PrgU: A Suppressor of Sex Pheromone Toxicity in *Enterococcus faecalis*

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Running Title: PrgU suppression of PrgB toxicity in *E. faecalis*

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TABLE S1. Bacterial strains used in this study.

Strains or plasmids	Relevant features	Source or Reference
Strains		
E. coli		
DH5α	F-φ80d*lacZ* ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1hsdR17(rK-mK+)phoA supE44λ- thi-1gyrA96 relA1*	Gibco-BRL
BL21 (DE3)	F^-ompT r_b^- m_b^- DE3	Novagen
EC1000	*E. coli* cloning host, provides RepA in *trans*	(Leenhouts *et al.*, 1996)
E. faecalis		
OG1RF	Rif^r Fus^r	(Dunny *et al.*, 1978)
OG1ES	Ery^r Str^r	(Staddon *et al.*, 2006)
TX5537	Rif^r Fus^r, OG1RF deleted of *srtA* and *bps*	(Nallapareddy *et al.*, 2006)

TABLE S2. Plasmids used in this study.

Plasmids		
pCJK47	Spc^r, carries $oriT_{pCF10,}$ *lacZ,* and P-pheS* cassette	(Kristich *et al.*, 2007)
pDL278p23	Spc^r, pDL278 with *L. lactis* constitutive promoter P_{23}	(Chen *et al.*, 2007)
pCI372	Chl^r, *E. coli* - *E. faecalis* shuttle vector	(Hayes *et al.*, 1990)
pCF10	Tet^r, pheromone inducible conjugative plasmid	(Dunny *et al.*, 1981)
pCJK205	Erm^r, plasmid constitutively expressing *lacZ*	(Djoric & Kristich, 2015)
pCF10Δ*prgU*	Tet^r, pCF10 deleted of *prgU*	This study
p10-mini	Chl^r, pCI372 carrying the entire *prgQ* regulatory region and the *prgA-prgC* gene cassette	This study
p10-miniΔ*prgU*	Chl^r, p10-mini deleted of *prgU*	This study
pMCM3	Spc^r, pCJK47 with *prg*U flanking regions for construction of pCF10 Δ*prgU*	This study
pMB11	Spc^r, pDL278p23 expressing P_{23}::*prgU*	This study
pMC001	Spc^r, pDL278p23 expressing P_{23}::*prgA*	This study
pMC002	Spc^r, pDL278p23 expressing P_{23}::*prgB*	This study
pMB4	Spc^r, pDL278p23 expressing P_{23}::*prgC*	This study
pMC003	Spc^r, pDL278p23 expressing P_{23}::*prgR,prgS*	This study

TABLE S3. Oligonucleotides used in this study.

Primers	**Sequence (5' to 3')**	**Used for:**
F- prgU up-XbaI	gctctagaggacaatggtctgtgtttgc	pMCM3
R- prgU up-XmaI	taatttttcccgggtttttcccctccataactaa	pMCM3
F- prgU down-XmaI	gggaaaaacccggaaaaattatttggaggaaattac	pMCM3
R- prgU down-NcoI	catgccatgggcctccgctaagttgcttgtt	pMCM3
F-RBS prgU-BamHI	cgcgggatccttaaaggaggtattatctcgagatggaagcagtagtagcaga	pMB11
R-prgU- SphI	ccgcgcatgcttatgattttaaagtttcgcc	pMB11
F-prgU down 5'phos	ataaaaaattatttggaggaaattacaatg	p10miniΔ*prgU*
R-prgU up 5'phos	tttcccctccataactaaaaaaagaag	p10miniΔ*prgU*
F-prgA-BamHI	cgcggatccttaaaggaggtattatatgaaaaagattgcaagt	pMC001
R-prgA-SphI	acatgcatgcttaactatttttttttacg	pMC001
F-prgB-BamHI	cgcggatccttaaaggaggtattatatgaatcaacagactgaag	pMC002
R-prgB-SphI	acatgcatgcttattttgtttcttttctacg	pMC002
F-prgC-BamHI	cgcggatccttaaaggaggtattatatgaaaaaaattattttatcaagc	pMB4
R-prgC-SphI	acatgcatgcttaagctttttttcttattc	pMB4
F-prgRS-BamHI	cgcggatccttaaaggaggtattatatgattgaactgaaagcaactg	pMC003
R-prgRS-SphI	acatgcatgctcacgtaccgcctttgttctg	pMC003
qRT-PCR Primers	**Sequence (5' to 3')**	**Source or Reference**
gyrB forward	caagccaaaacaggtcgcc	(Bourgogne *et al.*, 2007)
gyrB reverse	accaacaccgtgcaagcc	(Bourgogne *et al.*, 2007)
QL forward	catgtatatgttccccgctttt	(Chatterjee *et al.*, 2013)
QL reverse	cggctcttacgagtagttcca	(Chatterjee *et al.*, 2013)
prgA-F RT	agtcaacaagcagtgactgacc	This study
prgA-R RT	aacagcctgtgtatccgtagc	This study
prgB-F RT	cgacaggcgtttctttcttc	(Chatterjee *et al.*, 2013)
prgB-R RT	ggtctttggcagaaatcgtc	(Chatterjee *et al.*, 2013)
prgC-F RT	cctgaacagcccactaaacc	This study
prgC-R RT	gtgttacctccgctaagttgc	This study

prgJ-F RT	accccaatgactggcttagag	(Bhatty *et al.*, 2015)
prgJ-R RT	tagacgttagccctgatacgg	(Bhatty *et al.*, 2015)
pcfC-F RT	gcgcttattggaggagacgag	(Chatterjee *et al.*, 2013)
pcfC-R RT	cggcgccacgtataccac	(Chatterjee *et al.*, 2013)

SUPPLEMENTARY FIGURE LEGENDS

FIG. S1. Pheromone induction results in enhanced *prgQ* transcript levels in a Δ*prgU* mutant. qRT-PCR results showing the relative expression levels of regions of the *prgQ* operon in OG1RF strains carrying pCF10, pCF10Δ*prgU,* and the ΔU-Res *R1* variant at 30 and 60 min following cCF10 pheromone induction with 5 ng ml^{-1} cCF10. The data shown are from one biological replicate, which was repeated with similar results.

