SUPPLEMENTAL MATERIAL FOR:

Title: *Streptococcus mutans* requires mature rhamnose-glucose polysaccharides for proper pathophysiology, morphogenesis and cellular division

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Growth curves

Growth rates were determined using a Bioscreen C plate reader (Growth Curves USA, Piscataway, NJ). Overnight cultures of *S. mutans* and mutant strains grown in BHI medium were subcultured into fresh BHI medium and incubated at 37°C in a 5% (v/v) CO_2/95% air atmosphere until cultures reached an OD_{600} of 0.3. A 10 µL aliquot was used to inoculate wells of a microtiter plate containing 300 µL test medium. A mineral oil overlay was added to wells to create a microaerobic environment. Assays were performed at 37°C (unless indicated differently) and OD_{600} was continually read at 30 minute intervals following 10 seconds of shaking at medium amplitude. Assays were performed in three independent experiments with 5 replicates each and are displayed as mean OD_{600} values ±SD. Generation times were calculated using the formula $0.3/[(N–N_0)/(T–T_0)]$, where N represents the mean OD_{600} value at the end of exponential phase and N_0 represents the mean OD_{600} at the beginning of exponential phase. T and T_0 refer to the times in minutes that correspond to the OD_{600} values for N and N_0, respectively. Statistical significance ($p \leq 0.001$) was determined by pairwise comparison using Student's *t*-test.

Minimum inhibitory concentration (MIC) testing

MIC testing was performed as per Clinical and Laboratory Standards Institute guidelines to assess antimicrobial efficacy of ampicillin, oxacillin, ceftriaxone, vancomycin, bacitracin, tunicamycin, nisin, cycloserine and

kanamycin against *S. mutans* UA159, Δ*rgpE*, *rgpE*$^{+}$, Δ*rgpF*, *rgpF*$^{+}$ and Δ*rgpG* (Clinical Laboratory Standards Institute, 2018). Briefly, two-fold serial dilutions of test article were made across wells of a 96-well microtiter plate in 100 µl of BHI medium, to which 100 µl of BHI containing ~3.0 x 10^5 cells were added. Plates were incubated overnight at 37°C in a 5% (v/v) CO_2/95% air atmosphere and MIC values were determined as the lowest concentration within a dilution scheme that lacked evident growth. Data are derived from consensus MIC values of three independent experiments, performed in triplicate.

Antibiotic drug combinations and isobolograms

Combination indices of tunicamycin and ampicillin co-administration against *S. mutans* UA159, Δ*rgpE*, *rgpE*$^{+}$, Δ*rgpF*, *rgpF*$^{+}$ and Δ*rgpG* displayed as isobolograms (Zhao *et al.*, 2004). On the plots, connecting diagonal solid lines correspond to the MIC of tunicamycin (Y-axis) and the MIC of ampicillin (X-axis), respectively. Plotted points connected by dotted lines represent combinatorial MIC values (used to calculate the FIC). Points that fall on the solid line demonstrate indifferent effects of drug concentration combinations, those above the line are antagonistic, while points below the line show synergistic interactions of drugs. Data are derived from consensus MIC values of two independent experiments, performed in triplicate.

Quantitative real-time PCR (qRT-PCR)

RNA was extracted from three independent overnight batch cultures of bacteria, according to previously described methods (Abranches *et al*., 2006, Baker *et al*., 2014). The High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) was used to generate cDNA from RNA samples using random primers. Primers specific to genes of interest (listed in Table S3) were used with Power SYBR Green Master Mix (Applied Biosystems) and reactions were carried out in a Step One Plus Real-time PCR System (Applied Biosystems). The mRNA copy number was quantified based on a standard curve of PCR products for specific gene targets. Statistical significance ($p \leq 0.05$) was determined by pairwise comparison using Student's *t*-test.

REFERENCES

Abranches J, Candella MM, Wen ZT, Baker HV, Burne RA. 2006. Different roles of EIIABMan and EIIGlc in regulation of energy metabolism, biofilm development, and competence in *Streptococcus mutans*. J Bacteriol 188:3748-3756.

Baker, J.L., A.M. Derr, K. Karuppaiah, M.E. MacGilvray, J.K. Kajfasz, R.C. Faustoferri, I. Rivera-Ramos, J.P. Bitoun, J.A. Lemos, Z.T. Wen & R.G. Quivey, Jr., (2014) *Streptococcus mutans* NADH oxidase lies at the intersection of overlapping regulons controlled by oxygen and NAD^+ levels. *J Bacteriol* **196**: 2166-2177.

