

Supplementary Material

A ComRS competence pathway in the oral pathogen *Streptococcus sobrinus*

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Supplementary Table S1. Selected NCBI BLAST (tblastn) results of relevant ComR homologs

Template	Species, Strain	Locus ID	Punitive Function(?)	Chromosome Indexing	Query Coverage	Coverage Indexing	E-Value	% Identity
SSO_SL1_ComR1	SSO_SL1	DK182_00280	ComR2	43319-44221	99%	43319-44218	7e-131	72% (217/300)
	SSO_6715-15	DLJ52_00265	ComR1	38804-39709	100%	38804-39709	0.0	99% (298/302)
	SSO_6715-15	DLJ52_00275	ComR2	42279-43178	99%	42279-43178	4e-132	72% (217/300)
	SSO_10919	DK181_00265	ComR	38716-39618	99%	38716-39615	5e-146	79% (237/300)
	SMU_UA159	SMU_381c	Unknown	359781-360683	99%	359784-360683	3e-127	75% (226/300)
	SMU_UA159	SMU_61	ComR	61631-62545	60%	61640-62173	7e-29	37% (67/179)
	STL_LMD-9	STER_0316	ComR	272013-272912	99%	272013-272909	6e-63	41% (122/299)
	SSL_NCTC8618	NCTC8618_02749	ComR	319683-320582	99%	319683-320579	2e-58	42% (125/299)
SSO_SL1_ComR2	SSO_SL1	DK182_00270	ComR1	39843-40748	99%	39843-40742	2e-120	72% (217/300)
	SSO_6715-15	DLJ52_00275	ComR2	42279-43178	99%	42279-43181	2e-173	99% (299/301)
	SSO_6715-15	DLJ52_00265	ComR1	38804-39709	99%	38804-39703	2e-119	71% (214/300)
	SSO_10919	DK181_00265	ComR	38716-39618	100%	38716-39618	6e-154	90% (270/301)
	SMU_UA159	SMU_381c	Unknown	359781-360683	100%	359781-360683	4e-109	70% (210/301)
	SMU_UA159	SMU_61	ComR	61631-62545	60%	61643-62176	3e-20	34% (61/178)
	STL_LMD-9	STER_0316	ComR	272013-272912	99%	272013-272909	2e-57	41% (123/299)
	SSL_NCTC8618	NCTC8618_02749	ComR	319683-320582	99%	319683-320579	1e-52	42% (125/299)
	SSL_NCTC8618	NCTC8618_02909	DNA-binding protein	483624-484547	55%	483981-484463	1e-9	30% (49/164)
SSO_6715-15_ComR1	SSO_6715-15	DLJ52_00275	ComR2	42279-43178	100%	42279-43178	2e-130	71% (214/300)
	SSO_SL1	DK182_00270	ComR1	39843-40748	100%	39843-40748	0.0	99% (298/302)
	SSO_SL1	DK182_00280	ComR2	43319-44221	100%	43319-44218	5e-129	71% (214/300)
	SSO_10919	DK181_00265	ComR	38716-39618	100%	38716-39615	2e-146	79% (237/300)
	SMU_UA159	SMU_381c	Unknown	359781-360683	99%	359784-360683	1e-128	76% (227/300)
	SMU_UA159	SMU_61	ComR	61631-62545	60%	61640-62173	3e-29	38% (68/179)
	STL_LMD-9	STER_0316	ComR	272013-272912	99%	272013-272909	2e-63	41% (123/299)
	SSL_NCTC8618	NCTC8618_02749	ComR	319683-320582	99%	319683-320579	6e-59	42% (126/299)
SSO_6715-15_ComR2	SSO_6715-15	DLJ52_00265	ComR1	38804-39709	100%	38804-39703	1e-120	71% (214/300)
	SSO_SL1	DK182_00280	ComR2	43319-44221	100%	43319-44221	2e-173	99% (300/301)
	SSO_SL1	DK182_00270	ComR1	39843-40748	100%	39843-40741	9e-122	72% (217/300)
	SSO_10919	DK181_00265	ComR	38716-39618	100%	38716-39618	1e-155	90% (272/301)
	SMU_UA159	SMU_381c	Unknown	359781-360683	100%	359781-360683	1e-109	69% (209/301)
	SMU_UA159	SMU_61	ComR	61631-62545	60%	61643-62176	5e-20	34% (60/178)
	STL_LMD-9	STER_0316	ComR	272013-272912	99%	272013-272909	5e-59	42% (125/299)
	SSL_NCTC8618	NCTC8618_02749	ComR	319683-320582	99%	319683-320579	2e-54	42% (127/299)