FIG. S2. The Δ*prgU* mutant, but not other Δ*prg/pcf* mutant strains displays pheromone sensitive growth. A) OG1RF strains carrying pCF10 or pCF10 variants deleted of the genes shown were assayed for growth in the presence of pheromone. Overnight cultures were diluted 1:100 in fresh BHI and incubated for 1 h at 37°C in the absence of pheromone. Cultures were spread on BHI media, allowed to dry, and cCF10 pheromone (10 ng ml^{-1}) was added to the center of the plate. Plates were incubated overnight at 37°C and assessed for growth. cCF10 pheromone is solubilized in DMSO, which inhibits *E. faecalis* growth and causes small zones of clearance independently of cCF10-induced toxicity. B) Pheromone spot assays showing that the Δ*prgU* mutant is growth inhibited only when exposed to pheromone at an early log phase of growth.

FIG. S3. ΔU-Res mutations suppress production of PrgB and PrgC adhesins and accumulate mutations in the *prgQ* regulatory region. **A)** Representative pheromone spot assays showing pheromone sensitivity of the **Δ*prgU*** mutant, OG1RF(pCF10Δ*prgU*), and pheromone resistance of a **ΔU-Res** mutant strain isolated from within the zone of pheromone inhibition. **B)** Steady-state levels of Prg proteins in 10 ΔU-Res variants induced for 1 h with cCF10 pheromone (10 ng ml^{-1}). Strains: OG1RF with **pCF10** (WT plasmid), **Δ*prgU*** (pCF10Δ*prgU*), or ΔU-Res variants *R1- R10.* Immunoblots were developed with antibodies to the Prg proteins shown or to RNA polymerase β subunit as a loading control. **MW**, Molecular weights of proteins in kDa are indicated. Protein extracts were loaded on a per-cell equivalent basis. **C)** Upper: Schematic of the *prgQ* regulatory region showing the locations of the P_Q and P_X promoters, inverted repeats IRS1 and IRS2 predicted to form stem-loop transcription terminators, and the putative regulatory genes *prgR* and *prgS*. Numbers refer to distances (in base pairs) from the P_Q promoter start-site. Below: Positions of mutations identified in the 10 ΔU-Res variants. Symbols: open triangles, single base-pair (bp) deletion mutations; inverted filled triangles, single bp insertion mutations. Right: Transfer frequencies of the ΔU-Res variant plasmids in 2 h filter matings presented as the number of transconjugants per donor cell (Tcs/D). Transfer frequencies of donors harboring WT pCF10 and pCF10Δ*prgU* are shown for comparison. Experiments were repeated at least three times in duplicate, and results from a representative experiment are shown.

FIG. S4. Growth defect of OG1RF(pCF10Δ*prgU*) in the presence of bile salts. Overnight cultures were diluted into fresh BHI medium and cultured to an OD_{600} of ~0.1. Tenfold serial dilutions were plated on BHI medium with or without cCF10 pheromone (2 ng ml^{-1}), or these media additionally containing 4 % sodium cholate or 0.06 % sodium deoxycholate. Strains: OG1RF harboring **pCF10** or **Δ*prgU*** (pCF10Δ*prgU*) alone or together with the P_{23}::*prgU* expression plasmid (**P_{23}::U**, pMB11).

FIG. S5. Deletion of *prgU* from a miniaturized pCF10 plasmid confers enhanced production of the Prg surface adhesins and pheromone toxicity. **A)** Steady-state levels of Prg/Pcf proteins in strains induced for 1 h

with cCF10 pheromone (10 ng ml^{-1}). Strains: OG1RF with **pCF10** (WT plasmid); **Δ*prgU*** (pCF10Δ*prgU*), **p10-mini** (encodes the *prgQ* regulatory region and the *prgA-C* gene cassette; see Fig. 1A), and **p10-miniΔ*prgU*** alone **(-)** or with the P_{23}::*prgU* expression plasmid (**P_{23}:U**; pMB11). Immunoblots were developed with antibodies to the Prg/Pcf proteins shown or to RNA polymerase β subunit as a loading control. Protein sizes (in kilodaltons, kDa) are listed at the right. Protein extracts were loaded on a per-cell equivalent basis. **B)** Pheromone spot assay. OG1RF carrying p10-miniΔ*prgU* is inhibited by pheromone; P_{23}::*prgU* expression *in trans* restores pheromone insensitive growth. **C)** Pheromone-mediated antibiotic sensitivity of OG1RF(p10-miniΔ*prgU*). Strains: OG1RF with p10-mini alone or with the P_{23}::*prgU* expression plasmid (**P_{23}:U**, pMB11). Strains were inoculated from glycerol stocks into BHI lacking **(-)** or containing **(+)** cCF10 and in the absence (**No AB**) or presence of the antibiotics at final concentrations listed (in μg ml^{-1}). Cultures were incubated overnight at 37 ^{o}C without shaking and culture densities (OD_{600}) were measured. **D)** PrgB overproduction confers severe growth defects. Freshly transformed cells were inoculated in fresh BHI supplemented with pheromone (10 ng ml^{-1}) and incubated without shaking for 1 h at 37^{o}C. Cells were processed for imaging as described in the Experimental procedures. Strains: OG1RF with p10-mini alone or with the P_{23}::*prgU* expression plasmid (**P_{23}:U**, pMB11), or with p10-miniΔ*prgU*.