Clinical Laboratory Standards Institute (2018) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—10th ed. M07-A11. Clinical and Laboratory Standards Institute, Wayne, PA.

Zhao, L., M.G. Wientjes & J.L. Au, (2004) Evaluation of combination chemotherapy: integration of nonlinear regression, curve shift, isobologram, and combination index analyses. *Clin Cancer Res* **10**: 7994-8004.

SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1: Disruption of RGP causes growth impairment in *S. mutans*. Cultures of *S. mutans* UA159 (■), Δ*rgpE* (■), *rgpE*$^{+}$ (■), Δ*rgpF* (■), *rgpF*$^{+}$ (■) and Δ*rgpG* (■) were grown (A) in BHI medium at 37°C, (B) in BHI medium at 42°C, (C) in BHI medium supplemented with 1% NaCl at 37°C, and (D) in BHI medium supplemented with 20% sorbitol at 37°C. Growth was assessed by measuring optical density at 600 nm in a Bioscreen C plate reader (as described in Experimental Procedures). Assays were performed in three independent experiments with 5 replicates each and are displayed as mean OD_{600} values ±SD.

FIGURE S2. Antibiotic combination treatment. Susceptibilities of *S. mutans* (A) UA159 (■), (B) Δ*rgpE* (■), (C) *rgpE*$^{+}$(■), (D) Δ*rgpF* (■), (E) *rgpF*$^{+}$ (■) and (F) Δ*rgpG* (■) against combinations of tunicamycin and ampicillin were performed using methodology for fractional inhibitory concentration testing as described in Experimental Procedures. Data are plotted as isobolograms, with solid connecting diagonal lines correspond to the MIC of tunicamycin (Y-axis) and the MIC of ampicillin (X-axis), respectively. Plotted points connected by dotted lines represent combinatorial MIC values (used to calculate the FIC). Points that fall on the solid line demonstrate indifferent effects of drug concentration combinations, those above the line are antagonistic, while points below the line show synergistic interactions of drugs.

FIGURE S3. Polar effects of *rgpE*, *rgpF* and *rgpG* deletion. RNA was isolated from overnight batch cultures and used to measure expression of genes surrounding *rgpG* (A). Gene expression within the *rgp* operon was also measured for the Δ*rgpE* strain (B) and the Δ*rgpF* strain (C). Samples from three independent cultures were measured in triplicate and are represented as mean values ± SD. Statistical significance was determined by pairwise comparison using Student's *t*-test (* $p \leq 0.05$).

FIGURE S4. Growth characteristics of *rgp* mutant strains. Representative images of overnight growth for *S. mutans* UA159, Δ*rgpE, rgpE*$^{+}$, Δ*rgpF*, *rgpF*$^{+}$, Δ*rgpG,* and the "*rgpG* reverse complement" mutants UA159+[*rgpG*$^{+}$] and UA159+[*rgpG*+/Δ*rgpG*] ("*G*$^{+}$/Δ*G*") grown in liquid BHI medium. Strains harboring most severe RGP disruption tend to flocculate and settle to bottom of culture tube. Notably, the UA159+[*rgpG*+/Δ*rgpG*] strain fails to display phenotypic restoration and resembles the Δ*rgpG* mutant.

FIGURE S5. Characterization of Δ*rgpG* genetic complementation mutants. (A) Transcription of *rgpG* was determined by qRT-PCR using RNA isolated from an overnight batch culture of *S. mutans* UA159, Δ*rgpG*, UA159+[*rgpG*$^{+}$] and UA159+[*rgpG*$^{+}$/Δ*rgpG*] ("*G*$^{+}$/Δ*G*"). Samples from three independent cultures were measured in triplicate and are represented as mean values ± SD. Statistical significance was determined by pairwise comparison using Student's *t*-test (* $p \leq 0.05$) versus UA159. (B) Antibiotic selection plating of UA159, Δ*rgpG*,