	SSL_NCTC8618	NCTC8618_02909	DNA-binding protein	483624-484547	95%	483639-484532	3e-11	24% (77/317)
SSO_10919_ComR	SSO_6715-15	DLJ52_00275	ComR2	42279-43178	99%	42279-43181	1e-163	90% (272/301)
	SSO_6715-15	DLJ52_00265	ComR1	38804-39709	100	38804-39703	5e-144	79% (237/300)
	SSO_SL1	DK182_00280	ComR2	43319-44221	100%	43319-44221	4e-162	90% (270/301)
	SSO_SL1	DK182_00270	ComR1	39843-40748	99%	39843-40742	1e-143	79% (237/300)
	SMU_UA159	SMU_381c	Unknown	359781-360683	100%	359781-360683	2e-125	73% (220/301)
	SMU_UA159	SMU_61	ComR	61631-62545	60%	61640-62161	1e-24	34% (60/175)
	STL_LMD-9	STER_0316	ComR	272013-272912	99%	272013-272909	5e-61	42% (125/299)
	SSL_NCTC8618	NCTC8618_02749	ComR	319683-320582	99%	319683-320579	1e-56	43% (128/299)

Supplementary Table S2. Genomic architecture of ComRS locus in selected organisms

Organism	Element Name	Size	Strand	Gene Name or Punitive Function
<i>Streptococcus sobrinus</i> SL1	DK182_00250	720	+	Permease
	comrxip1	20	+	ComR-XIP binding site
	promoter1	10	+	Promoter
	peptide	130	+	Unknown, unannotated
	DK182_00255	231	+	Unknown
	comrxip2	20	+	ComR-XIP binding site
	promoter2	10	+	Promoter
	DK182_00260	1338	+	Lipase
	DK182_00265	159	+	Bacteriocin
	promoter3	10	+	Promoter
	DK182_00270	906	+	ComR
	comrxip3	20	+	ComR-XIP binding site
	promoter4	10	+	Promoter
	ComS	70	+	ComS
	DK182_00275	2118	+	ABC transporter, peptide cleavage
	promoter5	10	+	Promoter
	DK182_00280	903	+	XRE transcription factor
	DK182_00285	873	-	XRE transcription factor
	promoter6	10	-	Promoter
	promoter7	10	+	Promoter
DK182_00290	759	+	ABC transporter	
<i>Streptococcus sobrinus</i> 6715-15	DLJ52_00245	720	+	Permease
	comrxip1	20	+	ComR-XIP binding site
	promoter1	10	+	Promoter
	peptide	129	+	Unknown, unannotated
	DLJ52_00250	231	+	Unknown
	comrxip2	20	+	ComR-XIP binding site
	promoter2	10	+	Promoter
	DLJ52_00255	1338	+	Lipase
	DLJ52_00260	159	+	Bacteriocin
	promoter3	10	+	Promoter
	DLJ52_00265	906	+	ComR
	comrxip3	20	+	ComR-XIP binding site
	promoter4	10	+	Promoter
	ComS	72	+	ComS
	DLJ52_00270	2118	+	ABC transporter, peptide cleavage
	promoter5	10	+	Promoter
	DLJ52_00275	903	+	XRE transcription factor
	DLJ52_00280	873	-	XRE transcription factor
	promoter6	10	-	Promoter
	promoter7	10	+	Promoter
DLJ52_00285	759	+	ABC transporter	
<i>Streptococcus sobrinus</i> 10919	DK181_00245	720	+	permease
	comrxip1	20	+	ComR-XIP binding site
	promoter1	10	+	Promoter
	peptide	129	+	Unknown, unannotated
	DK181_00250	231	+	Unknown
	comrxip2	20	+	ComR-XIP binding site
	promoter2	10	+	Promoter
	DK181_00255	1338	+	Lipase
	DK181_00260	159	+	Bacteriocin
	promoter3	10	+	Promoter
	DK181_00265	903	+	ComR
	DK181_00270	873	-	XRE transcription factor
	promoter4	10	-	Promoter
	promoter5	10	+	Promoter
	DK181_00275	759	+	ABC transporter
<i>Streptococcus thermophilus</i> LMD-9	STER_0314	558	+	ECF transporter
	STER_0315	651	+	Phospholipid phosphatase
	promoter1	10	+	Promoter
	STER_0316	900	+	ComR
	comrxip1	20	+	ComR-XIP binding site
promoter2	10	+	Promoter	

