Supplementary material for:

Multiplex genome editing by natural transformation (MuGENT) for synthetic biology in *Vibrio natriegens*

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Supplementary protocol: Natural transformation / MuGENT in *V. natriegens*

Materials:

1. LBv2 = Mix 400 mL LB + 32 mL sterile 5 M NaCl + 3.4 mL sterile 1 M KCl + 18.5 mL sterile 1 M MgCl2
2. 2XIO = 28 g/L of instant ocean sea salts (www.instantocean.com)
3. Transforming DNA (tDNA) –
    a. selected product = mutant construct that has a selectable marker (antibiotic resistance cassette) that replaces the gene of interest or a neutral locus (for MuGENT). Homology on each side of the mutation can be as little as 0.5 kb. Homology of 3 kb on either side of the mutation results in the highest transformation efficiencies.
    b. Unselected product (cotransformation / MuGENT) = mutant construct that lacks any selectable marker but has a mutation of interest (deletion, point mutation, promoter swap, etc.). Unselected products should have 3 kb of homology on each side of the mutation for the highest rates of cotransformation / MuGENT.

Notes:

1. Carb100 = Carbenicillin 100 μg/mL
2. SAD1306 = *V. natriegens* Rif^R 14048 pMMB67EH-tfoX
3. The pMMB67EH-tfoX plasmid is very stable in *V. natriegens*, therefore, Carbenicillin (Carb) is not needed to maintain it throughout the transformation protocol.

Procedure:

1. Inoculate 3 mL of LBv2+Carb100+100 μM IPTG in a culture tube with SAD1306. Grow at 30°C in rollerdrum overnight (12-18 hours).
2. Next day, take culture out of incubator and measure OD_{600}. Generally, our overnights are at an OD_{600} of between 7-10.
3. For each transformation reaction, take 3.5 μL of the overnight culture and dilute into 350 μL of 2XIO+100 μM IPTG (no Carb). Invert gently to mix. Be sure to also prep a "no DNA" control reaction.
4. Add tDNA to each reaction and invert gently to mix:
    a. For a selected product (i.e. a product that has an antibiotic resistance marker) = ~5-50 ng yields thousands of colonies.
    b. For cotransformation / MuGENT = use 50 ng of a selected product and ~200 ng of each unselected product
5. Incubate reactions at 30°C statically for 4-6 hours.
6. Next, add 1mL LBv2 (no drug) to each transformation reaction and outgrow at 30°C shaking for 1-2 hours
7. To determine the transformation efficiency:
    a. Plate all reactions for quantitative culture on media to select for the transformants (i.e. on antibiotic plates that select for integration of selected

product) and on plates without any drug to determine the total CFU in the culture.
   b. Transformation efficiency = transformants CFU / total CFU
8. For cotransformation / MuGENT:
   a. Plate onto media to select for integration of the selected product.
   b. Pick single colonies and screen by MASC-PCR to identify clones with the desired genome edits.
   c. To perform a subsequent round of MuGENT, ~200 μL of the outgrown transformation can be inoculated into 3 mL of LBv2+Carb100+100 μM IPTG+the antibiotic that selects for integration of the selected product (e.g. if Δ*dns*::Kan^R was used in the first cycle of MuGENT, then 50 μg/mL Kan would be included in this overnight culture). Start at "step 2" of this procedure to perform the next cycle of MuGENT being sure to use a selected product with a distinct Ab^R marker.

Supplementary Tables

Table S1 – *Strains used in this study*

Strain name	Genotype and antibiotic resistances	Description	Reference / (strain#)
WT	Rif^R	Spontaneous Rif^R derivative *V. natriegens* ATCC14048 that is the parent isolate for all strains used in this study.	This study (SAD1304)
pMMB-tfoX (Vc)	pMMB-*tfox* (Vc) Carb^R	SAD1304 containing pMMB-*tfoX* (Vc), a vector containing the *tfoX* gene from *V. cholerae* (VC1153) under the control of an IPTG-inducible P_{tac} promoter. Vector is derived from pMMB67EH and has a Carb^R gene for selection.	This study (SAD1306)
pMMB-tfoX (Vn)	pMMB-*tfox* (Vn) Carb^R	SAD1304 containing pMMB-*tfoX* (Vn), a vector containing the *tfoX* gene from *V. natriegens* (BA890_05980) under the control of an IPTG-inducible P_{tac} promoter. Vector is derived from pMMB67EH and has a Carb^R gene for selection.	This study (TND0322 / SAD1495)
pMMB	pMMB empty vector Carb^R	SAD1304 containing the pMMB67EH empty vector	This study (TND0321 / SAD1496)
WT (Fig. 1D)	pMMB-*tfoX* (Vc) Carb^R, Δ*dns*::Kan^R	SAD1306 with Δ*dns*::Kan^R (ΔBA890_12415)	This study (SAD1313)
Δ*mutS*	pMMB-tfoX (Vc) Carb^R, Δ*dns*::Kan^R, Δ*mutS*	Generated by cotransformation into SAD1306 with Δ*dns*::Kan^R and a product to delete ~500bp of the 5' end of the *mutS* gene	This study (TND0362 / SAD1497)