155 UA159+[*rgpG*$^{+}$] and UA159+[*rgpG*$^{+}$/Δ*rgpG*] ("*G*$^{+}$/Δ*G*") demonstrating expression
156 of appropriate resistance cassettes. Erm = erythromycin, Kan = kanamycin.
157
158 **FIGURE S6. Characterization of Δ*rgpE* genetic complementation mutant.**
159 (A) Transcription of *rgpE* was determined by qRT-PCR using RNA isolated from
160 an overnight batch culture of *S. mutans* UA159, Δ*rgpE* and *rgpE*$^{+}$. (B) Antibiotic
161 selection plating of UA159, Δ*rgpE* and *rgpE*$^{+}$ demonstrating expression of
162 appropriate resistance cassettes. Erm = erythromycin, Kan = kanamycin.
163

FIGURE S1

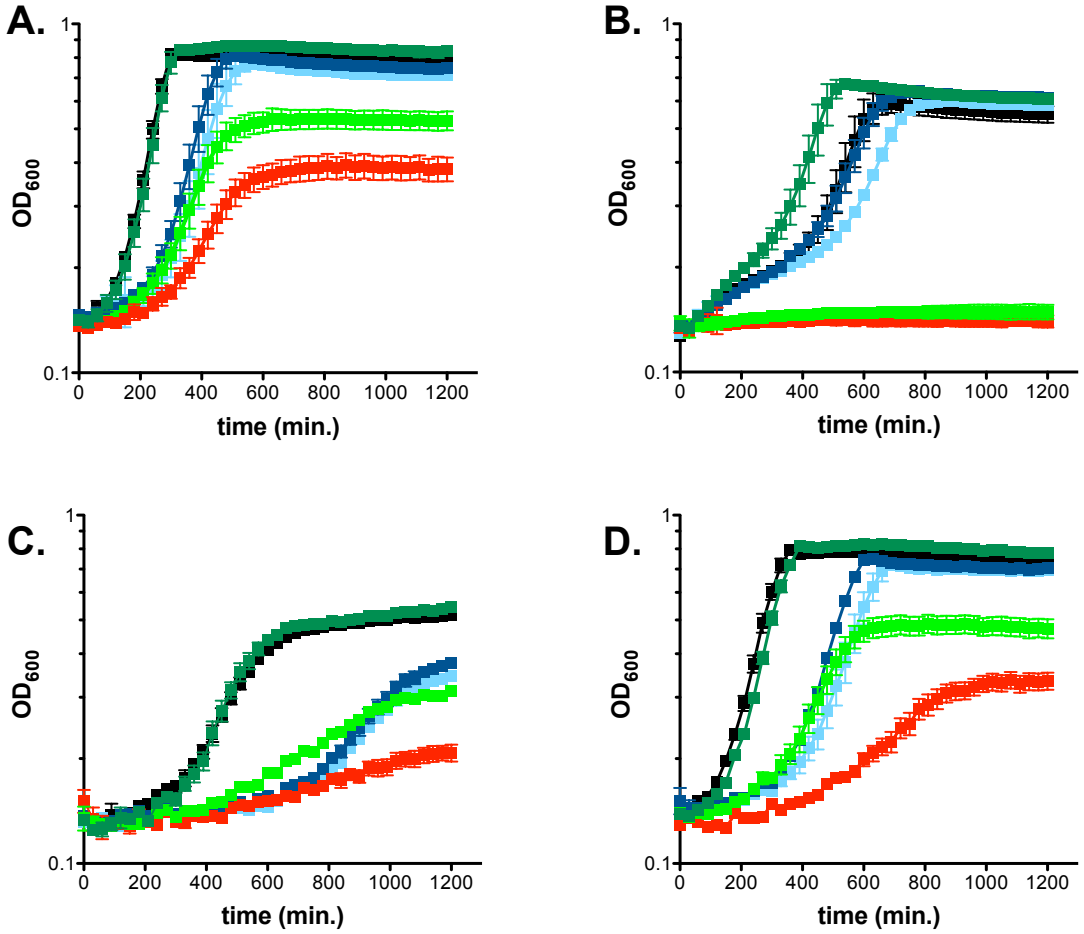


Table S1 Calculated doubling times# of *S. mutans* UA159, Δ*rgpE*, *rgpE*$^{+}$, Δ*rgpF*, *rgpF*$^{+}$ and Δ*rgpG*.

