The Role of *opuB* in Modulating Membrane Vesicles Composition and Function in *Streptococcus mutans* under Neutral and Acidic Conditions

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Preparation of *Smu_opuB*

1. Bacterial culture and extraction of bacterial genomic DNA (gDNA)

1) The UA159 strain stored at -80 ℃ was revived in BHI medium. After morphological and biochemical identification, the plate was stored at 4 ℃ for short-term seed preservation.

2) A single clone was picked from the plate and inoculated into BHI liquid medium for overnight culture.

3) The overnight culture solution was extracted from the bacteria according to the instructions of the bacterial genomic DNA extraction kit.

4) The purity and concentration were determined and stored at -20 ℃.

2. PCR amplification and connection of target fragments

1) PCR was used to amplify the fragments up, erm and down. The reaction conditions were 94 ℃ pre-denaturation for 3 min; 94 ℃ denaturation for 30 s, 55 ℃ annealing for 30 s, 72 ℃ extension for 1 min, 30 cycles, and 72 ℃ extension for another 5 min. The reaction was terminated at 4 ℃.

2) 1% agarose gel electrophoresis was used to identify the PCR amplification product, and the gel was recovered and the product concentration and purity were determined, and stored at -20 ℃.

3) Using up, erm, and down as templates, upF and dnR as primers, fusion PCR was used to connect the above three fragments (recorded as up-erm-dn, with a size of about 1.6 kb). The reaction conditions were 94 ℃ pre-denaturation for 3 min; 94 ℃ denaturation for 30 s, 55 ℃ annealing for 30 s, 72 ℃ extension for 2 min, 30 cycles; 72 ℃ extension for another 5 min. The reaction was terminated at 4 ℃.

4) 1% agarose gel electrophoresis was used to identify the PCR amplification product, and stored at -20 ℃.

3. Transformation and screening

1) Pick UA159 monoclonal clones and inoculate them in BHI liquid medium. Take the overnight culture solution and dilute it in BHI medium at 1:20 and culture it for 2-3 h until the bacterial solution OD_{600} = 0.2-0.3.

2) Take bacterial solution and add samples according to the following groups: group a: 500 μL bacterial solution + 5 μL up-erm-dn + 0.5 μL CSP (1 μg·μL-1); group b: 500 μL bacterial solution + 0.5 μL CSP (1 $\mu g \cdot \mu L^{-1}$); group c (negative control): 500 μL bacterial solution. After adding and mixing, incubate in a 37℃ incubator for 2 h in microanaerobic culture.

3) Take 200 μL of bacterial solution from groups a, b, and c and spread them on BHI agar plates containing 12.5 $\mu g \cdot mL^{-1}$ erythromycin, and incubate in a 37℃ incubator for 48 h in microanaerobic culture.

4) Send the obtained positive monoclonal clones to the Guangzhou Branch of Beijing Liuhe BGI Gene Technology Co., Ltd. for sequencing.

Table S1: Strain and Primer Sequences Used in the Preparation of *Smu_opuB*

Strain	**Source**
S. mutans UA159（ATCC®700610™）	Guangdong Microbiology Research Institute Culture Collection Center
Primer Name	**Nucleotide Sequence (5'-3')**
upF	CGCGACTGTCAGAAAAGGAG
up-erm	TATTTTATATTTTTGTTCATCTTTATTCCTCTTTTCTAAT
ermF	ATGAACAAAAATATAAAATATTCTCA
ermR	TTATTTCCTCCCGTTAAATAAT
dnF-erm	ATTATTTAACGGGAGGAAATAAGAATAAGGAGGTTAGAACAC
dnR	ATCAAAAATTTCACTGCGGA

Erythromycin resistance gene (738 bp)

ATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACGAGTGAAAAAGTA
CTCAACCAAATAATAAAACAATTGAATTT
AAAAGAAACCGATACCGTTTACGAAATTGGAACAGGTAAAGGGCATTTAA
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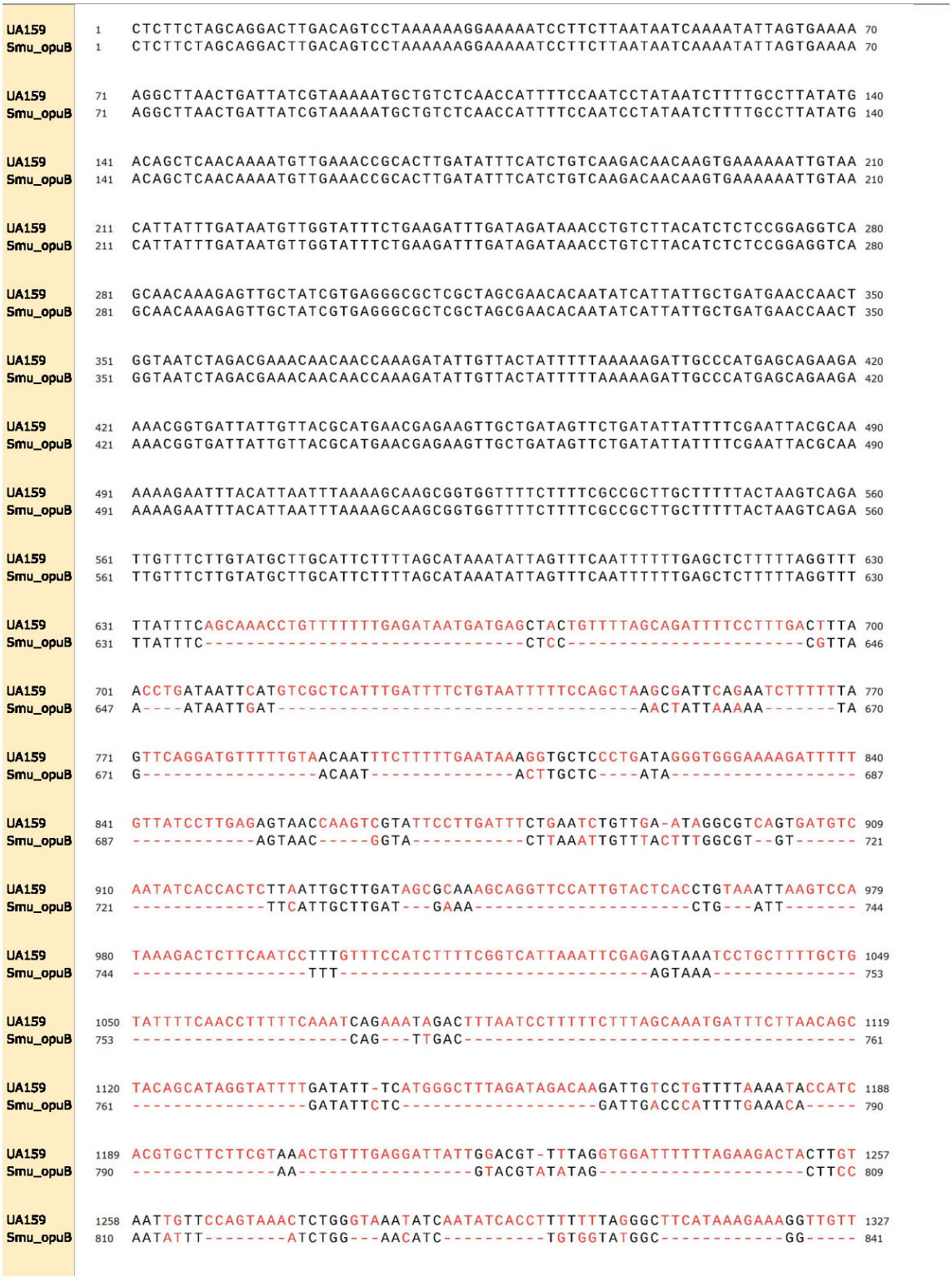
