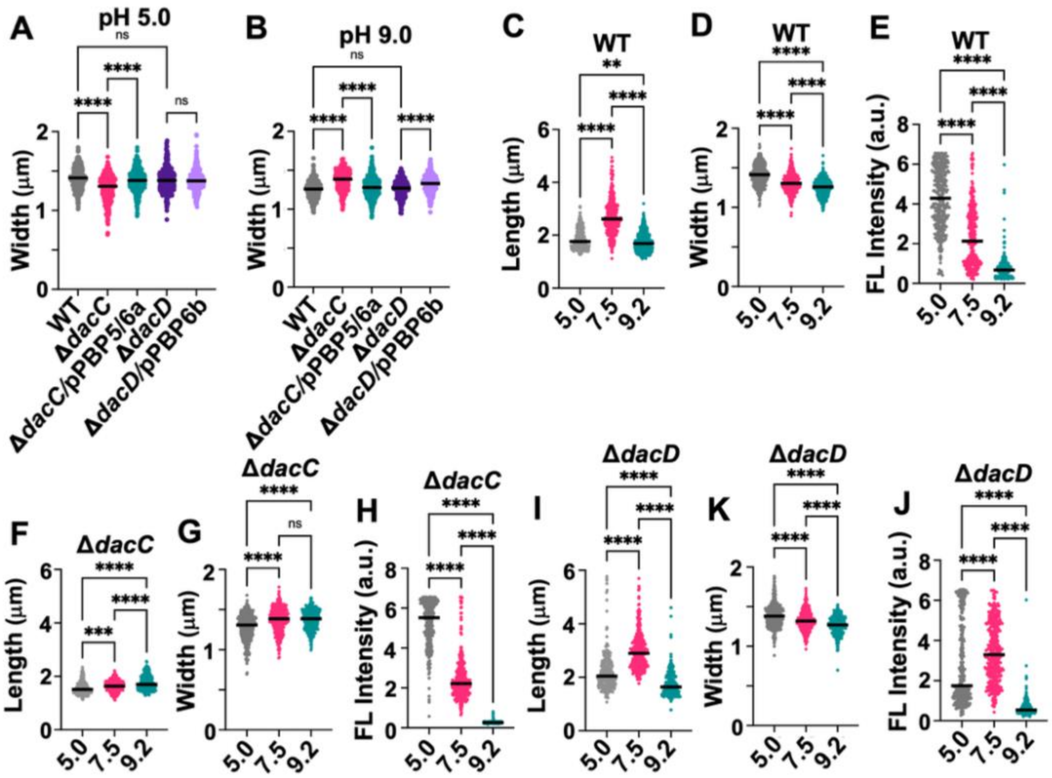

Figure S5: Quantitative analysis of *A. baumannii* strain ATCC 17978 cell morphology and fluorescence under acidic and alkaline conditions. (A) Quantifications of width in wild type (WT), Δ*dacC*, Δ*dacC*/pPBP5/6a, Δ*dacD*, and Δ*dacD*/pPBP6b strains grown at pH 5.0. **(B)** Same as (A), but for cells grown in pH 9.0. Quantifications (*n* $\geq$300) were performed using ImageJ with the MicrobeJ plugin. Each dot represents a single cell. Error bars represent standard deviation. Statistical significance was determined using one-way ANOVA (** *P* <0.01, *** *P* <0.001, **** *P* <0.0001, ns = not significant). **(C)** Quantification of length, **(D)** width, and **(E)** fluorescence intensity in WT cells grown at pH 5.0, 7.5, and 9.0. **(F, G, H)** Same as described for (C-E), but for Δ*dacC* cells. **(I, J, K)** Same as described for (C-E), but for Δ*dacD* cells.