	Doubling time (min.)							
S. mutans	**untreated, 37ºC**		**untreated, 42ºC**		**1% NaCl, 37ºC**		**20% sorbitol, 37ºC**	
strain	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
UA159	83.6	1.9	346.6	6.5	118.8	5.9	342.0	12.6
Δ*rgpE*	147.0$^{\dagger, a}$	8.9	482.5$^{\dagger, c}$	16.8	183.1$^{\dagger, d}$	7.0	694.2$^{\dagger, f}$	24.6
rgpE^{+}	141.5$^{\dagger, a}$	10.1	372.0$^{\dagger, c}$	12.6	164.3$^{\dagger, d}$	10.9	640.9$^{\dagger, f}$	26.6
Δ*rgpF*	244.8$^{\dagger, b}$	31.2	NA		341.0$^{\dagger, e}$	59.2	1419$^{\dagger, g}$	98.1
rgpF^{+}	82.2 ^{b}	3.4	258.4†	47.3	120.9 ^{e}	6.1	311.6$^{\dagger, g}$	14.6
Δ*rgpG*	438.1†	49.6	NA		1005.6†	124.9	NA	

data derived from growth curves in Figure S2

† $p \leq 0.01$ versus UA159

$p \leq 0.01$ where like letters are compared

Table S2 Minimum inhibitory concentration (MIC) testing for antibiotic susceptibilities.

	MIC* (µg ml^{-1})					
antibiotic	UA159	Δ*rgpE*	*rgpE*$^{+}$	Δ*rgpF*	*rgpF*$^{+}$	Δ*rgpG*
ampicillin	4	4	4	2	4	1
oxacillin	0.08	0.16	0.08	0.08	0.16	0.04
ceftriaxone	0.16	0.16	0.16	0.08	0.08	0.02
vancomycin	1	0.5	0.5	0.25	1	0.25
bacitracin	128	32	32	0.5	128	4
tunicamycin	1	4	4	16	1	8
nisin	512	512	512	32	512	64
cycloserine	512	512	512	256	512	256
kanamycin	128	64	128	8	128	16

* consensus value based on three independent experiments

Highlighted values were greater than 2-fold different compared to UA159 (green and red for reduced and enhanced susceptibility, respectively)