FIG. S6. PrgB overproduction confers pheromone toxicity. **A)** Plasmid curing assay. Colonies from transformation plates were inoculated into antibiotic-free BHI and incubated without shaking overnight at 37^{o}C. Overnight cultures were then serially diluted and spotted onto BHI agar plates containing or lacking spectinomycin (500 μg ml^{-1}) to which the P_{23} plasmid confers resistance. **B)** Levels of Prg proteins in the Δ*prgA-C* mutants engineered to express *prgA*, *prgB* or *prgC* from the P_{23} promote, as monitored by immunostaining with antibodies to PrgA, PrgB, or PrgC. Protein extracts were loaded on a per-cell equivalent basis and RNA polymerase β subunit as a loading control. **C)** PrgB overproduction confers severe growth defects. Freshly transformed cells were inoculated in fresh BHI supplemented with pheromone (10 ng ml^{-1}) and incubated without shaking for 1 h at 37^{o}C. Cells were processed for imaging as described in the Experimental procedures. Strains analyzed: OG1RF carrying pCF10 or pCF10Δ*prgA-C* alone or with the P_{23}::*prgA*, P_{23}::*prgB*, or P_{23}::*prgC* expression plasmids. **D)** Pheromone spot assay showing OG1RF harboring the pCF10Δ*prgA-C* mutant plasmid (**Δ*prgA-C***) exhibit pheromone-insensitive growth, while production of PrgB restores pheromone-mediated growth suppression. Strains: OG1RF(Δ*prgA-C*) carrying the P_{23} vector plasmid (pDL278P_{23}), P_{23}:*prgA* (pMC001), P_{23}::*prgB* (pMC002), or p*23*::*prgC* (pMB4).

FIG. S7. PrgU Structure. **A)** An X-ray structure of a PrgU (V583; EF0046) tetramer. **B)** Ribbon representation of a PrgU monomer, determined by Phyre2 modeling. **C, D)** Ribbon representation and topology diagram of the PUA domain from archaeosine tRNA-guanine transglycosylase (ArcTGT) of *P. horikoshii* (Protein Data Bank code 1J2B) (reprinted with permission from (Perez-Arellano *et al.*, 2007).

FIG. S8. Phylogenetic distributions and genetic linkages of *prgU* and *prgB*-like surface adhesins. *prgU* genes are distributed among *E. faecalis* and other enterococcal species, as well as *Staphylococcus aureus*, invariably linked to *prgB*-like genes and genes encoding other surface adhesins or cell wall modifying proteins. In *E. faecalis* and *S. agalactiae* species, *prgU* genes are linked to *prgB* genes that encode conserved GbpC (Glucan-binding protein C) and isopep_sspB_C2 (adhesin isopeptide-forming domain, sspB-C2 type) domains. In *E. faecium*, *E. raffinosus*, and *S. aureus* species, *prgU* genes are instead linked to genes (provisionally designated "prgB-like") that encode adhesins with Cna-B peptidase (Cna protein B-type) domains. Species/strain/plasmid names with genome and *prgU* accession numbers in parantheses: pCF10 (NC_006827.2, pCF10-17); V583 PAI (NC_004668.1, EF0486), pTEF1 (NC_004669.1, EFA0046); pTEF2 (NC_004671.1; unannotated), pEF62pC (CP002494.1, EF62_RS15500); strain 19 (JTKW01000029.1,KII47007.1); EnGen0418 (JAHX01000008.1, ETU39552.1); *S. agalactiae* DK-PW-092 (LBKE01000032.1, KLL26985.1); *Enterococcus faecium* VRE0576 (JAAK01000014.1, EZP99904.1); *Enterococcus raffinosus* ATCC 49464 (AJAL01000017.1, EOH75571.1); *Staphylococcus aureus* VRS11b (AHBV01000001.1, EIK36346.1).

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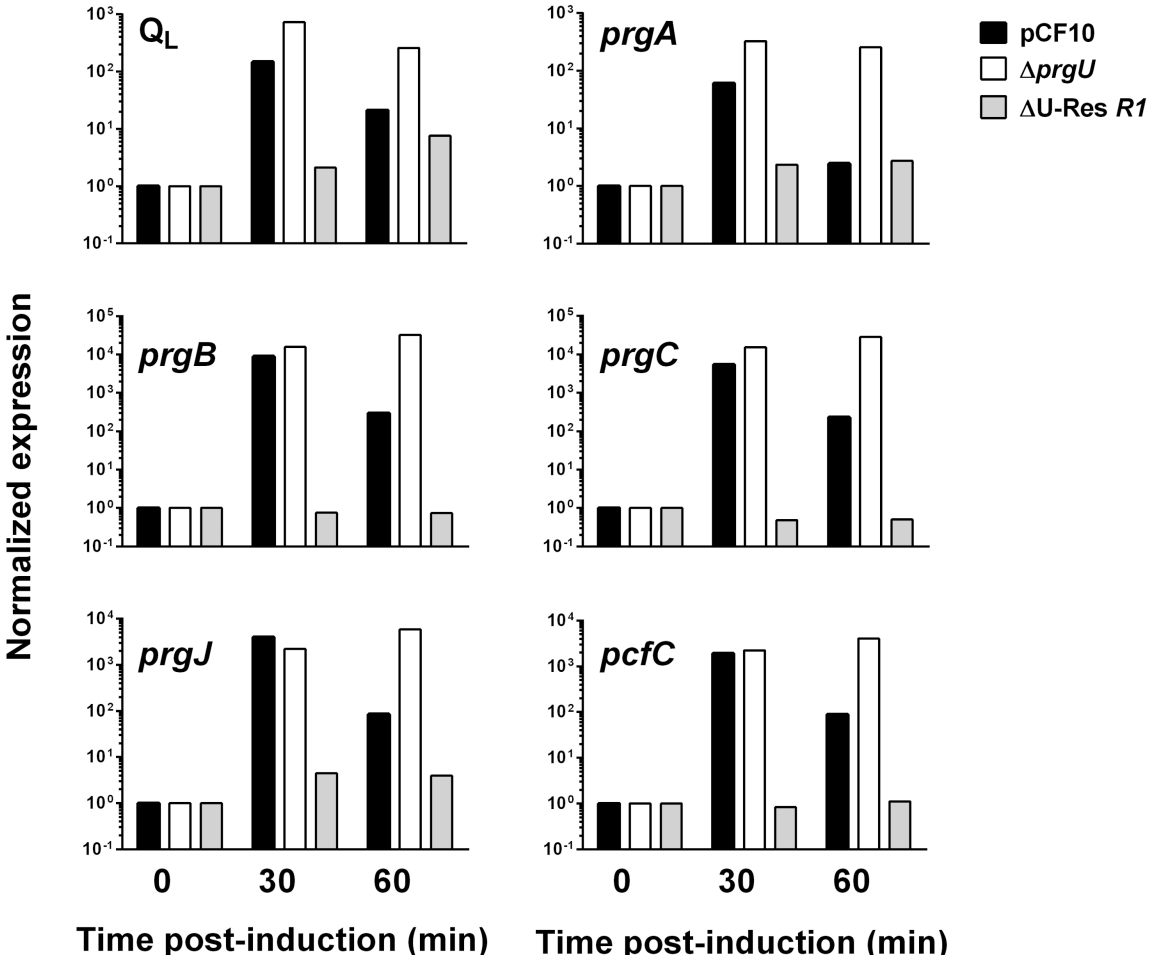