		(BA890_12150).	
Δ*wbfF*	pMMB-tfoX (Vc) Carb^R, Δ*wbfF*::Kan^R	Introduced a Δ*wbfF*::Kan^R mutation (ΔBA890_01135) into the SAD1306 strain background.	This study (CAH509 / SAD1498)
MuGENT quadruple mutant	pMMB-tfoX (Vc) Carb^R, Δ*wbfF*::Kan^R, ΔBA890_01815 (mannitol transporter), ΔBA890_19540 (sucrose transporter), ΔBA890_16410 (fructose transporter), Δ*dns*	MuGENT into SAD1306 strain with 5 unselected genome edits. This quadruple mutant was whole genome sequenced and no off target mutations were identified.	This study (TND0338 / SAD1499)
Fig. 4E, second bar	pMMB-tfoX (Vc) Carb^R, Δ*dns*::Kan^R, P$_{tac}$-*phaBAC*	MuGENT into SAD1306 to enhance PHB production. The strain contains the genome edits indicated.	This study (TND0364 / SAD1500)
Fig. 4E, third bar	pMMB-tfoX (Vc) Carb^R, Δ*dns*::Kan^R, P$_{tac}$-*phaBAC*, P$_{tac}$-*nadK,* Δ*ldhA*	MuGENT into SAD1306 to enhance PHB production. The strain contains the genome edits indicated.	This study (SAD1501)
Fig. 4E, fourth bar	pMMB-tfoX (Vc) Carb^R, Δ*dns*::Kan^R, P$_{tac}$-*phaBAC*, P$_{tac}$-*pntAB,* Δ*pta*, Δ*gltA,* Δ*aceA*	MuGENT into SAD1306 to enhance PHB production. The strain contains the genome edits indicated.	This study (SAD1502)
Fig. 4E, fifth bar	pMMB-tfoX (Vc) Carb^R, Δ*dns*::Spec^R, P$_{tac}$-*phaBAC*, P$_{tac}$-*nadK,* Δ*pta*, Δ*gltA,* Δ*ldhA*	MuGENT into SAD1306 to enhance PHB production. The strain contains the genome edits indicated.	This study (SAD1503)
Fig. 2E, sixth bar	pMMB-tfoX (Vc) Carb^R, Δ*dns*::Spec^R, P$_{tac}$-*phaBAC*, P$_{tac}$-*nadK,* Δ*pta*, Δ*gltA,* Δ*ldhA,* Δ*aceA*	MuGENT into SAD1306 to enhance PHB production. The strain contains the genome edits indicated.	This study (SAD1504)

Table S2 – *Primers used in this study*

Primer Name	**Primer Sequence (5'→3')***	**Description**
Primers for Mutant constructs		
ABD123	ATTCCGGGGATCCGTCGAC	Amplify MIDDLE Ab^R (Kan^R, Spec^R, or Tm^R cassettes) F
ABD124	TGTAGGCTGGAGCTGCTTC	Amplify MIDDLE Ab^R (Kan^R, Spec^R, or Tm^R cassettes) R
BBC1264	CTAACATGGCTAAGCACCTG	Δ*dns* F1 (3kb)
BBC1605	GCACTTCTTCGCGAATTCGC	Δ*dns* F1 (2kb)
BBC1607	AGTGATTGGGTCACTCATTGG	Δ*dns* F1 (1kb)
BBC1609	AATGAGATTCGCCTTAACCC	Δ*dns* F1 (0.5kb)
BBC1265	gtcgacggatccccggaatAGAGAACAGGTATTTCATAGTTAAAGTC	Δ*dns* R1
BBC1266	gaagcagctccagcctacaTAATCCTCACCAATCGCGAC	Δ*dns* F2
BBC1610	TCGAGCTTTACGCCACAACG	Δ*dns* R1 (0.5kb)
BBC1608	ACACCTTGGTCGAGGTGAAG	Δ*dns* R1 (1kb)
BBC1606	ATAACGCAGTAGAAAGTATCCAC	Δ*dns* R1 (2kb)
BBC1267	ACTGGTAAGCCATAACGACC	Δ*dns* R1 (3kb)
DOG0246	AGGCTCGTGTTGCATGTGAG	Δ*dns* 501bp F1
DOG0247	gctaattcagtttaagcggccatCATAGTTAAAGTCTTTAAAAAGTA	Δ*dns* 501bp R1