FIGURE S2

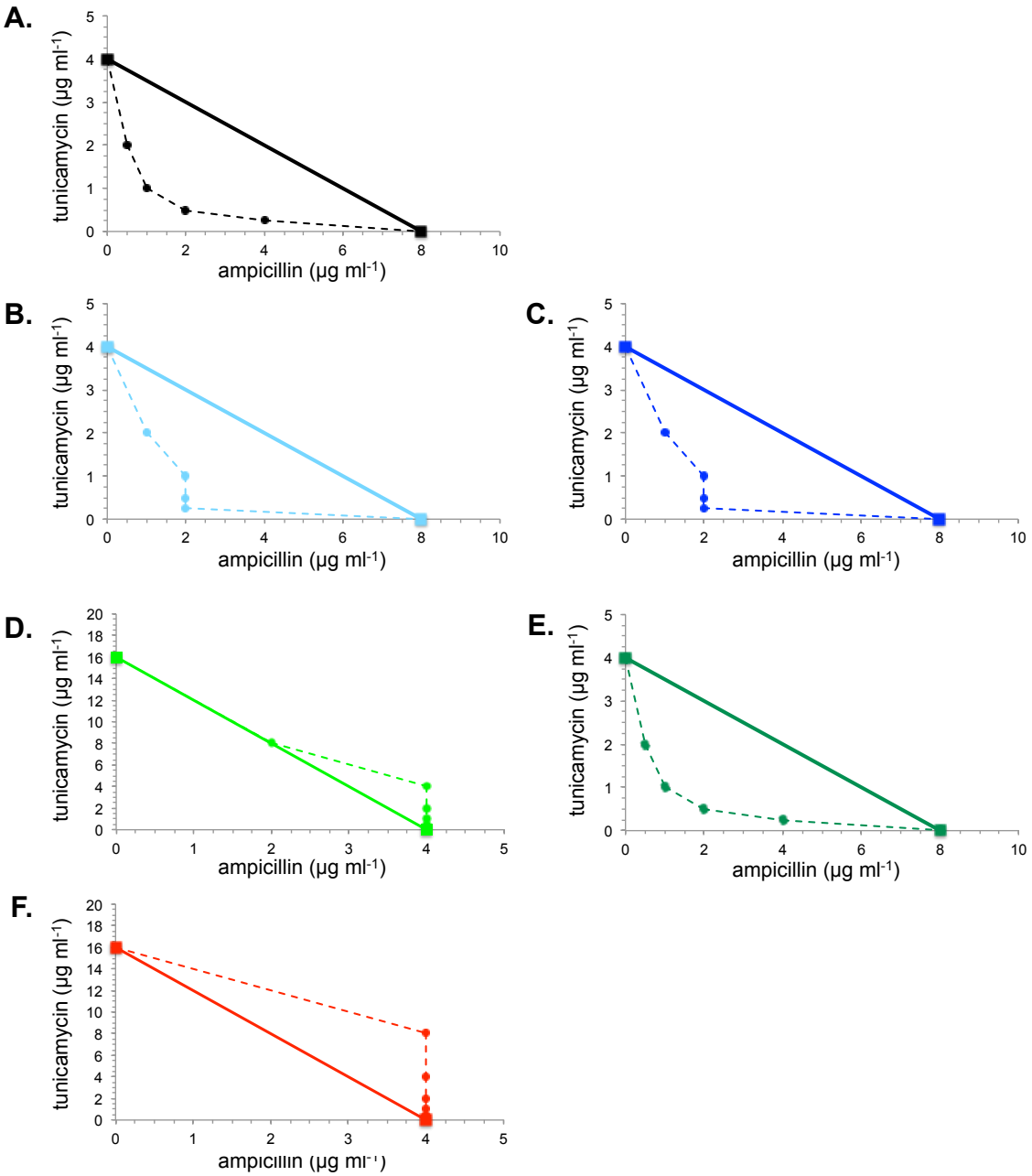
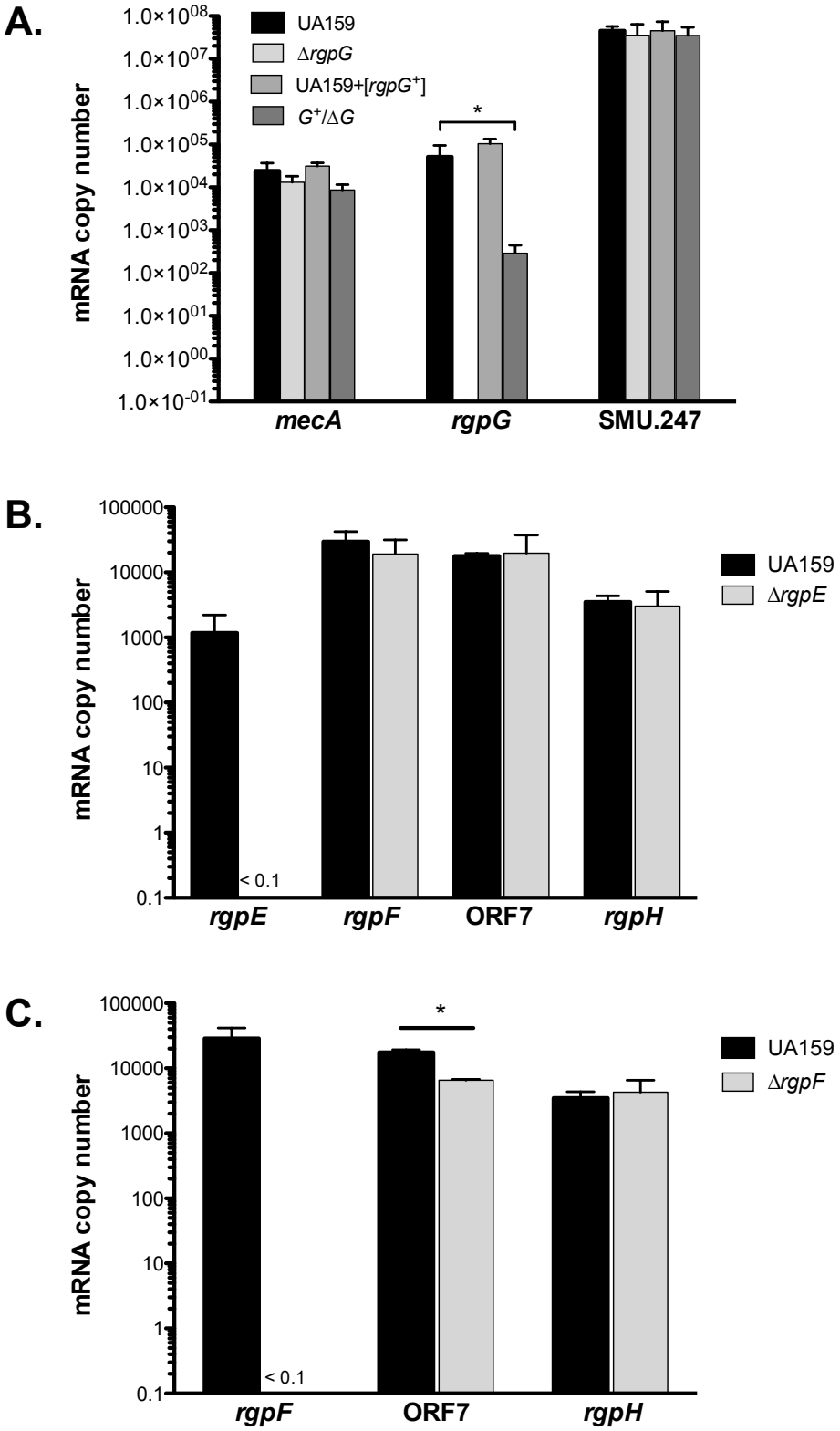