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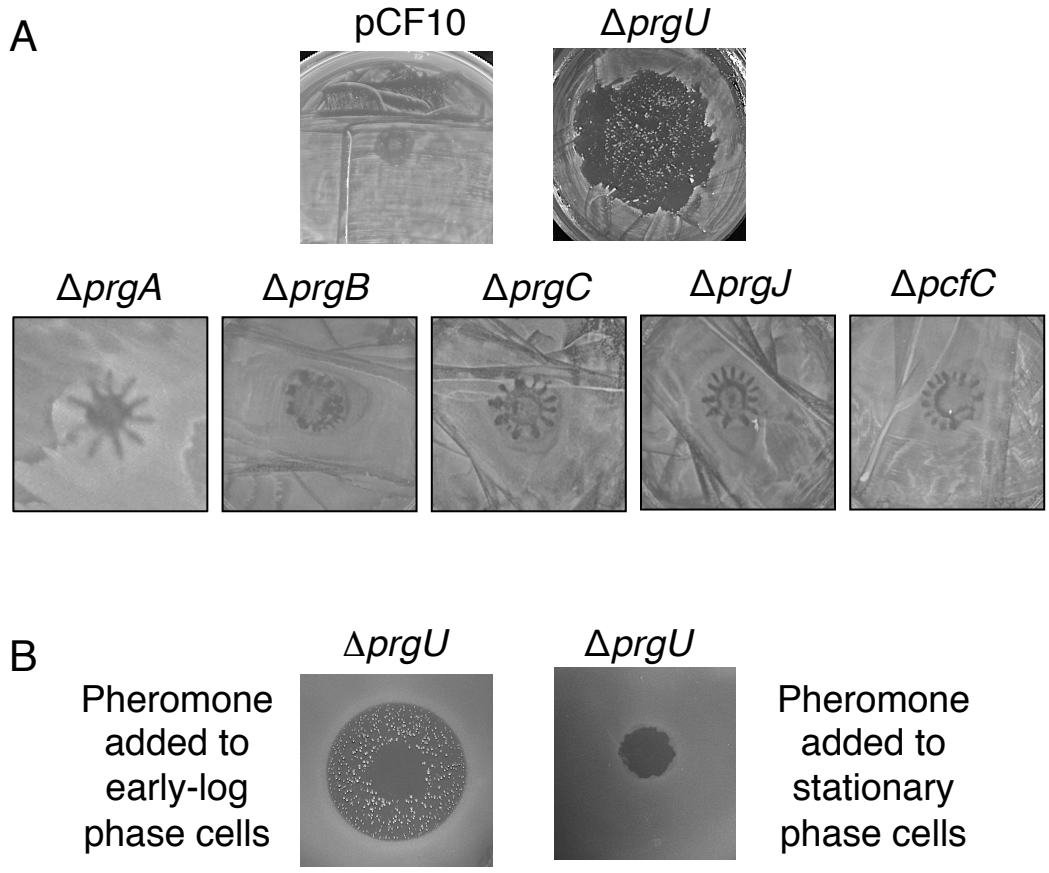

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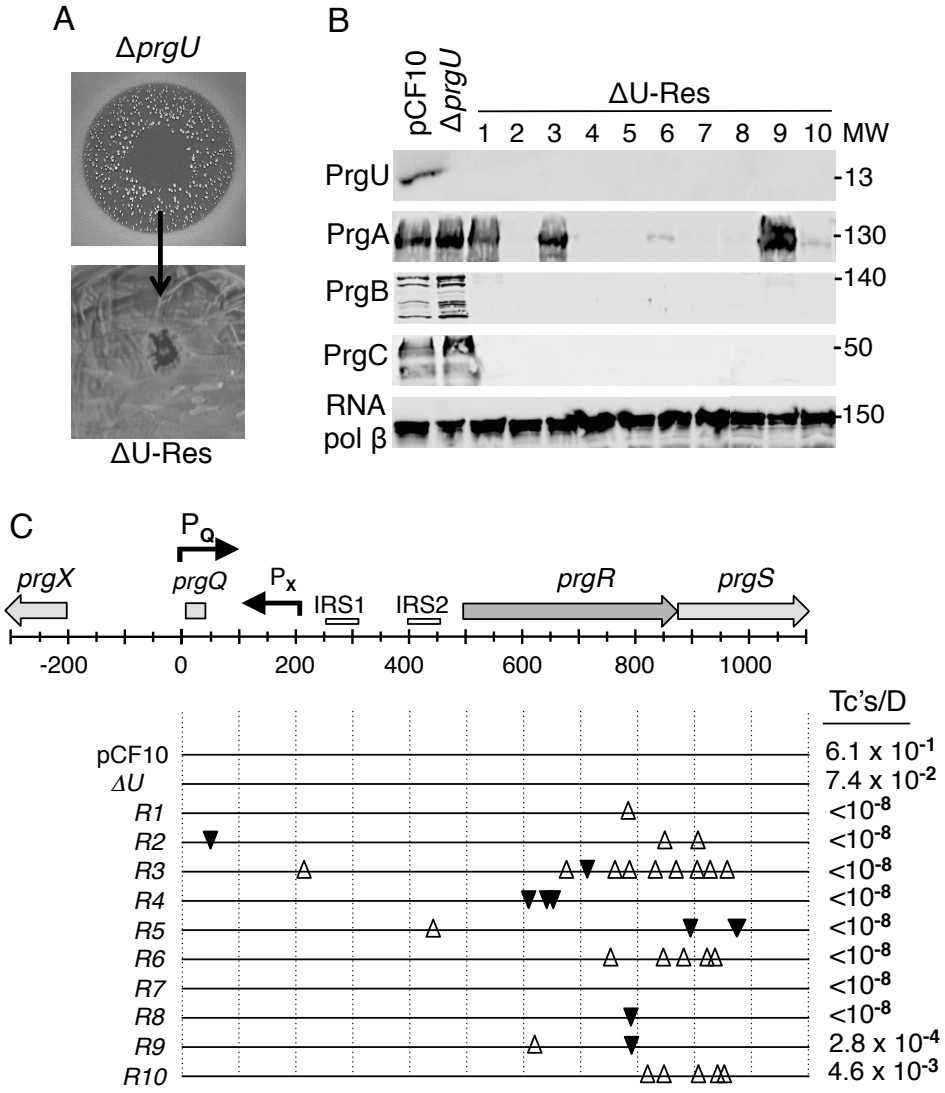