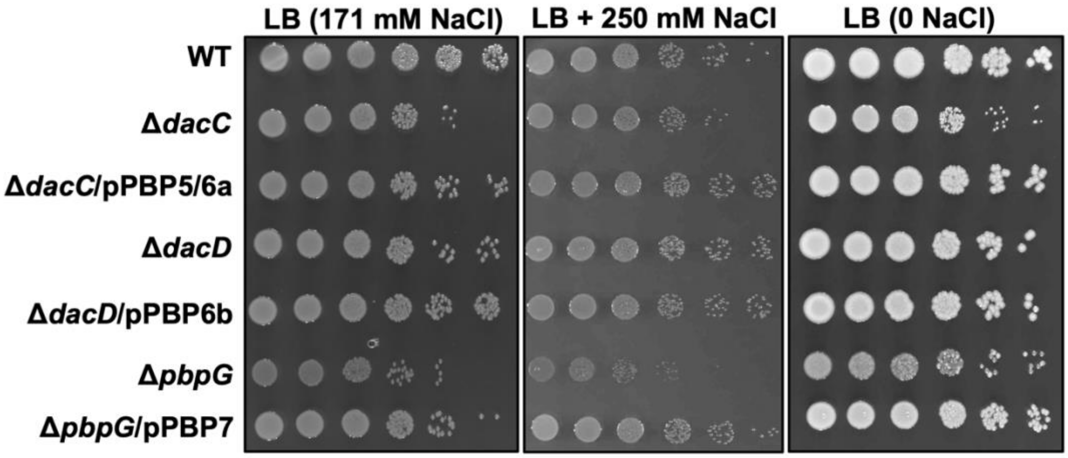

Figure S6: Colony growth of *A. baumannii* strain ATCC 17978 mutants under osmotic stress. (A) Colony spot assays of wild-type (WT) and DD-CPase mutant strains. Cultures were serially diluted 10-fold starting at OD_{600} 0.05, plated on LB agar supplemented with the indicated NaCl concentrations to impose hypo- or hyperosmotic conditions.