TABLE S3. Primers used for qRT-PCR.

Primer name	Sequence (5'-3')	Application
rgpE-qPCR-For	TGCTTCAACAGATCATTCAG	forward primer for *rgpE* qPCR
rgpE-qPCR-Rev	ATCCAATAATCATCACCATCAC	reverse primer for *rgpE* qPCR
rgpF-qPCR-For	GCGAGATGGAATGGTCTT	forward primer for *rgpF* qPCR
rgpF-qPCR-Rev	CACGGTTGTTGGTCAATC	reverse primer for *rgpF* qPCR
ORF7-qPCR-For	GATGAGCCAAGTGATGTG	forward primer for ORF7 qPCR
ORF7-qPCR-Rev	TTATCTGTATAGGTCAGCAATC	reverse primer for ORF7 qPCR
rgpG-qPCR-For	GGTCCCATGCTCCACTTTAAT	forward primer for *rgpG* qPCR
rgpG-qPCR-Rev	CCAAACCATCCAAACCATCAATC	reverse primer for *rgpG* qPCR
mecA-qPCR-For	AGGAGTCTGACGCTTATCAC	forward primer for *mecA* qPCR
mecA-qPCR-Rev	CGTGTCTTTGTTCCTCTACC	reverse primer for *mecA* qPCR
SMU.247-qPCR-For	TGCCATGCAATATCCGTCTG	forward primer for SMU.247 qPCR
SMU.247-qPCR-Rev	AGCGCTCTGCCATTTCTTC	reverse primer for SMU.247 qPCR

FIGURE S3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UA159
ΔrgpG
UA159+[rgpG+]
G+/ΔG
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FIGURE S4