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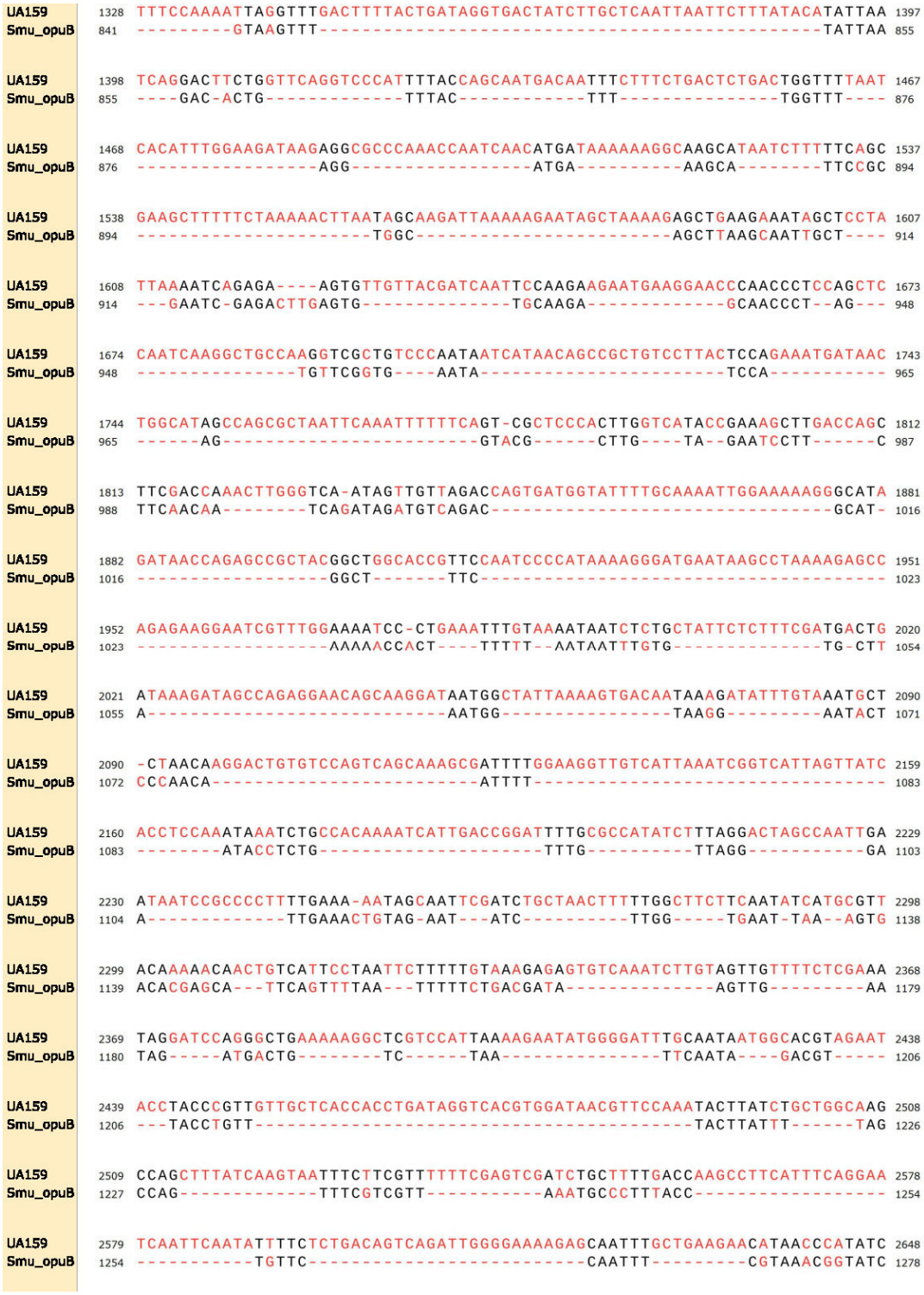
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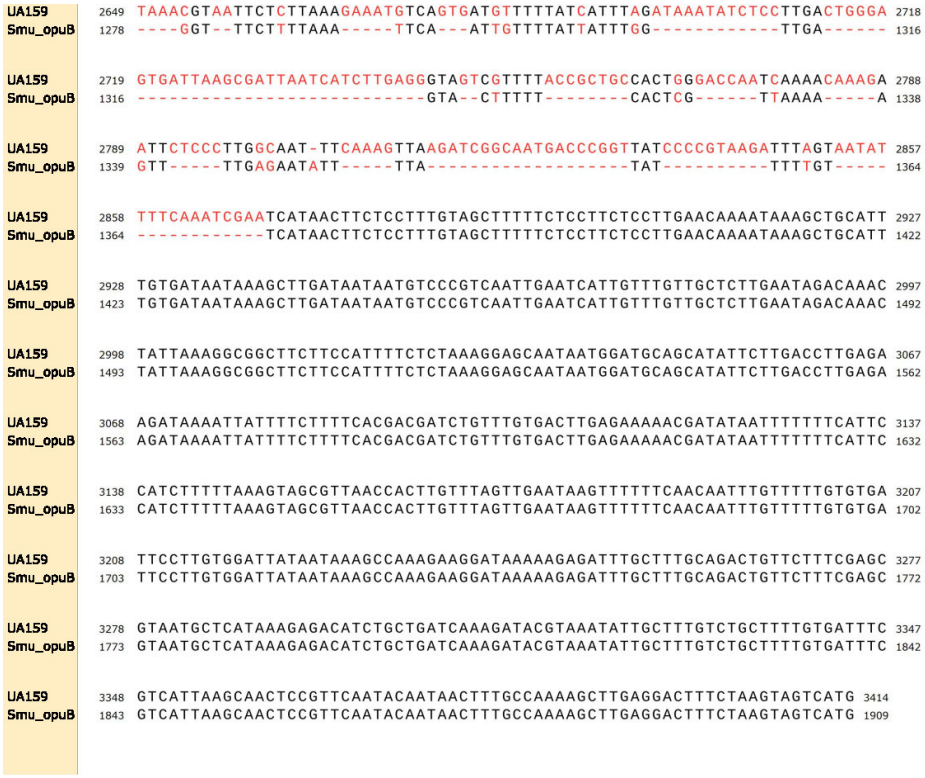