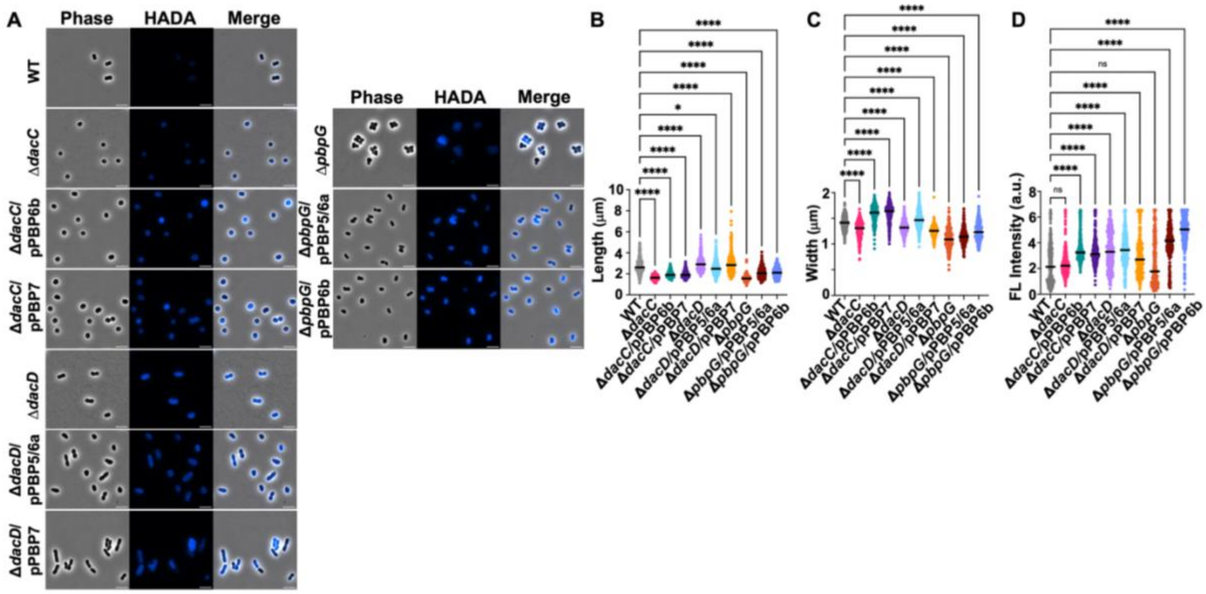

Figure S7: DD-CPase mutants cannot be complemented by other DD-CPases. (A) Representative phase-contrast (left), fluorescence (middle), and merged images of wild-type (WT), and DD-CPase mutants: Δ*dacC*, Δ*dacC*/pPBP6b, Δ*dacC*/pPBP7, Δ*dacD*, Δ*dacD*/pPBP5/6a, Δ*dacD*/pPBP7, Δ*pbpG,* Δ*pbpG*/pPBP5/6a, and Δ*pbpG*/pPBP6b. Scale bar: 5 μm. **(B)** Cell length measurements (pole to pole) for each strain (*n* ≥ 300), quantified using ImageJ with the MicrobeJ plugin. Each dot represents a single cell; error bars indicate standard deviation. Statistical significance was determined using one-way ANOVA ($p < 0.05$, ns = not significant). **(C)** Quantifications of cell width. **(D)** Quantification of fluorescence intensity for the same cell populations. (a.u. - arbitrary units).

Supplementary Tables

Table S1. Established DD-peptidases in *E. coli vs* homologs in *A. baumannii*

E. coli					***A. baumannii***			
Gene	**Protein**	**Length (aa)**	**Function**		**Gene**	**Protein**	**Length (aa)**	**Predicted function**
dacB	PBP4	477	DD-CPase; DD-EPase		n/d	n/d	n/d	-
dacA	PBP5	403	DD-CPase		*dacC*	PBP5/6a	382	DD-CPase
dacC	PBP6a	400	DD-CPase					
*dac*D	PBP6b	388	DD-CPase		*dacD*	PBP6b	439	DD-CPase; DD-EPase
pbpG	PBP7/8	479	DD-EPase		*pbpG*	PBP7	348	DD-CPase; DD-EPase
ampC	AmpC	377	β-lactamase; DD-CPase		*bla*$_{ADC\text{-}26}$	AmpC	383	β-lactamase; DD-CPase?
ampH	AmpH/PBP4b	385	DD-CPase; DD-EPase		n/d	n/d	n/d	-

DD-CPase: DD-carboxypeptidase

DD-EPase: DD-endopeptidase

n/d: no homolog detected

Table S2: Muropeptide composition of wild type (WT), Δ*dacC*, and Δ*dacD A. baumannii* strain ATCC 17978