	ComS	78	+	ComS
	STER_0317	1697	+	ABC transporter, peptide cleavage
	STER_0318	390	+	ABC transporter
<i>Streptococcus salivarius</i> SK126	STRSA0001_1792	558	+	ECF transporter
	STRSA0001_1791	651	+	Phospholipid phosphatase
	promoter1	10	+	Promoter
	STRSA0001_1790	900	+	ComR
	comrxip1	20	+	ComR-XIP binding site
	promoter2	10	+	Promoter
	ComS	78	+	ComS
	STRSA0001_1789	2097	+	ABC transporter, peptide cleavage
<i>Streptococcus mutans</i> UA159	SMU_60	687	+	DNA repair enzyme
	promoter1	10	+	Promoter
	SMU_61	915	+	ComR
	comrxip1	20	+	ComR-XIP binding site
	promoter2	10	+	Promoter
	ComS	54	+	ComS
	SMU_63c	1842	-	Cell-wall associated protein

Supplementary Table S3. List of Strains and Plasmids

Strain or plasmid	Characteristic(s)	Source
<i>S. sobrinus</i>		
NCTC 10919	Wild type	ATCC 33402
NIDR 6715-7	Wild type	ATCC 27351
SL1 (DSM 20742)	Wild type	ATCC 33478
NCTC 10919:: <i>comR</i>	NCTC 10919 pComR	This study
NCTC 10919:: <i>comR</i> Δ <i>comR</i>	NCTC 10919 Δ <i>comR</i> :: <i>cat</i> pComR	This study
NCTC 10919 Δ <i>comR</i>	NCTC 10919 Δ <i>comR</i> :: <i>cat</i> (38715...39601)	This study
NIDR 6715-7:: <i>comR1</i>	NIDR 6715-7 pWL52 (<i>comR1-comS</i>)	This study
NIDR 6715-7:: <i>comR2</i>	NIDR 6715-7 pWL53 (<i>comR2</i>)	This study
NIDR 6715-7 Δ <i>comR1-comS</i>	NIDR 6715-7 Δ <i>comR1-comS</i> :: <i>cat</i> (38803...39926)	This study
NIDR 6715-7 Δ <i>comR2</i>	NIDR 6715-7 Δ <i>comR2</i> :: <i>cat</i> (42284...43164)	This study
NIDR 6715-7 Δ <i>comR1-comR2</i>	NIDR 6715-7 Δ <i>comR1-comR2</i> :: <i>cat</i>	This study
NIDR 6715-7:: <i>comR1</i> Δ <i>comR1-comS</i>	NIDR 6715-7 Δ <i>comR1-comS</i> :: <i>cat</i> pComR1	This study
NIDR 6715-7:: <i>comR2</i> Δ <i>comR1-comS</i>	NIDR 6715-7 Δ <i>comR1-comS</i> :: <i>cat</i> pComR2	This study
NIDR 6715-7:: <i>comR1</i> Δ <i>comR2</i>	NIDR 6715-7 Δ <i>comR2</i> :: <i>cat</i> pComR1	This study
NIDR 6715-7:: <i>comR2</i> Δ <i>comR2</i>	NIDR 6715-7 Δ <i>comR2</i> :: <i>cat</i> pComR2	This study
NIDR 6715-7:: <i>comR1</i> Δ <i>comR1-comR2</i>	NIDR 6715-7 Δ <i>comR1-comR2</i> :: <i>cat</i> pComR1	This study
NIDR 6715-7:: <i>comR2</i> Δ <i>comR1-comR2</i>	NIDR 6715-7 Δ <i>comR1-comR2</i> :: <i>cat</i> pComR2	This study
Plasmids		
pLacZ	Sp ^c ; pRW17::P23- <i>lacZ</i>	This study
pComR	Sp ^c ; pRW17:: <i>comR</i> (from NCTC 10919, 38498...39787)	This study
pComR1	Sp ^c ; pRW17:: <i>comR1-comS</i> (from NIDR 6715-7, 38585...40006)	This study
pComR2	Sp ^c ; pRW17:: <i>comR2</i> (from NIDR 6715-7, 42099...43350)	This study