Figure S1. Sequencing results of positive monoclonal.

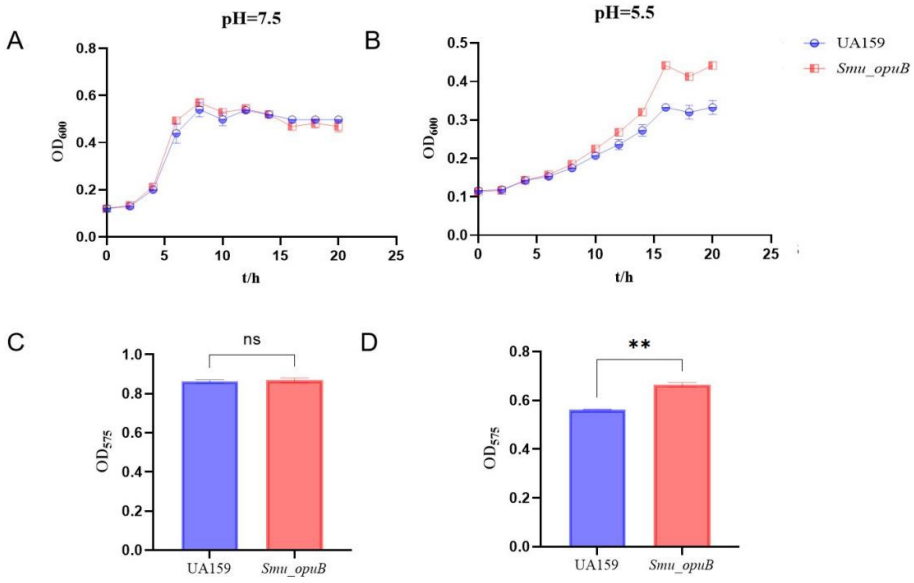

Figure S2. Growth and biofilm formation of *Smu_opuB* and UA159 under acidic and neutral conditions. Growth curves of *Smu_opuB* and UA159 under (A) neutral and (B) acidic conditions. Biofilm formation of *Smu_opuB* and UA159 after 24 hours under (C) neutral and (D) acidic conditions., assessed by crystal violet staining.