	TGACTT	
DOG0248	atggccgcttaaactgaattagcATCGCTCGTACCTATCTTTATATG	Δ*dns* 501bp F2
DOG0249	TAAGGTGTCTCAAATCTCAATCTAGG	Δ*dns* 501bp R2
BBC1255	TGAGAAATTCTTTGCATCACATC	*rpsL* K43R (Sm^R) F1
BBC1256	GAAGTGCTGAGTTAGGTTTTcTAGGTGTAGTAGTGTAAACAC	*rpsL* K43R (Sm^R) R1
BBC1257	GTGTTTACACTACTACACCTAgAAAACCTAACTCAGCACTTC	*rpsL* K43R (Sm^R) F2
BBC1258	GTAGTGACGAGTTGGAGTG	*rpsL* K43R (Sm^R) R2
BBC1552	GAACTGCATGAATACGTTGTTCC	Δ*mutS* 501bp F1
BBC1553	gctaattcagtttaagcggcCACAGGTAAGTTCTTTTGTTTATTTCC	Δ*mutS* 501bp R1
BBC1554	GTGgccgcttaaactgaattagcCGCACCGCACCACGTGAG	Δ*mutS* 501bp F2
BBC1555	GAGTATCAGCAACACAGTAACC	Δ*mutS* 501bp R2
BBC1347	TAGCAACTGTTTTAGCGCTG	Δ*wbfF* F1
BBC1348	gtcgacggatccccggaatCTTTTATCATCATACTCATTCATTAAAG	Δ*wbfF* R1
BBC1349	gaagcagctccagcctacaTGATGTATAAGCGTCATTTATTCG	Δ*wbfF* F2
BBC1350	GTTCCTGTCGATAAGTATTGATC	Δ*wbfF* R2
DOG0353	AATGTCGGCCTTCTGATTAG	Δ*wbfF* 501bp F1 (3kb)
BBC1612	TAAACTTTATCAGCGACGTCAG	Δ*wbfF* 501bp F1 (2kb)
BBC1614	TTCAGGAACGATGTCGACAG	Δ*wbfF* 501bp F1 (1kb)
DOG0354	gctaattcagtttaagcggccatTATCATCATACTCATTCATTAAAGTTTTAA	Δ*wbfF* 501bp R1
DOG0355	atggccgcttaaactgaattagcACTAATAACGTCAGTGTATACGTAAAC	Δ*wbfF* 501bp F2
BBC1615	CCACGCAATGTAGTCATCAATC	Δ*wbfF* 501bp R2 (1kb)
BBC1613	GGATACGCAGCATACCTTG	Δ*wbfF* 501bp R2 (2kb)
BBC1611	TTAATTGTGCCTGAGCAAGC	Δ*wbfF* 501bp R2 (3kb)
DOG0271	AAGTAGTGATGATCCGAAGCG	ΔBA890_01815 501bp (mannitol transporter) F1
DOG0272	gctaattcagtttaagcggccatCATAACAATTCCCCGTTCGATG	ΔBA890_01815 501bp (mannitol transporter) R1
DOG0273	atggccgcttaaactgaattagcCTTGTATCAGCGCACCTTCTAC	ΔBA890_01815 501bp (mannitol transporter) F2
DOG0274	ATCGTGGTAAATATCGTCAGGTAG	ΔBA890_01815 501bp (mannitol transporter) R2
DOG0266	ATCTCGGCTTGTCTACACCAG	ΔBA890_19540 (sucrose transporter) F1
DOG0267	gctaattcagtttaagcggccatCATTGCACACCCCGATTGG	ΔBA890_19540 (sucrose transporter) R1
DOG0268	atggccgcttaaactgaattagcTATTTACCTGTTTTATTGGCGTTTTC	ΔBA890_19540 (sucrose transporter) F2
DOG0269	TGAACTGAATCCTCGCAGG	ΔBA890_19540 (sucrose transporter) R2
DOG0256	ATGCTCGTCATCCATGGGAC	ΔBA890_16410 (fructose transporter) F1
DOG0257	gctaattcagtttaagcggccatCATACTGATAACCTTCTGTTCCTTAG	ΔBA890_16410 (fructose transporter) R1
DOG0258	atggccgcttaaactgaattagcACCGCGCAAGAGATCGAAG	ΔBA890_16410 (fructose transporter) F2
DOG0259	TTGGGTGCTTTGCTTCTCG	ΔBA890_16410 (fructose transporter) R2