Supplementary Table S4. List of Primers

Primer	Sequence	Note
O1-KRMIT-aph3-F	gaaggtctctatagggcaaacataaaaactgcatggact	<i>aph3</i> cloning
O2-KRMIT-aph3-R	gaaggtctctgattacatcagagtatggacagttgcgga	<i>aph3</i> cloning
O1-cat-F2	gaaggtctctatagagcttgatttcgtcgtgaatacatgt	<i>cat</i> cloning
O2-cat-R2	gaaggtctctgattcaataatcgcatccgattgcagt	<i>cat</i> cloning
<i>comR</i> deletion		
10-comR-U-F	cttgactcttgagttgaaaaggctcgt	NCTC 10919 <i>comR</i> , NIDR 6715-7 <i>comR12</i> deletion upstream arm
O1-10-comR-U-R	gaaggtctctatactgggccctccaatttagtct	NCTC 10919 <i>comR</i> , NIDR 6715-7 <i>comR12</i> deletion upstream arm
O2-10-comR-D-F	gaaggtctctaatcaacgatggattgagtaagagcgagtt	NCTC 10919 <i>comR</i> , NIDR 6715-7 <i>comR12</i> deletion downstream arm
10-comR-D-R	tggcacttgaacaaccctcgt	NCTC 10919 <i>comR</i> , NIDR 6715-7 <i>comR12</i> deletion downstream arm
67-comR2-U-F	cgtggtaactcicaatagggcagtt	NIDR 6715-7 <i>comR2</i> deletion upstream arm
O1-67-comR2-U-R	gaaggtctctatcgtcataatcgtcctccaattctggtt	NIDR 6715-7 <i>comR2</i> deletion upstream arm
comR-check-F2	agtggttcctctgggattca	NCTC 10919 <i>comR</i> , NIDR 6715-7 <i>comR12</i> deletion check
10comR-check-R2	tggtaaccaactagcgactttca	NCTC 10919 <i>comR</i> , NIDR 6715-7 <i>comR12</i> deletion check
67-comR2-check-F2	accaacaaaaaactcagggtgta	NIDR 6715-7 <i>comR2</i> deletion check
cat-i-F	attgtcagataggcctaattgactggct	<i>comR</i> deletion check
cat-i-R	acctaactcctcgtcgtattgtaacca	<i>comR</i> deletion check
10comR-o-F	cagtcctactcaagaaggacagaatga	NCTC 10919 <i>comR</i> , NIDR 6715-7 <i>comR12</i> deletion check
10comR-o-R	tgctacgctttgtagcgcaattcttt	NCTC 10919 <i>comR</i> , NIDR 6715-7 <i>comR12</i> deletion check
67R2-o-F	agcggcgttactcttcaattgaagg	NIDR 6715-7 <i>comR2</i> deletion check
<i>comR</i> plasmid complementation		
pRW17-seq-R	tgagccagtgtagcttagtagaga	Insertion check primer for pRW17
pRW17-seq-F	taccaatgcttaacagtgaggac	Insertion check primer for pRW17
O1-10comR-F	gaaggtctctataggggtgatccacgtattggaattcca	NCTC 10919 <i>comR</i> cloning
O1-67-comR2-F	gaaggtctctatagtcctfatagataagacaagcactctcaacaac	NIDR 6715-7 <i>comR2</i> cloning
O4-10comR-R	gaaggtctctagccaatggtatcaagagtgagctgacca	NCTC 10919 <i>comR</i> cloning
<i>lox</i> deletion		
lox-5-F	accaatcctcgcgaaattgct	<i>lox</i> deletion upstream arm
O1-lox-5-R	gaaggtctctatggactgtcaattatcctgctcagct	<i>lox</i> deletion upstream arm
O2-lox-3-F	gaaggtctctaatcgtcccaaaccttgaagaagt	<i>lox</i> deletion downstream arm
lox-3-R	agcaatcaaggcaactaggatagca	<i>lox</i> deletion downstream arm
lox-check-R	agagcctgatggctccctatca	<i>lox</i> deletion check
lox-check-F	atcttctatggagtcgggccagt	<i>lox</i> deletion check

