Supporting Information for:

A cardiolipin-deficient mutant of *Rhodobacter sphaeroides* has an altered cell shape and is impaired in biofilm formation

Ti-Yu Lin[1], Thiago M. A. Santos[1], Wayne S. Kontur[2,3], Timothy J. Donohue[2,3], Douglas B. Weibel[1,4,5]*

[1] *Department of Biochemistry, University of Wisconsin–Madison, Madison, Wisconsin, USA*
[2] *Department of Bacteriology, University of Wisconsin–Madison, Madison, Wisconsin, USA*
[3] *DOE Great Lakes Bioenergy Research Center, Madison, Wisconsin, USA*
[4] *Department of Chemistry, University of Wisconsin–Madison, Madison, Wisconsin, USA*
[5] *Department of Biomedical Engineering, University of Wisconsin–Madison, Madison, Wisconsin, USA*

* Author to whom correspondence should be addressed:
Douglas B. Weibel
Department of Biochemistry
6424 Biochemical Sciences Building
440 Henry Mall
Phone: +1 (608) 890-1342
Fax: +1 (608) 265-0764
E-mail: weibel@biochem.wisc.edu

Running title: Cardiolipin affects cell shape and biofilm formation

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Results

RSP_0113 does not encode a cardiolipin synthase in *Rhodobacter sphaeroides*

To investigate the origins of the residual 10% of the cardiolipin (CL) in the CL3 mutant, we performed a BLAST search and found another candidate *CL synthase* (*cls*) gene in *R. sphaeroides*: *RSP_0113* (GenBank entry YP_353188) (1, 2). RSP_0113 is 25% identical and 41% similar to the amino acid sequence of *Escherichia coli* CL synthase, and contains a phospholipase D domain that is a characteristic motif found in CL synthases. We cloned the *RSP_0113* gene into an *E. coli* expression plasmid pTrc99A and an *R. sphaeroides* expression plasmid pIND4, and expressed it in the *E. coli* CL-deficient mutant BKT12 (3) and *R. sphaeroides* CL3, respectively. We were unable to detect CL in phospholipids extracted from BKT12 cells harboring a plasmid-encoded *RSP_0113* gene or nor were we able to detect increased CL in analogous CL3 transformants (data not shown) by thin-layer chromatogram. These results suggest that RSP_0113 is not a CL synthase in either bacterium and leave us uncertain of the origins of the residual 10% CL in *R. sphaeroides* CL3.

Materials and Methods

Confocal scanning laser microscopy of biofilms

Stationary phase cell cultures were standardized to an absorbance of 1.0 (λ, 600 nm). The standardized cultures were inoculated 1/100 in Sistrom's succinate medium containing 5 μg/ml Nile Red in a chamber slide with hydrophobic plastic surfaces (ibidi, Verona, WI) and incubated for 72 h at 30°C. After washed with water to remove planktonic cells, biofilms were imaged on a Nikon A1R- Si confocal microscope. Images were processed with NIS-Elements AR software.

References

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3. **Tan BK, Bogdanov M, Zhao J, Dowhan W, Raetz CR, Guan Z.** 2012. Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as substrates. Proc Natl Acad Sci U S A **109:**16504-16509.

Figures and Legends

Figure S1. Nile Red does not change cell shape and impede cell growth of *R. sphaeroides*. (A) Images depicting the morphology of *R. sphaeroides* wild-type (WT) and CL3 cells grown aerobically in Sistrom's succinate medium containing 5 μg/mL Nile Red at 30°C with shaking at 200 rpm. Each data point was determined by imaging 300 cells using phase contrast brightfield microscopy and using ImageJ to determine cell width and length. The values represent mean values ± standard deviations. Differences of the cell shape parameters between the two strains were analyzed by Student's *t test*. The P value for all parameters measured was < 0.001. Scale bar, 2 μm. (B) Growth curves of *R. sphaeroides* WT and CL3 cells grown with shaking in glass test tubes at 30°C in Sistrom's succinate medium containing 5 μg/mL Nile Red. The values represent mean values ± standard deviations obtained from three independent experiments. Although a ~0.1 difference in absorbance (λ, 600 nm) was observed at stationary phase, colony-forming units (CFUs) of the WT and CL3 strains were not significantly different (WT: 1.6×10^9 CFU/mL, CL3: 1.5×10^9 CFU/mL).