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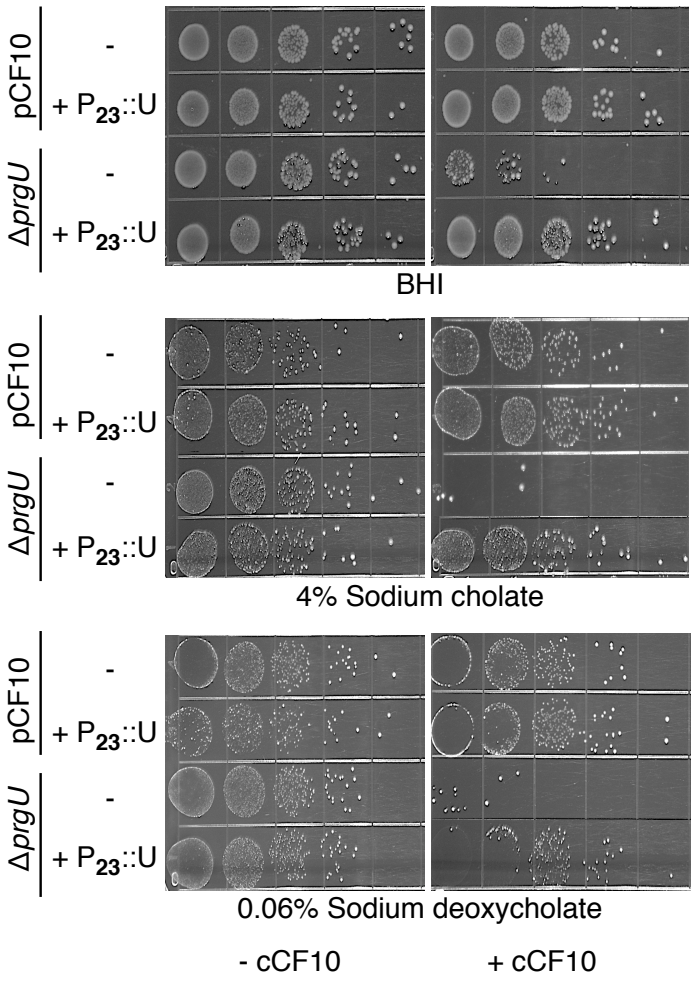

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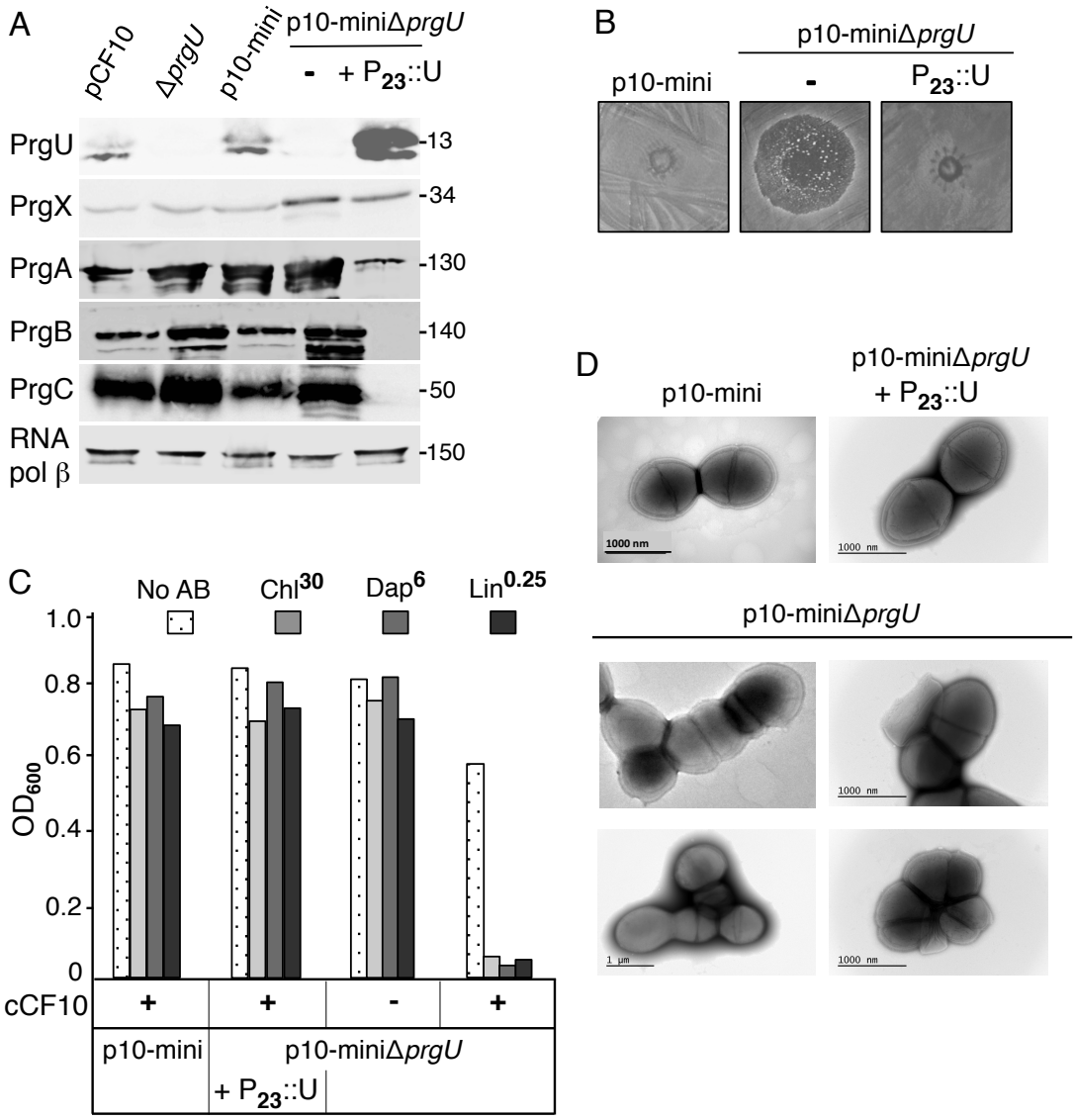