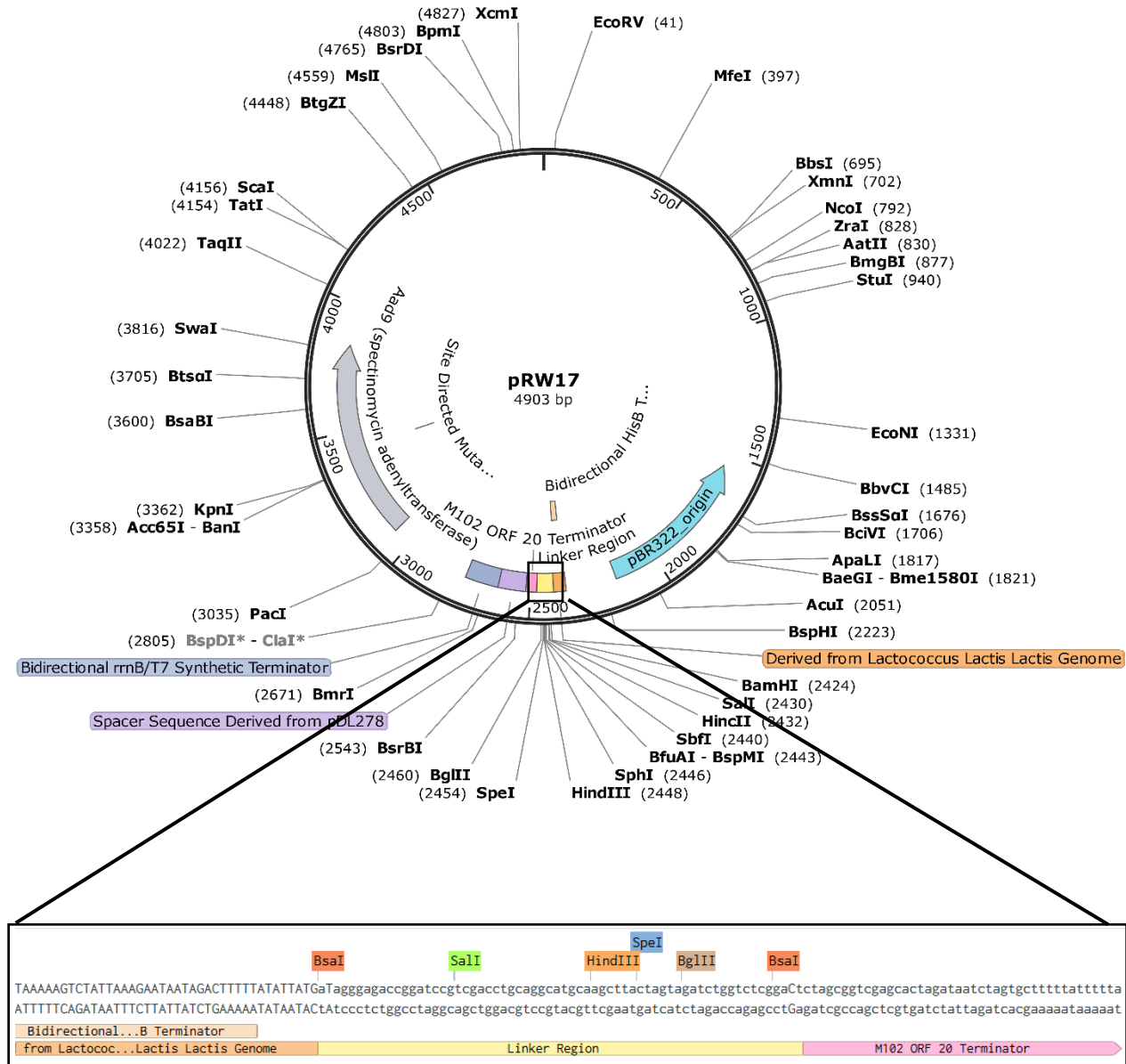
Supplementary Methods

Transformation Protocol for *Streptococcus sobrinus*

1. Inoculate a culture of *S. sobrinus* from a frozen glycerol stock or an agar plate into a 15 ml tube with 10 ml CDM. The CDM should be fresh, as we found that transformation efficiency drops noticeably after CDM has been kept in the fridge for over 3 weeks. Incubate overnight without shaking in an anaerobic chamber at 37°C.
2. At the same time, incubate enough CDM for the next day's dilution in 15 ml or 50 ml tubes with a loosened cap. This serves to prewarm and deoxygenate the media.
3. Dilute the overnight culture 150-fold into a culture tube with the pre-incubated CDM. The higher dilution seems to increase transformation efficiency. Incubate anaerobically at 37°C while monitoring the OD₆₀₀ with a portable cell density meter.
4. When the OD₆₀₀ reaches about 0.6, add XIP (LMCTIAR for NIDR 6715-7 or LMCTIVR for SL1 dissolved in DMSO at 10 mM) to a final concentration of 10 µM. Mix well by vigorously shaking the culture tube.
5. Immediately aliquot 200 µl of the culture into a microcentrifuge tube. Add appropriate amount of DNA (as a reference, 400 ng purified PCR product was used for our strain characterization experiments, but efficiency varies depending on the DNA sequence). Mix well by flicking the tube 15 times. (We found that flicking produces noticeably better results than mixing with a pipette; for this reason, we use 500 µl tubes instead of 1.5 ml tubes as the former are easier to flick.)
6. Incubate the capped tubes for 2 h.
7. Prewarm two THY agar plates with appropriate antibiotic selection.
8. Plate 150 µl of the culture onto a selection plate. Plate a small amount of culture that did not have added DNA to another selection plate as a negative control.
9. Incubate the plates at 37°C in a 5% CO₂ chamber for 24 h or until colonies are visible.

Plasmid Construction

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Supplementary Figure S1. pRW17 plasmid map and linker region.

pRW17 is a streptococcal expression vector designed to enable Golden Gate assembly of modular gene expression cassettes. The backbone of pRW17 was derived from pRW06, a derivative of pZX10 (Xie et al. 2013) with the Gram-negative origin of replication from pDL278 (LeBlanc et al. 1992) inserted into the BbvCI site. pRW17BB.for (5'-cggatgggtctcggagtagaagatcgatcttctg-3') and pRW17BB.rev (5'-tgctcgggtctcgtcctcactgattaagcattgg-3') were used to perform PCR (Q5 Hot Start High Fidelity, NEB) using pRW06 as template. The resulting amplicon contained