Peak No.	Muropeptide	Relative % of each muropeptide[a]					
		WT Logarithmic	WT Stationary	Δ*dacC* Logarithmic	Δ*dacC* Stationary	Δ*dacD* Logarithmic	Δ*dacD* Stationary
1	**Tri**	2.5 ± 0.0	2.9 ± 0.0	3.3 ± 0.2	4.0 ± 0.1	2.6 ± 0.0	2.9 ± 0.1
2	**Tri-D-Asn**	0.7 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	1.1 ± 2.2
3	**Tri-D-Lys**	0.1 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	1.2 ± 1.0	0.7 ± 0.0	1.5 ± 1.3
4	**TetraGly4**	1.8 ± 0.0	1.5 ± 0.0	2.6 ± 0.0	1.3 ± 0.8	1.6 ± 0.0	1.9 ± 3.1
5	**Tetra-D-Lys**	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.3	0.0 ± 0.0
6	**Tetra**	19.0 ± 0.4	20.7 ± 0.0	16.9 ± 7.6	19.1 ± 0.1	16.0 ± 0.4	17.6 ± 0.0
7	**Tetra-D-Arg**	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	1.5 ± 0.0
7B	**Penta**	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
8	**TetraTriDapGly4**	0.2 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
9	**TriTri(Dap)/TriTriDap-D-Lys**	0.5 ± 0.0	0.9 ± 0.1	0.6 ± 0.0	1.3 ± 0.1	0.5 ± 0.0	0.8 ± 0.2
10	**TetraTri(Dap)**	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.8 ± 1.1
11	**TetraTri**	3.7 ± 0.1	4.0 ± 0.2	4.0 ± 0.1	4.9 ± 0.6	3.1 ± 0.0	3.7 ± 0.7
12	**TetraTri-D-Lys**	0.3 ± 0.1	1.9 ± 0.7	1.5 ± 0.6	2.5 ± 2.0	0.9 ± 0.3	1.9 ± 1.8
13	**TetraTri-D-Lys**	0.9 ± 0.1	1.0 ± 0.6	0.4 ± 0.2	1.6 ± 0.2	0.4 ± 0.0	1.6 ± 0.4
14	**TetraTri-D-Arg**	0.5 ± 0.0	1.0 ± 0.4	1.4 ± 0.4	1.1 ± 1.3	2.1 ± 0.1	3.4 ± 19.2
15	**TetraTetra**	37.7 ± 1.4	31.5 ± 1.0	32.5 ± 5.8	25.8 ± 0.8	34.7 ± 0.6	25.4 ± 24.5
15B	**TetraPenta**	0.4 ± 0.0	0.7 ± 0.1	1.6 ± 0.0	1.8 ± 0.0	1.6 ± 0.4	2.8 ± 0.0
16	**TetraTetraTri or TetraTetraTriDap**	1.1 ± 0.0	1.7 ± 0.0	1.0 ± 0.5	2.0 ± 0.0	1.1 ± 0.0	1.4 ± 0.0
17	**TetraTetraTri or TetraTetraTriDap**	0.2 ± 0.0	0.6 ± 0.1	0.8 ± 0.2	1.7 ± 1.0	0.6 ± 0.1	0.6 ± 0.1
18	**TriTriDap-D-Met**	0.8 ± 0.0	1.1 ± 0.2	1.0 ± 0.8	0.7 ± 0.2	0.3 ± 0.2	1.1 ± 0.1
19	**TetraTetraTetra**	18.5 ± 0.0	16.1 ± 0.1	15.2 ± 7.2	12.8 ± 0.0	17.8 ± 0.0	13.6 ± 0.1
20	**TetraTri-D-Met**	0.4 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	1.0 ± 0.1	0.3 ± 0.0	0.5 ± 0.1
21	**TetraTriAnh / TetraTetraTetraTri**	4.8 ± 0.0	4.2 ± 0.0	3.7 ± 1.8	3.3 ± 0.0	4.7 ± 0.0	3.5 ± 0.1
22	**TetraTetraAnh I**	1.7 ± 0.2	1.3 ± 0.1	1.8 ± 0.8	1.1 ± 0.2	1.6 ± 0.3	1.2 ± 0.2
23	**TetraTetraAnh II**	0.7 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.7 ± 0.0	0.9 ± 0.1
24	**TetraTetraTetraAnh**	2.0 ± 0.0	2.1 ± 0.0	2.0 ± 0.0	1.7 ± 0.1	2.1 ± 0.0	2.0 ± 0.0
Sum of known peaks		97.7 ± 1.1	96.0 ± 3.9	93.2 ± 27.4	91.5 ± 0.0	94.7 ± 0.7	90.9 ± 0.3
Monomers (Total)		24.5 ± 0.7	27.7 ± 0.0	26.2 ± 19.8	29.6 ± 0.0	23.5 ± 0.0	28.9 ± 5.4
Monomers with modification		2.6 ± 0.0	3.2 ± 0.0	4.0 ± 0.0	3.7 ± 0.2	3.8 ± 0.1	6.4 ± 4.5
Monomer tri		2.5 ± 0.0	3.0 ± 0.0	3.5 ± 0.5	4.4 ± 0.1	2.7 ± 0.1	3.2 ± 0.1
Monomer tri-D-Asn		0.7 ± 0.0	0.4 ± 0.0	0.8 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	1.1 ± 2.4
Monomer tri-D-Lys		0.1 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	1.3 ± 1.3	0.7 ± 0.0	1.7 ± 1.6
Monomer tetraGly4		1.9 ± 0.0	1.5 ± 0.0	2.7 ± 0.0	1.4 ± 1.0	1.7 ± 0.0	2.1 ± 3.6
Monomer tetra-D-Lys		0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.3	0.0 ± 0.0
Monomer tetra		19.4 ± 0.7	21.5 ± 0.1	18.2 ± 15.7	20.9 ± 0.2	16.9 ± 0.2	19.3 ± 0.0
Monomer tetra-D-Arg		0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	1.6 ± 0.0
Monomer penta		0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.0	0.8 ± 0.1	0.1 ± 0.0	0.0 ± 0.0