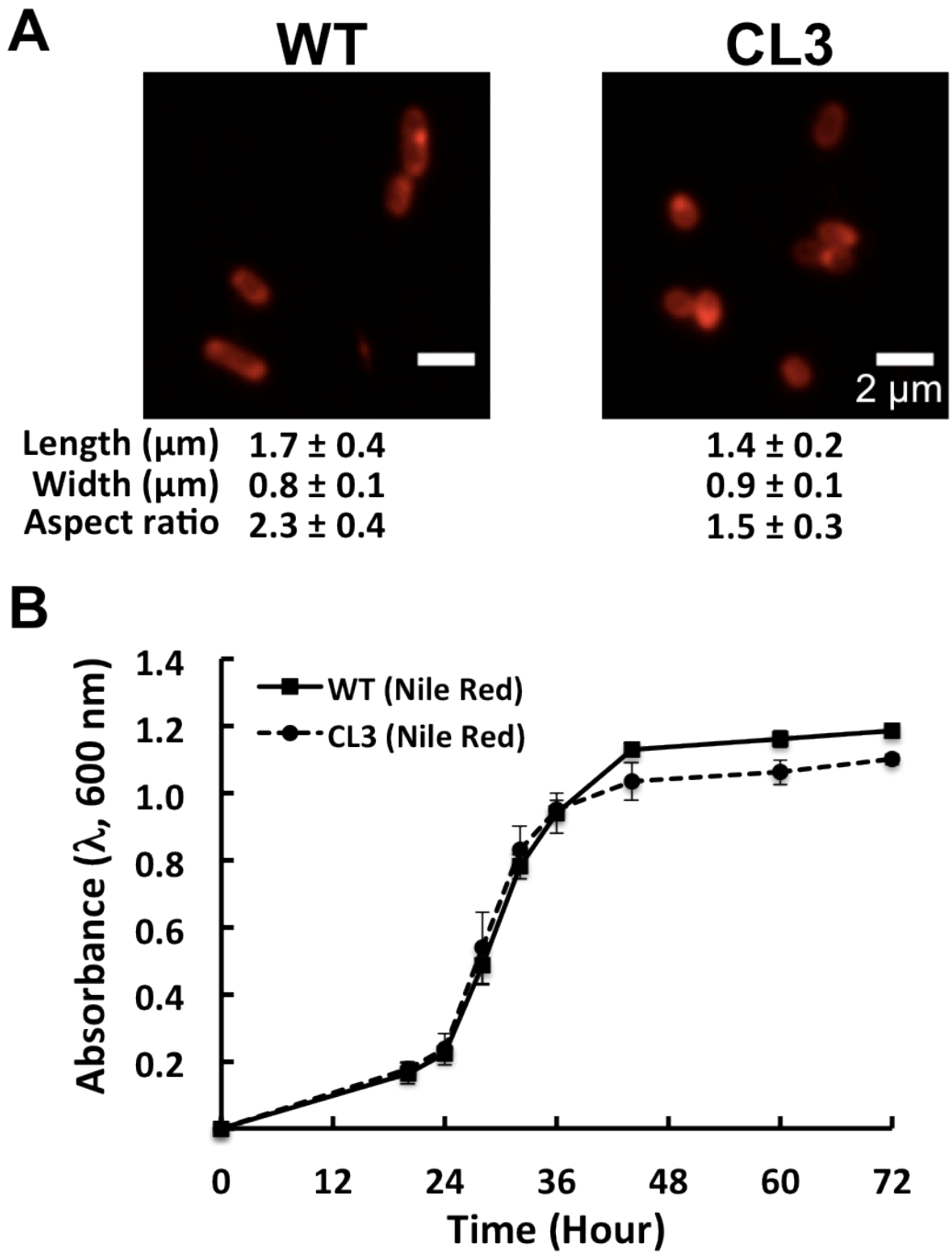
A
WT
CL3
2 µm
Length (µm) 1.7 ± 0.4 1.4 ± 0.2
Width (µm) 0.8 ± 0.1 0.9 ± 0.1
Aspect ratio 2.3 ± 0.4 1.5 ± 0.3
B
Absorbance (λ, 600 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
WT (Nile Red)
CL3 (Nile Red)
0
12
24
36
48
60
72
Time (Hour)

Figure S1

Figure S2. Confocal laser scanning micrographs of *R. sphaeroides* WT and CL3 biofilms. *R. sphaeroides* WT and CL3 biofilms were grown on a chamber slide with hydrophobic plastic surfaces for 72 h at 30°C in Sistrom's succinate medium containing 5 μg/ml Nile Red and imaged using a confocal microscope. Upper panels: orthogonal views. Scale bar, 10 μm. Lower panels: 3-D reconstructions of confocal microscopy images. The thickness of *R. sphaeroides* WT biofilms was 20 μm. In contrast, the CL3 strain formed biofilms that were typically ~9-μm thick.