FIG. S5. Deletion of *prgU* from a miniaturized pCF10 plasmid confers enhanced production of the Prg surface adhesins and pheromone toxicity. **A)** Steady-state levels of Prg/Pcf proteins in strains induced for 1 h with cCF10 pheromone (10 ng ml^{-1}). Strains: OG1RF with **pCF10** (WT plasmid); **Δ*prgU*** (pCF10Δ*prgU*), **p10-mini** (encodes the *prgQ* regulatory region and the *prgA-C* gene cassette; see Fig. 1A), and **p10-miniΔ*prgU*** alone **(-)** or with the P_{23}::*prgU* expression plasmid (**P_{23}:U**; pMB11). Immunoblots were developed with antibodies to the Prg/Pcf proteins shown or to RNA polymerase β subunit as a loading control. Protein sizes (in kilodaltons, kDa) are listed at the right. Protein extracts were loaded on a per-cell equivalent basis. **B)** Pheromone spot assay. OG1RF carrying p10-miniΔ*prgU* is inhibited by pheromone; P_{23}::*prgU* expression *in trans* restores pheromone insensitive growth. **C)** Pheromone-mediated antibiotic sensitivity of OG1RF(p10-miniΔ*prgU*). Strains: OG1RF with p10-mini or p10-miniΔ*prgU* alone or with the P_{23}::*prgU* expression plasmid (**P_{23}:U**, pMB11). Strains were inoculated from glycerol stocks into BHI lacking **(-)** or containing **(+)** cCF10 and in the absence (**No AB**) or presence of the antibiotics at final concentrations listed (in μg ml^{-1}). Cultures were incubated overnight at 37 °C without shaking and culture densities (OD_{600}) were measured. **D)** PrgB overproduction confers severe growth defects. Freshly transformed cells were inoculated in fresh BHI supplemented with pheromone (10 ng ml^{-1}) and incubated without shaking for 1 h at 37°C. Cells were processed for imaging as described in the Experimental procedures. Strains: OG1RF with p10-mini or p10-miniΔ*prgU* alone or with the P_{23}::*prgU* expression plasmid (**P_{23}::U**, pMB11).