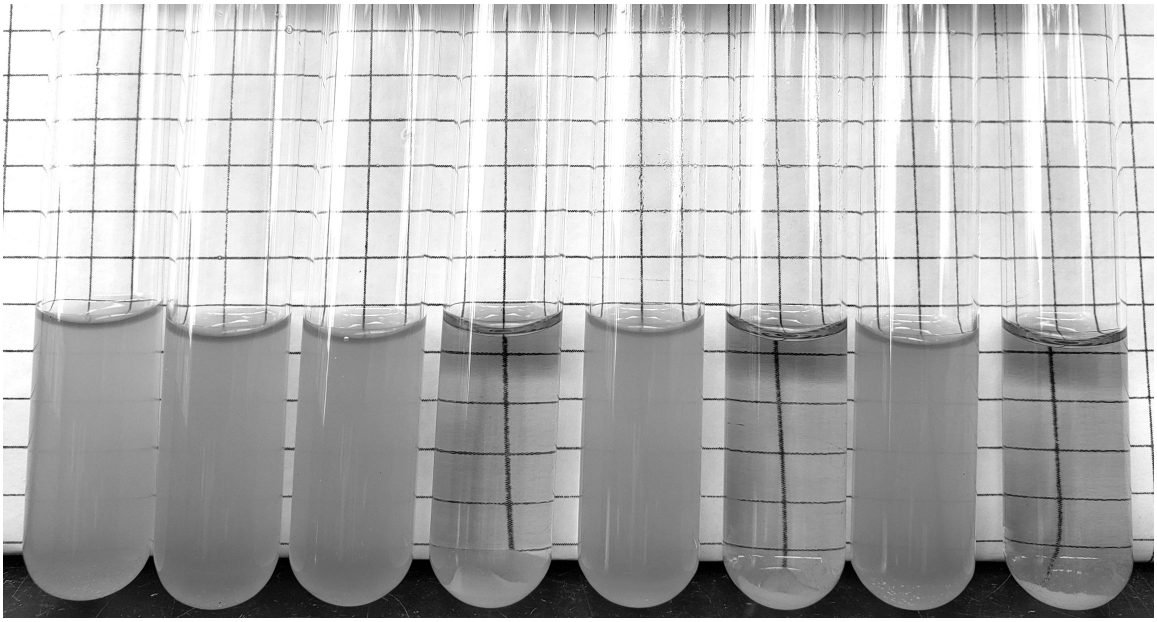
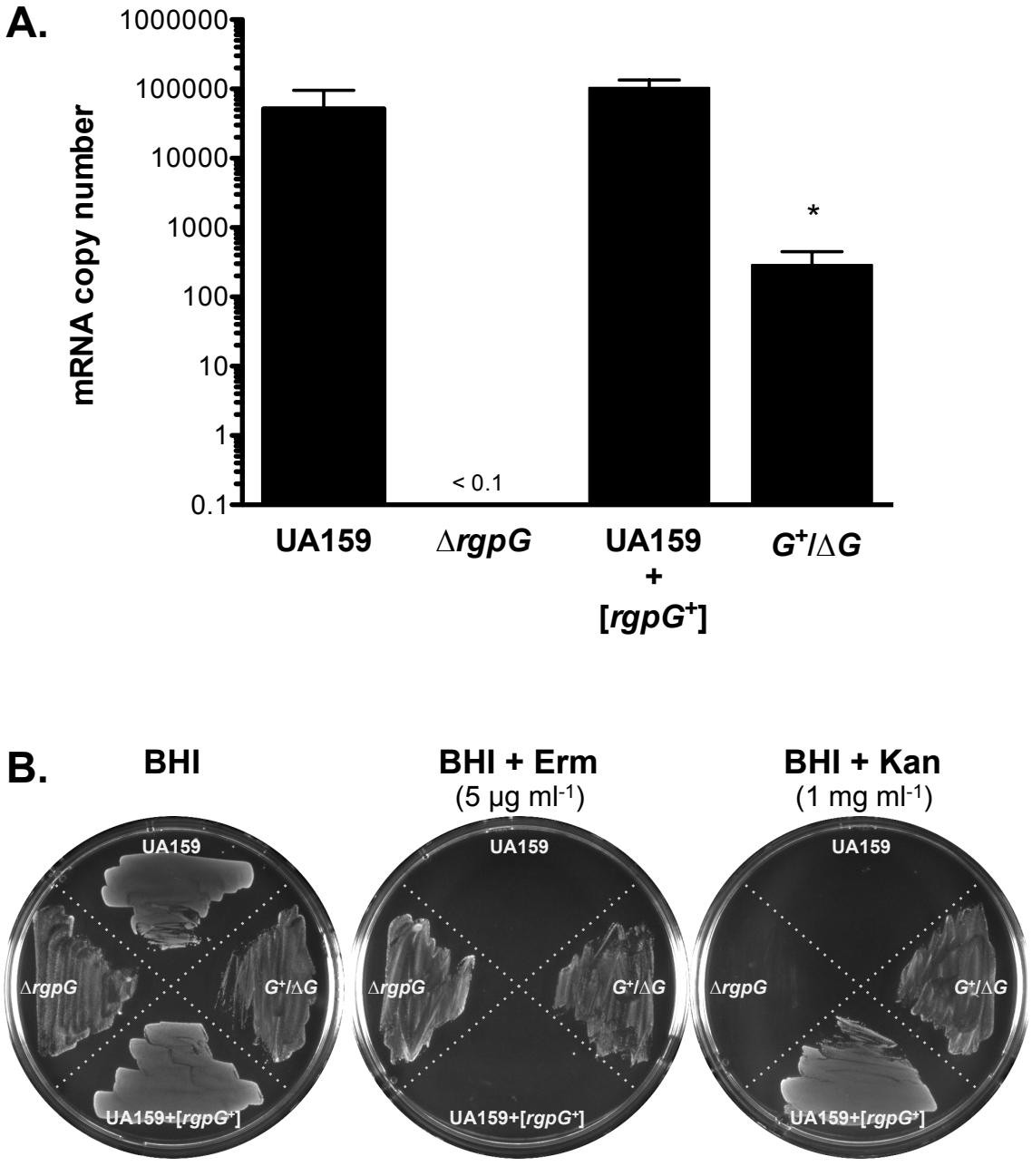