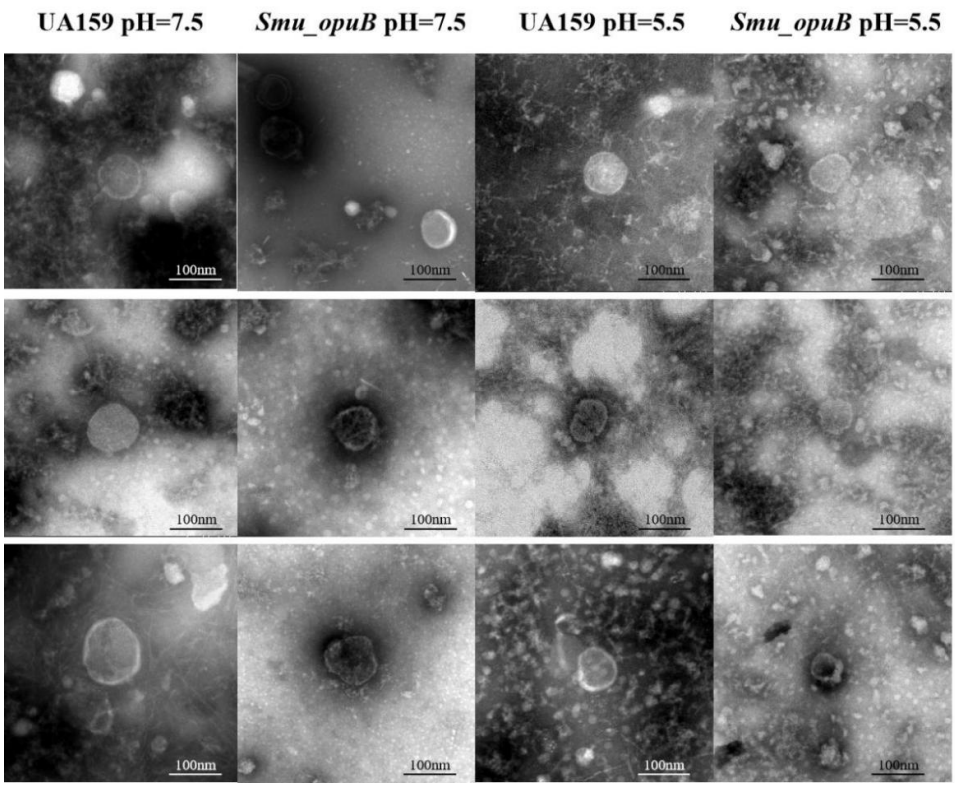

Figure S3. TEM images of MVs produced by *Smu_opuB* and UA159 under neutral (pH 7.5) and acidic (pH 5.5) conditions. For each group, three representative images are shown to provide a more comprehensive view of MV morphology. Scale bars are indicated in each image.