DOG0261	ATCTGAACTTAGGATACTCACATC	ΔBA890_03375 (trehalose transporter) F1
DOG0262	gctaattcagtttaagcggccatCATAACTTTGCCCACCCTGTATTG	ΔBA890_03375 (trehalose transporter) R1
DOG0263	atggccgcttaaactgaattagcTTCTTCCTGCCTGTTGGC	ΔBA890_03375 (trehalose transporter) F2
DOG0264	AGTCAGATGGCGATTGATGTG	ΔBA890_03375 (trehalose transporter) R2
ABD840	TTAATTGCGTTGCGCTCACTGCCCGACTCCCGTTCTGGATAATGTTTTTTGC	Amplify MIDDLE P_{tac} construct F
ABD625	CTGATGAATCCCCTAATGATTTTGG	Amplify MIDDLE P_{tac} construct R
BBC1536	GTAACGAACGTGTCATCAGTG	P_{tac}-*phaBAC* F1
BBC1540	CGGGCAGTGAGCGCAACGCAATTAATGCAAGCGCACTAATATGAC	P_{tac}-*phaBAC* R1
BBC1541	CAAAATCATTAGGGGATTCATCAGAAAGAATGGAGTCGTCAATGAATAAAG	P_{tac}-*phaBAC* F2
BBC1577	CGACATCTTCACCAACACG	P_{tac}-*phaBAC* R2
BBC1621	TCTGGAGAGTATGTTGGCC	P_{tac}-*pntAB* F1
BBC1622	cgggcagtgagcgcaacgcaattaaCCTTGTATACATATCAATTAATTAGTCCC	P_{tac}-*pntAB* R1
BBC1623	caaaatcattaggggattcatcagAggaggTTGCGTTTTGCAAATCGGTGTAC	P_{tac}-*pntAB* F2
BBC1624	AGACTACGCCAAACTATACAGC	P_{tac}-*pntAB* R2
BBC1616	CTTCTTCGTCTTCAAAACGACG	P_{tac}-*nadK* F1
BBC1617	cgggcagtgagcgcaacgcaattaaGCATTAAAGAGGCTTGAATCAGG	P_{tac}-*nadK* R1
BBC1618	caaaatcattaggggattcatcagaggaggtAATGCTATGAAAAATCCATGTAACG	P_{tac}-*nadK* F2
BBC1619	CTGCGCTGATAATAAACAAGC	P_{tac}-*nadK* R2
BBC1626	CACAAATAGCGAAGCTAACTG	P_{tac}-*udhA* F1
BBC1627	cgggcagtgagcgcaacgcaattaaTATTTGCTTAACATTGCCTTAGC	P_{tac}-*udhA* R1
BBC1628	caaaatcattaggggattcatcagAggaggtTACATCATGGCGCATGTAAATC	P_{tac}-*udhA* F2
BBC1629	GTGAAAGTATTTTCGCCTTTCG	P_{tac}-*udhA* R2
BBC1636	GACAAGTCAGAAAGTCCAGTCAC	Δ*pta* 501bp F1
BBC1637	gctaattcagtttaagcggccatAGACATTCGTAGAGTACCTTTGC	Δ*pta* 501bp R1
BBC1638	atggccgcttaaactgaattagcGTTATCATCAACAAGCTAAACGCAC	Δ*pta* 501bp F2
BBC1639	GATATCAACGAGTTTGCATCTG	Δ*pta* 501bp R2
BBC1646	GCTAACATCAATGCGTATGCC	Δ*pgi* 501bp F1
BBC1647	gctaattcagtttaagcggccatCAACATGGTCTTTATCCCGATG	Δ*pgi* 501bp R1
BBC1648	atggccgcttaaactgaattagcGCACTGGCACCATACAAAAAC	Δ*pgi* 501bp F2
BBC1649	CCTTTCTCAGACACTATCGACAC	Δ*pgi* 501bp R2
BBC1641	AGCCTTCTTCTACATCAAGTGTG	Δ*gltA* 501bp F1
BBC1642	gctaattcagtttaagcggccatATCCGCCATAACAATCTCCTTTG	Δ*gltA* 501bp R1
BBC1643	atggccgcttaaactgaattagcACACTGGCGGCAATGTGTTAC	Δ*gltA* 501bp F2
BBC1644	CAAGAGTACTACGAAGAGCTG	Δ*gltA* 501bp R2
BBC1651	CTTGTAACACTGCCGCTAAGAG	Δ*ldhA* 501bp F1
BBC1652	gctaattcagtttaagcggccatCATGGTTCTCTCTCGAAATCATTG	Δ*ldhA* 501bp R1
BBC1653	atggccgcttaaactgaattagcATGGAAATTCTTTGCCATGATCC	Δ*ldhA* 501bp F2
BBC1654	AGTGTGTTACTTATTTGGAGGATG	Δ*ldhA* 501bp R2
BBC1631	TGAACTGCTGGCGAAAGGAC	Δ*aceA* 501bp F1