FIGURE S5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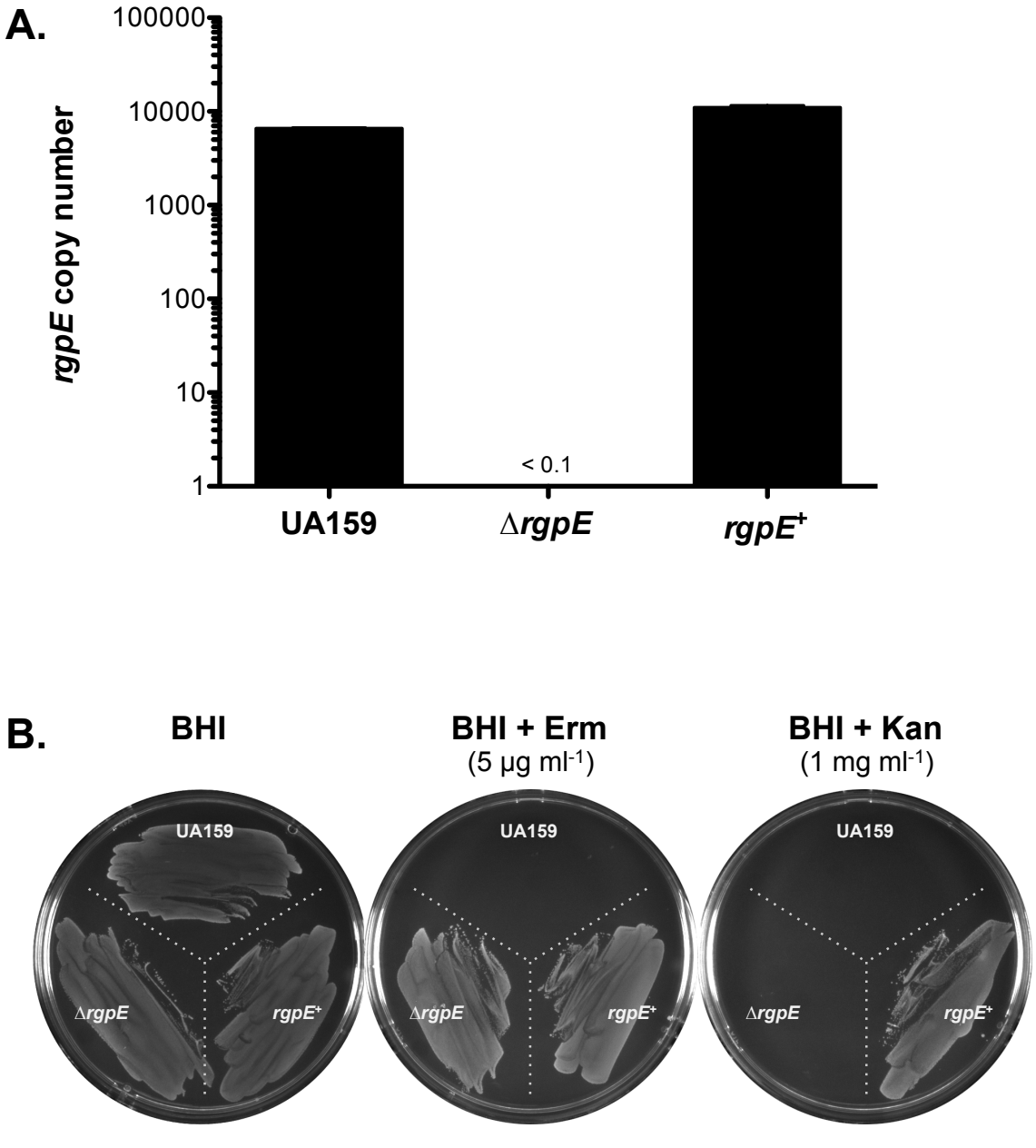
*
UA159
ΔrgpG
UA159 + [rgpG+]
G+/ΔG
B.
BHI
BHI + Erm
(5 µg ml-1)
BHI + Kan
(1 mg ml-1)
UA159
ΔrgpG
G+/ΔG
UA159+[rgpG+]

FIGURE S6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ΔrgpE
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