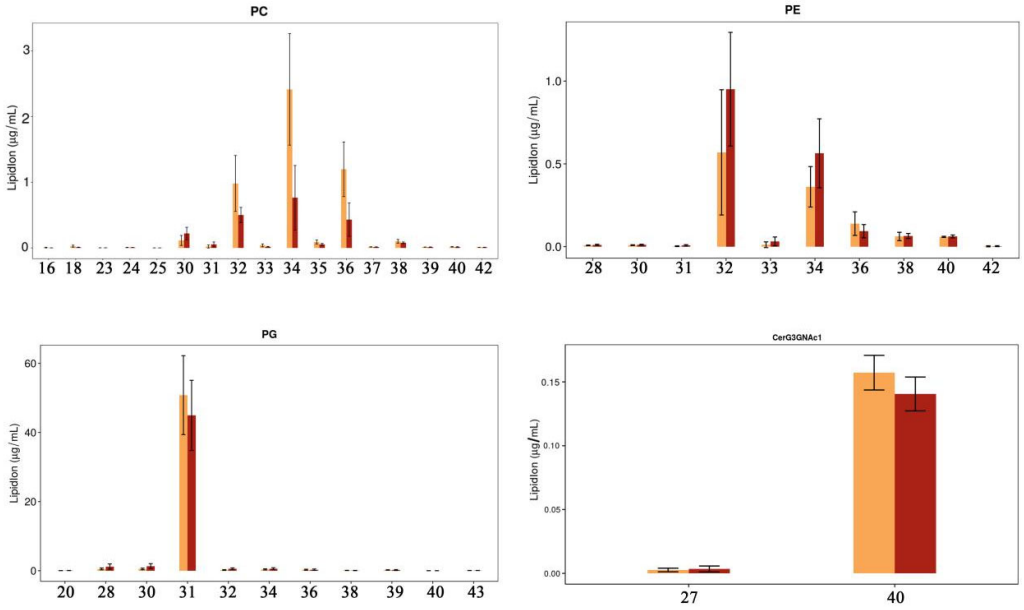

Figure S4. Chain length analysis of some lipids in UA159 and *Smu_opuB* MVs under neutral conditions. n=6. Data for all panels are mean ± S.D. *P < 0.05 (Student's *t*-test).

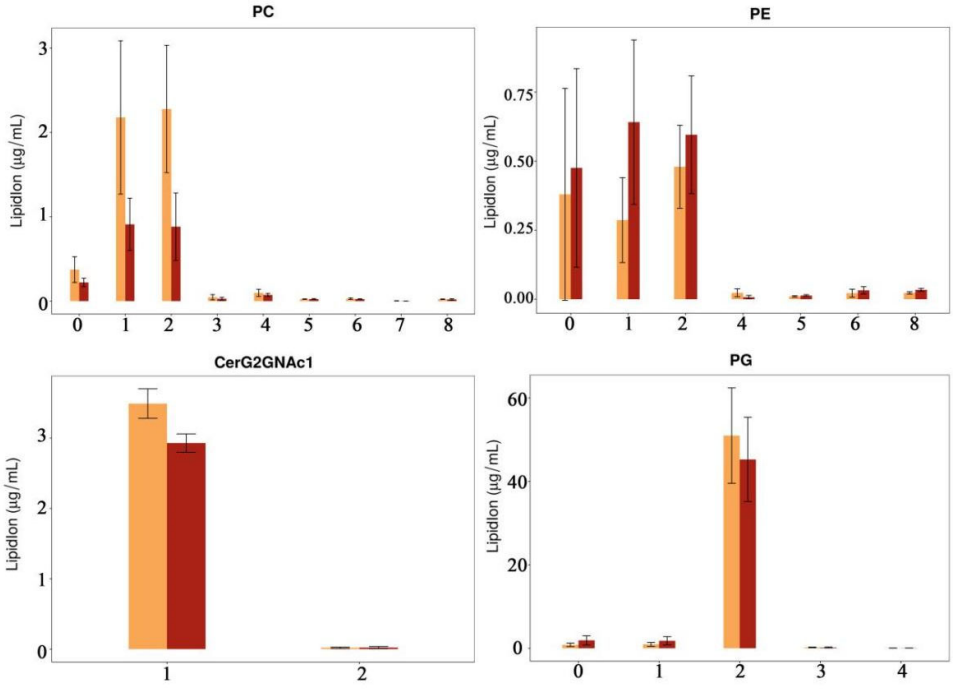

Figure S5. Saturation analysis of some lipids in UA159 and *Smu_opuB* MVs under neutral conditions. n=6. Data for all panels are mean ± S.D. *P < 0.05(Student's *t*-test).