BBC1632	GCTAATTCAGTTTAAGCGGCCATTGGTCTATCCCTCTTTATAATTTGC	Δ*aceA* 501bp R1
BBC1633	ATGGCCGCTTAAACTGAATTAGCCTAAATGCTTACGAACTGATGAAATC	Δ*aceA* 501bp F2
BBC1634	CGATTGAAGCTTGAAGAACAAGC	Δ*aceA* 501bp R2
Primers for MASC-PCR		
ABD969	ATGGCCGCTTAAACTGAATTAGC	Universal F primer for all Δ501 bp genome edits
DOG0250	TGGTTGCCTTGTACTTTGGC	R detect for Δ*dns* 501 bp (152bp product)
BBC1556	AGTGATCGAGAACAGCGG	R detect for Δ*mutS* 501bp (402bp product)
DOG0356	ATAGCTACCGCGTTCAGGG	R detect for Δ*wbfF* 501bp (165bp product)
DOG0275	AGTGACGTGGATGTTCAGAC	R detect for ΔBA890_01815 501bp (mannitol transporter) (750bp product)
DOG0270	AACCCAGTGATACCAGATGG	R detect for ΔBA890_19540 (sucrose transporter) (650bp product)
DOG0260	TATTCATCAGTGCAGCGGC	R detect for ΔBA890_16410 (fructose transporter) (352 bp product)
DOG0265	TCTTGCATTAACTGTAAATCCACG	R detect for ΔBA890_03375 (trehalose transporter) (500 bp product)
BBC435	ACACTCTTTGGGGGCCAAAATCATTAGGGGATTCATCAG	Universal F primer to detect all P_{tac} genome edits
BBC1551	GGTAAACCCTTTGCTGTTAAACC	R detect for P_{tac}-*phaBAC* (170bp product)
BBC1625	CTTGAGCTCGAGAGATACG	R detect for P_{tac}-*pntAB* (400bp product)
BBC1620	GATAAAATTCGTGCGGCTC	R detect for P_{tac}-*nadK* (260bp product)
BBC1630	AGATAATGATATGACGAGGGTC	R detect for P_{tac}-*udhA* (550bp product)
BBC1640	CGAATTGGAGAAGTGTTGAAG	R detect for Δ*pta* (140bp product)
BBC1650	AACCCAGTCCCAGAATTCAAAC	R detect for Δ*pgi* (300bp product)
BBC1645	GATGTTGACGCGTTTTGTTCG	R detect for Δ*gltA* (200bp product)
BBC1655	GGCTTCTACGTTATTTAGTGTC	R detect for Δ*ldhA* (450bp product)
BBC1656	TGTTGTGAATACCCGCTAGAG	R detect for Δ*aceA* (600bp product)

*Lower case nucleotides specify overlap regions for SOE PCR