Dimers (Total)	53.2 ± 1.0	51.3 ± 0.2	53.7 ± 6.1	50.7 ± 0.6	53.9 ± 0.0	52.0 ± 5.4
Dimers with modification	3.4 ± 0.2	6.9 ± 0.0	6.0 ± 3.4	9.1 ± 0.2	4.9 ± 0.1	10.1 ± 8.4
Dimer chain ends (anhydroMur*N*Ac)	7.3 ± 0.5	6.5 ± 0.1	6.6 ± 0.0	5.7 ± 0.2	7.4 ± 0.4	6.1 ± 0.2
Trimers (Total)	22.3 ± 0.0	21.2 ± 0.0	20.3 ± 4.2	19.9 ± 0.6	22.8 ± 0.0	19.2 ± 0.0
Trimer chain ends (anhydroMur*N*Ac)	2.1 ± 0.0	2.1 ± 0.0	2.2 ± 0.0	1.9 ± 0.1	2.3 ± 0.0	2.2 ± 0.0
Tripeptides (Total)	10.0 ± 0.1	13.0 ± 0.3	12.9 ± 1.8	17.0 ± 4.5	11.2 ± 0.1	16.5 ± 3.4
Tripeptides with modifications	3.1 ± 0.1	5.6 ± 0.4	5.1 ± 2.2	7.5 ± 1.0	4.1 ± 0.0	8.8 ± 1.6
Tetrapeptides (Total)	89.5 ± 0.1	86.0 ± 0.7	85.3 ± 2.4	80.5 ± 3.9	87.6 ± 0.0	81.5 ± 2.4
Tetrapeptides with modifications	2.9 ± 0.1	4.5 ± 0.4	4.9 ± 0.2	5.3 ± 1.0	4.7 ± 0.2	7.7 ± 14.0
Pentapeptides	0.2 ± 0.0	0.3 ± 0.0	1.3 ± 0.0	1.7 ± 0.0	1.0 ± 0.0	1.5 ± 0.0
3-3 Crosslinks	1.1 ± 0.0	2.1 ± 0.0	1.8 ± 0.6	2.8 ± 0.0	1.2 ± 0.1	2.2 ± 0.1
Chain ends (anhydroMur*N*Ac)	4.3 ± 0.1	4.0 ± 0.0	4.0 ± 0.0	3.5 ± 0.1	4.4 ± 0.1	3.8 ± 0.0
Degree of crosslinkage	41.4 ± 0.2	39.7 ± 0.0	40.3 ± 6.5	38.6 ± 0.0	42.1 ± 0.0	38.8 ± 1.4
% peptides in cross-links	75.5 ± 0.7	72.4 ± 0.0	73.9 ± 19.8	70.5 ± 0.0	76.6 ± 0.0	71.2 ± 5.4

[a]Values are mean ± variation of two biological repeats.

Table S3: Strains and plasmids used in this study.