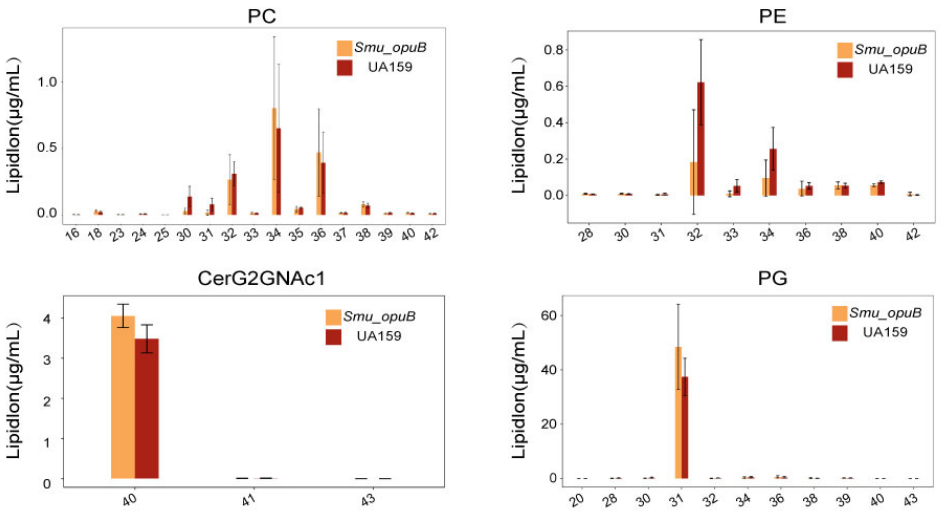

Figure S6. Chain length analysis of some lipids in UA159 and *Smu_opuB* MVs under acidic conditions. n=6. Data for all panels are mean ± S.D. *P < 0.05 (Student's *t*-test).

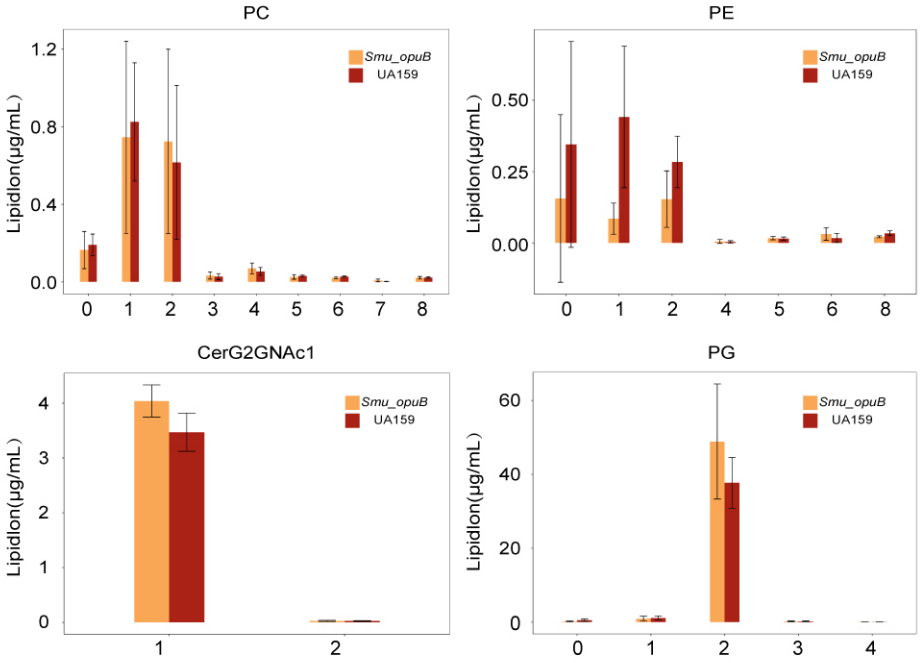

Figure S7. Saturation analysis of some lipids in UA159 and *Smu_opuB* MVs under acidic conditions. n=6. Data for all panels are mean ± S.D. *P < 0.05 (Student's *t*-test).