terminal 5' and 3' BsaI restriction sites flanking the *aad9* spectinomycin resistance marker, the Gram-positive origin of replication, and the Gram-negative origin of replication. The insert molecule for the construction of pRW17 was designed to include the several components and synthesized (GeneBlock, IDT). A polylinker including restriction sites for many commonly utilized restriction enzymes, including BsaI, was included between two intrinsic, transcriptional terminators. The *hisB* bidirectional terminator from the histidine biosynthesis cluster of *Lactococcus lactis* subsp. *lactis* (Delorme et al. 1992) was placed on the 5' flank of the polylinker, while a predicted, unidirectional terminator associated with ORF 20 of the *Streptococcus mutans* UA159 bacteriophage M102 (van der Ploeg , 2007) was placed in series with the synthetic bidirectional *rrnB* T1/T7-TE terminator on the 3' flank (Glass and Riedel-Kruse 2018). The synthesized insert was amplified upon arrival with PCR using the following primers: Insert.GG.Comp.for (5'-gtatacgaagacacggactacaacagaccttgct -3') and Insert.GG.Comp.rev (5'-acggetgaagacacactcctttgtttatcctctc -3'). The resulting amplicon had BbsI sites on the 5' and 3' end, which upon digestion yield overhangs compatible with the overhangs on the backbone produced from BsaI digestion. Each component was digested with its respective enzyme separately and then utilized in a standard T4 DNA Ligase-mediated ligation reaction using 40 fmol of digested insert and 20 fmol of digested backbone. After 1 hour, 5 uL of the 20 uL ligation mix was used to transform competent *E. coli* DH5a (Mix & Go, Zymo Research). After a 1-hour outgrowth, the cells were plated on LB/Spec100 and incubated overnight at 37 C. The next morning, colonies were screened with colony PCR. Subsequent plasmid isolation, restriction enzyme digest and Sanger sequencing validated successful pRW17 construction.

For use as a Golden Gate cloning platform a set of five, orthogonal four-nucleotide overhangs were developed by surveying previous literature using similar designs (Whitaker et al. 2017) and by referencing the T4 ligation fidelity and efficiency overhang profile developed by Potapov et al. 2018.

Overhang 1: 5' -*ATAG -3'
3' - TATC*-5'

Overhang 2: 5' -*TCTA -3'
3' - AGAT*-5'

Overhang 3: 5' -*AATC -3'
3' - TTAG*-5'

Overhang 4: 5' -*GACT -3'
3' - CTGA*-5'

Overhang 5: 5' -*CATA -3'
3' - GTAT*-5'