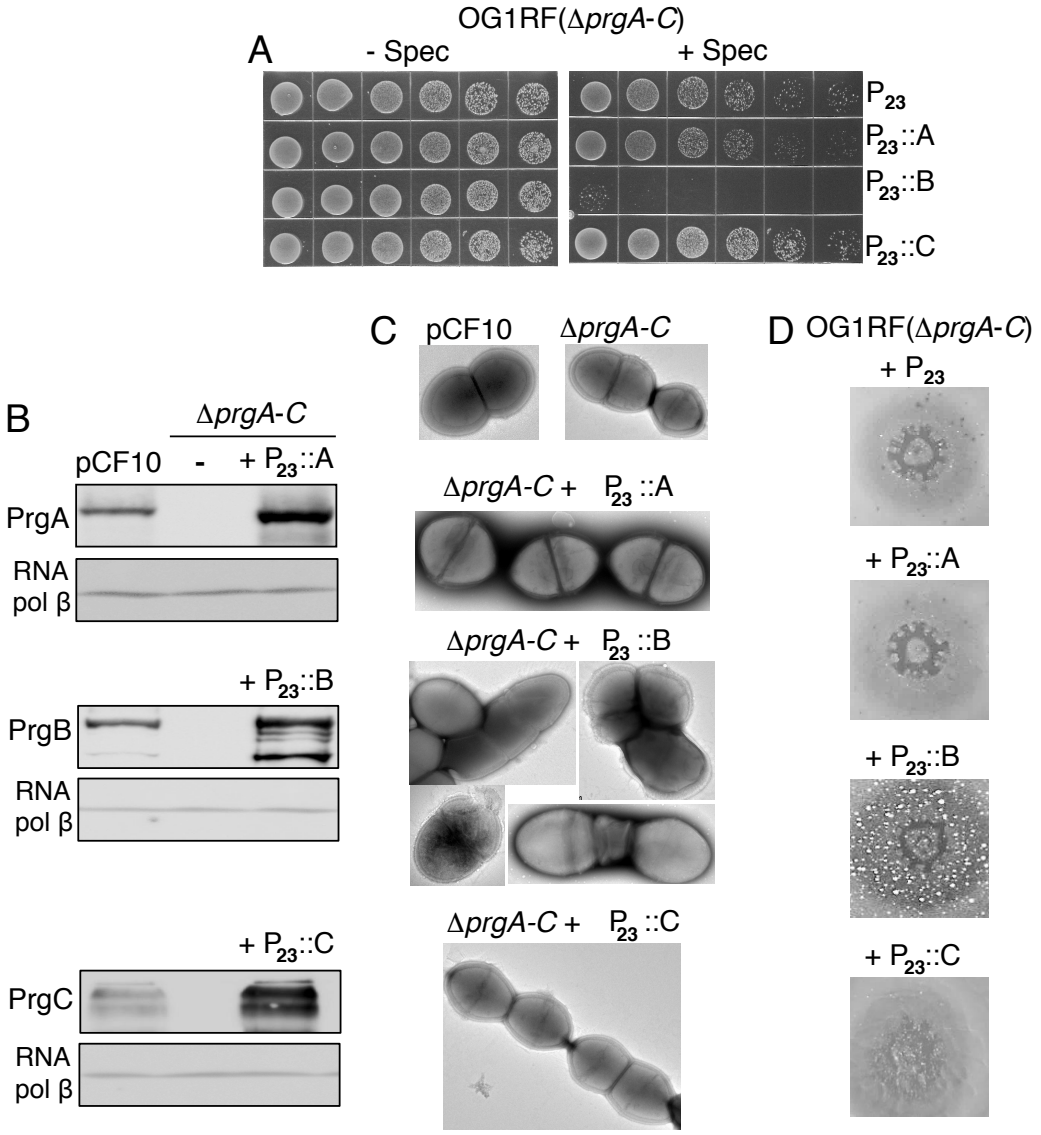

FIG. S6. PrgB overproduction confers pheromone toxicity in OG1RF(pCF10Δ*prgA-C*) cells. **A)** Plasmid curing assay. Colonies from transformation plates were inoculated into antibiotic-free BHI and incubated without shaking overnight at 37°C. Overnight cultures were then serially diluted and spotted onto BHI agar plates containing or lacking spectinomycin (500 μg ml^{-1}) to which the P_{23} plasmid confers resistance. **B)** Levels of Prg proteins in OG1RF(pCF10Δ*prgA-C*) strains engineered to express *prgA*, *prgB* or *prgC* from the P_{23} promoter, as monitored by immunostaining with antibodies to PrgA, PrgB, or PrgC. Protein extracts were loaded on a per-cell equivalent basis and RNA polymerase β subunit as a loading control. **C)** PrgB overproduction confers severe growth defects. Freshly transformed cells were inoculated in fresh BHI supplemented with pheromone (10 ng ml^{-1}) and incubated without shaking for 1 h at 37°C. Cells were processed for imaging as described in the Experimental procedures. Strains analyzed: OG1RF carrying pCF10 or pCF10Δ*prgA-C* alone or with the P_{23}::*prgA*, P_{23}::*prgB*, or P_{23}::*prgC* expression plasmids. **D)** Pheromone spot assay showing that OG1RF strains harboring the pCF10Δ*prgA-C* mutant plasmid (**Δ*prgA-C***) alone or with the P_{23}::*prgA* or P_{23}::*prgC* expression plasmids exhibit slight pheromone-sensitive growth, while a strain with the P_{23}::*prgB* expression plasmid exhibits strong pheromone suppression of growth. Strains: OG1RF(Δ*prgA-C*) carrying the P_{23} vector plasmid (pDL278P_{23}), P_{23}::*prgA* (pMC001), P_{23}::*prgB* (pMC002), or p*23*::*prgC* (pMB4).

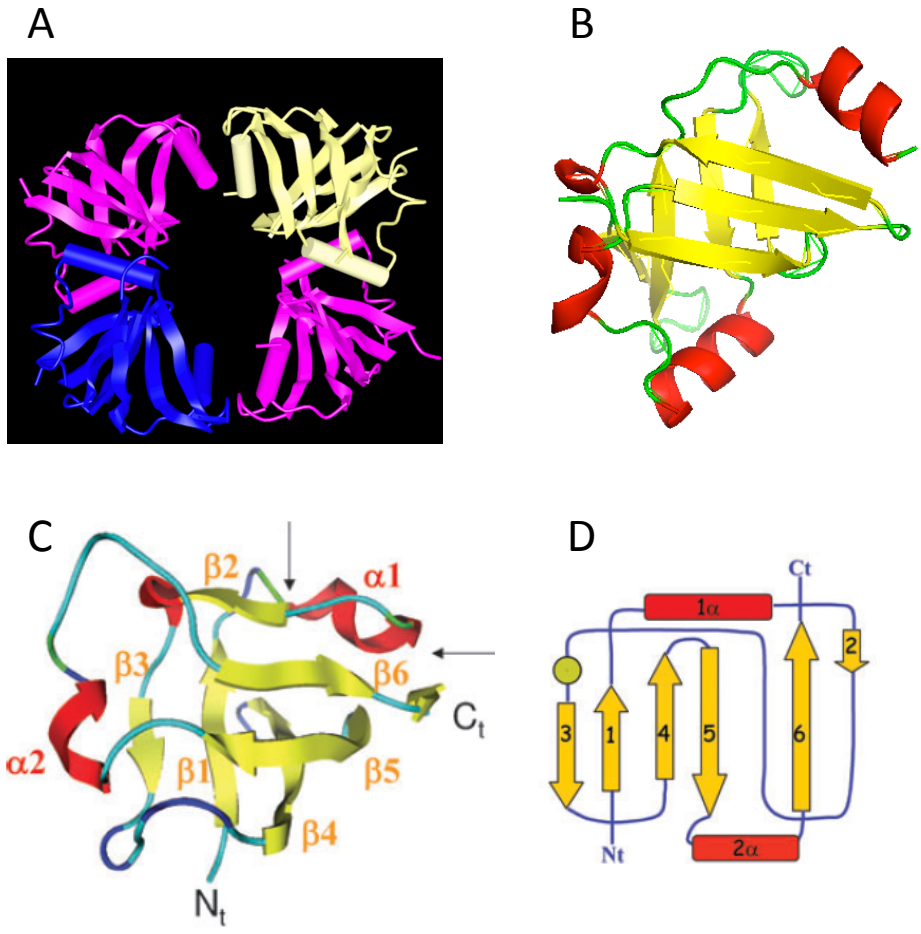

FIG. S7. PrgU Structure. **A)** An X-ray structure of a PrgU (V583; EF0046) tetramer. **B)** Ribbon representation of a PrgU monomer, determined by Phyre2 modeling. **C, D)** Ribbon representation and topology diagram of the PUA domain from archaeosine tRNA-guanine transglycosylase (ArcTGT) of *P. horikoshii* (Protein Data Bank code 1J2B) (reprinted with permission from (Perez-Arellano *et al.*, 2007).