Table S2 The top 20 most significantly altered MV proteins under pH7.5 condition

Protein Name	Gene Name	MolWeight	*Smu_opuB*/UA159	*p* value
Malonyl-CoA acyl-carrier-protein transacylase	*SMU_1344c*	47.086	40.696	0.019
rRNA adenine N-6-methyltransferase	*erm(B)*	28.795	34.575	0.036
	A6J86_0043			
DUF1307 domain-containing protein	*25*	19.683	27.666	0.018
Glucosamine-6-phosphate deaminase	*nagB*	25.473	19.354	0.040
HNH endonuclease	*SMU_1205c*	19.542	19.091	0.042
ABC transporter, amino acid binding protein	*SMU_459*	30.509	18.059	0.026
AgI/II	*spaP*	169.97	16.877	0.031
50S ribosomal protein L15	*rplO*	15.446	13.528	0.042
Dextranase	*dexA*	94.481	12.798	0.037
30S ribosomal protein S11	*rpsK*	13.399	12.751	0.042
(R)-2-hydroxyglutaryl-CoA dehydratase activator-related protein	*SMU_438c*	159.32	0.125	0.005
Elongation factor Ts	*tsf*	37.719	0.100	0.037
Amino acid ABC transporter, ATP-binding protein	*SMU_936*	28.357	0.095	0.041
ABC sugar transporter, permease protein	*SMU_1118c*	33.775	0.088	0.024
Ribosomal protein L11 methyltransferase	*prmA*	34.543	0.083	0.004
Metal-dependent transcriptional regulator	*sloR*	25.076	0.083	0.000
	SMU82_026			
Cytoplasmic protein	*71*	24.169	0.079	0.042
ABC transporter, ATP-binding protein	*SMU_923*	64.59	0.066	0.001
Oxidoreductase	*SMU_728*	32.188	0.064	0.006
ABC transporter, ATP-binding protein	*SMU_922*	66.968	0.039	0.002

Table S3 The top 20 most significantly altered MV proteins under pH5.5 condition

Protein Name	Gene Name	MolWeight	*Smu_opuB*/ UA159	p value
Probable transcriptional regulatory protein SMU82_03911	SMU82_03911	25.74	34.332	0.005
rRNA adenine N-6-methyltransferase	erm(B)	28.795	33.964	0.002
GTP cyclohydrolase 1 type 2 homolog	SMU_1463c	29.74	17.912	0.002
Acetolactate synthase AlsS	A6J86_0042 70	60.758	16.534	0.000
Ribulose-phosphate 3-epimerase	rpe	23.722	15.402	0.000
Glucosamine-6-phosphate deaminase	nagB	25.473	13.578	0.000
Histidinol dehydrogenase	hisD	46.45	12.250	0.000
Shikimate kinase	aroK	17.612	11.323	0.022
Ribonucleoside-diphosphate reductase subunit beta	nrdG	36.788	11.226	0.004
67 kDa myosin-crossreactive streptococcal antigen-like protein	SMU_1584c	67.347	11.034	0.000
Hydrolase	SMU_1171c	29.399	0.120	0.028
Transcriptional repressor	A6J86_0080 15	18.212	0.117	0.016
ABC transporter, ATP-binding protein	SMU_1078c	65.369	0.094	0.001
ABC transporter, ATP-binding protein	SMU_922	66.968	0.088	0.036
Homoserine O-acetyltransferase	metAA	36.746	0.063	0.022
Integral membrane protein	SMU_237c	45.139	0.057	0.035
Phosphate transport system permease protein PstA	pstC	31.773	0.043	0.021
UPF0246 protein SMU82_04358	SMU82_043 58	28.04	0.041	0.022
Mutans bacitracin-resistance related protein B	mbrB	75.823	0.038	0.028
Cell division protein FtsL	ftsL	12.298	0.026	0.000