Strain/Plasmid	Description	Reference/Source
Strains		
E. coli C2987	chemically competent wild type, K-12	New England Biolabs
E. coli C2527	chemically competent BL-21	New England Biolabs
A. baumannii ATCC 17978	wild type	ATCC (1)
A. baumannii 5075	wild type	(2)
A. baumannii ATCC 17978	Δ*dacC*	This Study
A. baumannii ATCC 17978	Δ*dacC*/pPBP5/6a	This Study
A. baumannii ATCC 17978	Δ*dacC*/pPBP6b	This Study
A. baumannii ATCC 17978	Δ*dacC*/pPBP7	This Study
A. baumannii ATCC 17978	Δ*dacD*	This Study
A. baumannii ATCC 17978	Δ*dacD*/pPBP6b	This Study
A. baumannii ATCC	Δ*dacD*/pPBP5/6a	This Study

17978		
A. baumannii ATCC 17978	Δ*dacD*/pPBP7	This Study
A. baumannii ATCC 17978	Δ*pbpG*	(3)
A. baumannii ATCC 17978	Δ*pbpG*/pPBP7	(3)
A. baumannii ATCC 17978	Δ*pbpG*/pPBP5/6a	This Study
A. baumannii ATCC 17978	Δ*pbpG*/pPBP6b	This Study
A. baumannii ATCC 17978	Δ*dacC* Δ*dacD*	This Study
A. baumannii ATCC 17978	Δ*dacC* Δ*pbpG*	This Study
A. baumannii ATCC 17978	Δ*dacD* Δ*pbpG*	This Study
A. baumannii 5075	Δ*dacC* Δ*dacD*	This Study
A. baumannii 5075	Δ*dacC* Δ*pbpG*	This Study
A. baumannii 5075	Δ*dacD* Δ*pbpG*	This Study
Plasmids		
pABBRKn	pABBR_MCS with the Kan^R gene from pKD4 inserted into the PvuI site, Kn^R	(5)
pAT03	pMMB67EH with FLP recombinase, Amp^R	(4)
pAT04	pMMB67EH with REC_{Ab} system, Tet^R	(4)
pKD4	Kan^R	(6)
pT7-7	Amp^R	(7)
pT7-7Kn	pT7-7 with the Kan^R gene from pKD4 inserted into the PvuI site, Kn^R	(3)
pPBP5/6a-His_{8X}	pT7-7 with *dacC* (A1S_2435) cloned into the NdeI and BamHI sites, Kn^R	This study
pPBP5/6a$_{\text{S121A}}$-His_{8X}	pT7-7 with *dacC*$_{\text{S121A}}$ cloned into the NdeI and BamHI sites, Kn^R	This study
pPBP7	pMMB67EHKn with the *pbpG* (A1S_0237) gene and native promoter inserted into the XhoI and KpnI sites, Kn^R	(3)
pPBP6b-His_{8X}	pT7-7 with *dacD* (A1S_2479) cloned into the NdeI and BamHI sites, Kn^R	This study
pPBP6b$_{\text{S122A}}$-His_{8X}	pT7-7 with *dacD*$_{\text{S122A}}$ cloned into the NdeI and BamHI sites, Kn^R	This study
pPBP5/6a	(A1S_2435) gene and native promoter inserted into the XhoI and KpnI sites, Kn^R	This study
pPBP6b	(A1S_2479) gene and native promoter inserted into the XhoI and KpnI sites, Kn^R	This study

Table S4: Oligonucleotides used in this study.