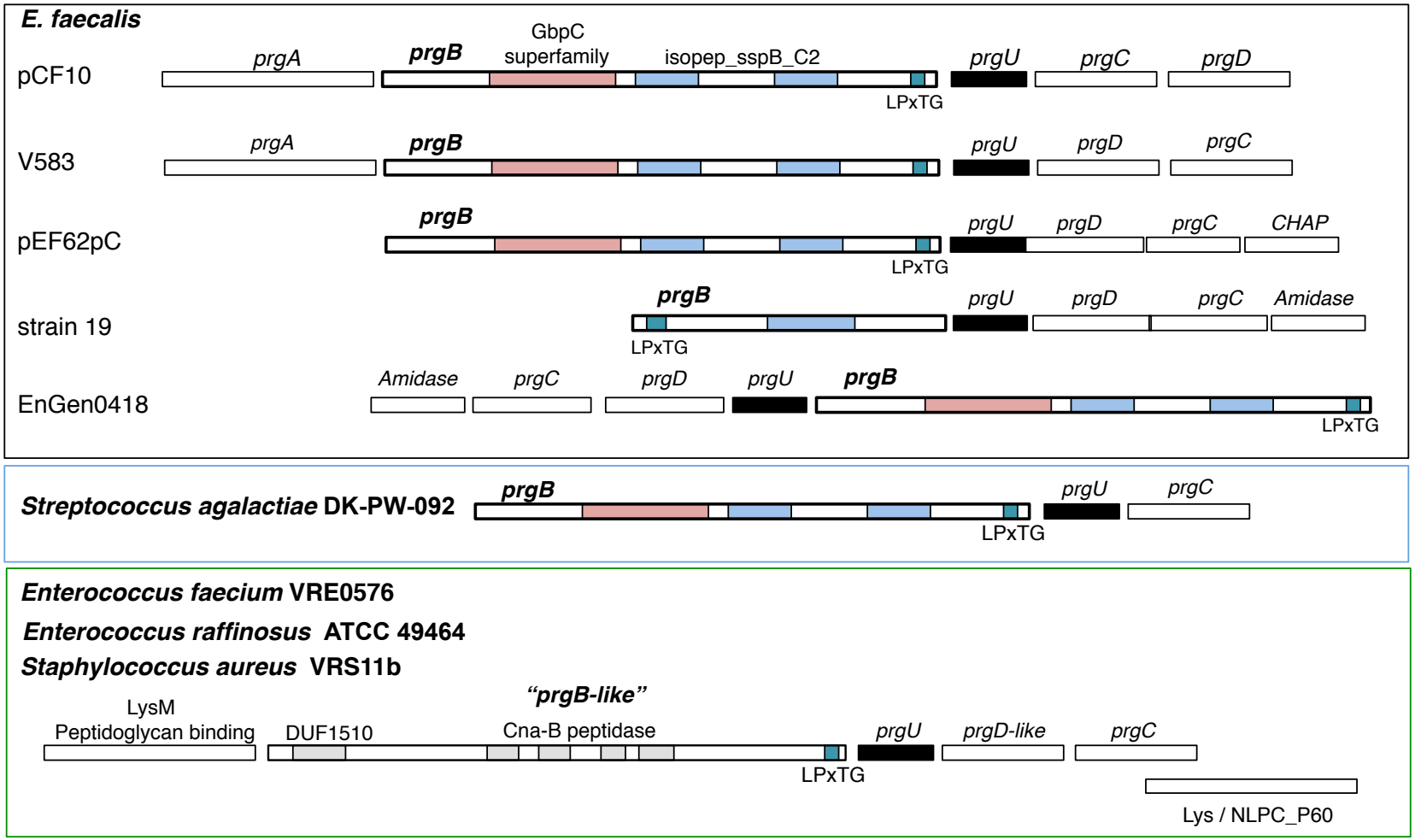

FIG. S8. Phylogenetic distributions and genetic linkages of *prgU* and *prgB*-like surface adhesins. *prgU* genes are distributed among *E. faecalis* and other enterococcal species, as well as *Staphylococcus aureus*, invariably linked to *prgB*-like genes and genes encoding other surface adhesins or cell wall modifying proteins. In *E. faecalis* and *S. agalactiae* species, *prgU* genes are linked to *prgB* genes that encode conserved GbpC (Glucan-binding protein C) and isopep_sspB_C2 (adhesin isopeptide-forming domain, sspB-C2 type) domains. In *E. faecium*, *E. raffinosus*, and *S aureus* species, *prgU* genes are instead linked to genes (provisionally designated "prgB-like") that encode adhesins with Cna-B peptidase (Cna protein B-type) domains. Species/strain/plasmid names with genome and *prgU* accession numbers in parantheses: pCF10 (NC_006827.2, pCF10-17); V583 PAI (NC_004668.1, EF0486), pTEF1 (NC_004669.1, EFA0046); pTEF2 (NC_004671.1; unannotated), pEF62pC (CP002494.1, EF62_RS15500); strain 19 (JTKW01000029.1,KII47007.1); EnGen0418 (JAHX01000008.1, ETU39552.1); *S. agalactiae* DK-PW-092 (LBKE01000032.1, KLL26985.1); *Enterococcus faecium* VRE0576 (JAAK01000014.1, EZP99904.1); *Enterococcus raffinosus* ATCC 49464 (AJAL01000017.1, EOH75571.1); *Staphylococcus aureus* VRS11b (AHBV01000001.1, EIK36346.1).