Oligo Name	**Sequence (5’ to 3’)**
Deletion Primers	
17978 *dacC* Kan-FRT 5’	GTTAAAAACTCTCTAGTTACAATACTGTTTGAAAAAGCCGACTTCCCCCATAGAAGTCGGCTTTGCTTTATCCTAAAATGCTAGGCTCTTCTGCTTCATACAAGATATTGGAATTACCTAGAATGATATCCTCCTTAGTTCCTATTCCG
17978 *dacC* Kan-FRT 3’	AACTTTACCATCTAAACTTGCAACAAGCTTACCAACGACTTGACCTTTTTGAAGTGGTGCATTTAGGTTCGGTTGAACAACCAATTGAGTTTTAATGCCGTCCGCTTTGCCTTTAGGCATAGTTAAGCGATTGTGTAGGCTGGAGCTGCTTCG
17978 *dacC*-kan verify 5’	AGCACGTAATGATGCAAAAGCTG
17978 *dacC*-kan verify 3’	CTTGGATCAATTTGTGGGTGATGGTC
17978 *dacD* Kan-FRT 5’	TTTGTATCGACTTGCGTAGATGTATTTTTTGGCAACAATTCACACTAGCCTTCTACAAAAAAAAGGCATAGCATGCTGGGCATCTGTGTTCCGTATTTGCCAGTAAACGTCAAGGTCACCTAGTG
17978 *dacD* Kan-FRT 3’	TTTGCTTCTTCAATGTGTACATCATTTTCAATTTGAAGACTGCGAATGAGCTGGTTGTTTTGATAAATTGAAACTGTTGCCAAATTCATCGCTTTCATTAACGGTGCTGTTAACTTTTGCTCATT
17978 *dacD*-kan verify 5’	GCGAATTGTCACGTGAACAAGG
17978 *dacD*-kan verify 3’	GCGTGGCGAGTTCTAAACCAC
17978 *pbpG*-Kan FRT 5’	AAAGCTTTATACCTTATATCTCAAATGTAAGGCATAATGATAGTAAGCGCAAATGTGTGTCACCCTGAGTCGAGTATTGTGCCGTGAAAAATTCTAAAAAGTCTTTAATGCATGTGCTAAGCATGatatcctccttagttcctattccg
17978 *pbpG* -Kan FRT 3’	TATCTAATATGTAAAATCTGAGTTTTTATAAAAAGCGGCTGTTTAATACAGCCGTTTTTTTATGCTTTTTAAATGGCATAAAAAAACGCTTCTTAAAAGAAGCGTTTTTAAAAATAATTAAATTAagcgattgtgtaggctggagctgcttcg
17978 *pbpG*-kan verify 5’	GCTTGCAATGGAATGACAAAATTAGCAATC
17978 *pbpG*-kan verify 3’	GATACAGCAATTAAACAATGTGCTGATGCAG
Complementation Primers	
pABBRKn-*dacC*-F XhoI	CGCCTCGAGatgcaaaagctgctggtaacgaac
pABBRKn-*dacC*-R KpnI	CGCGGTACCccacggacatttcatcaatgtaacagc
pABBRKn-*dacD*-F XhoI	CGCCTCGAGttcctgatttggattcgctaaaaagcg
pABBRKn-*dacD*-R KpnI	CGCGGTACCggctttaccatttttcccaaaagtttcacc
pABBRKn-*pbpG*-F XhoI	AAATTACTCGAGCTATTCTTCTATAGTGAGCGAATAGTTG
pABBRKn-*pbpG*-R KpnI	GTTGTCGGTACCTGCAACAATGGACCAAGTAAAAGATTCG

Overexpression Primers	
pT7-7::*dacC* NdeI	CGCCATATGACTCGAAAAAGCGCTATTGCTGCACTCCTCCTCTTAC
pT7-7::*dacC* BamHI 8X-his	CGCGGATCCTTAATGGTGATGGTGATGGTGATGGTGGTTGCTGAAGAATTGTTTGATATGG
pT7-7::*dacD* NdeI	CGCCATATGAAATTCTTCCTATCTCTTTTTACGCTGTTTAGTATTTTCTGTACTACTCTTACC
pT7-7::*dacD* BamHI 8X-his	CGCGGATCCTTAATGGTGATGGTGATGGTGATGGTGGTGCGAATCTATAGGG
Sequencing Primers	
pABBRKn confirm 1	GGGCTGACCGCTTCCT
pABBRKn confirm 2	CGCTAGCAGCACGCCATAG

References

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