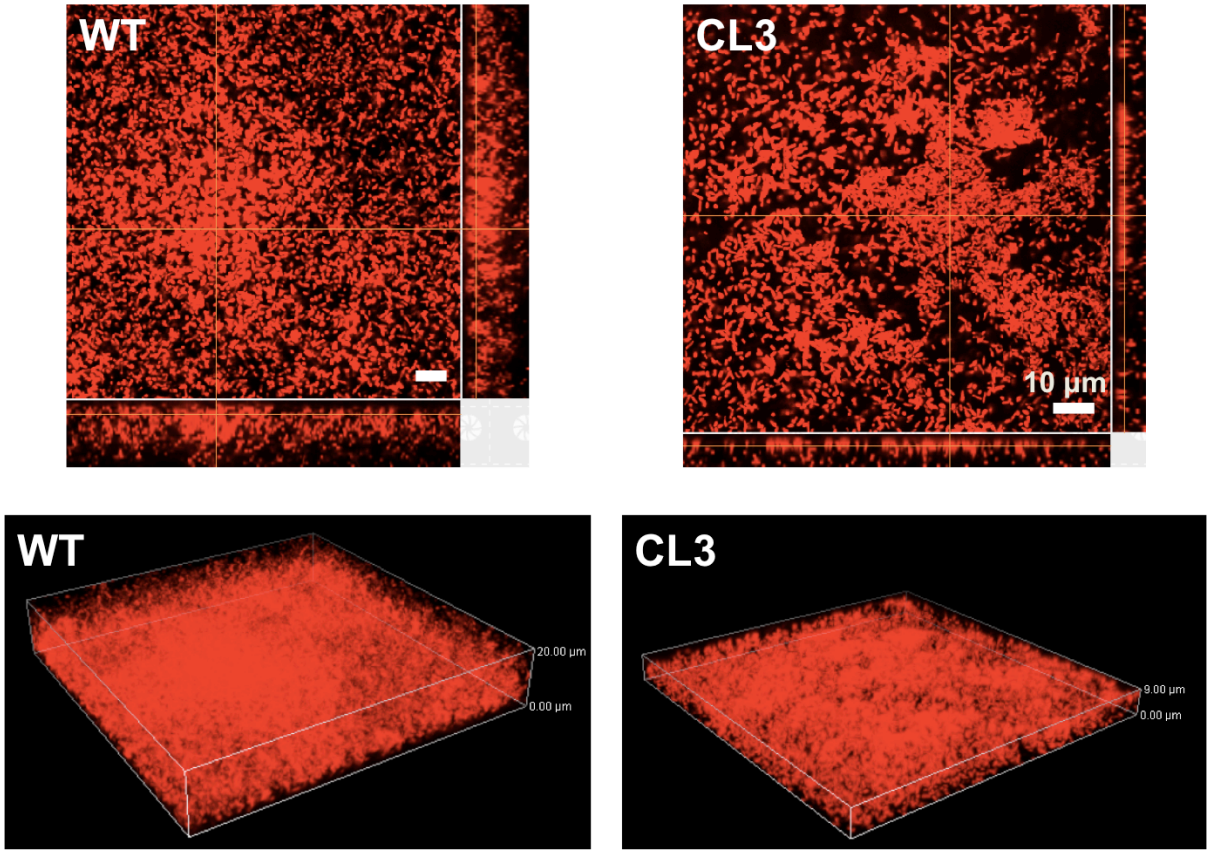

Figure S2

Figure S3. Representative micrographs of *R. sphaeroides* WT and CL3 cells attached to surfaces. *R. sphaeroides* WT and CL3 cells were grown on a chamber slide with hydrophobic plastic surfaces at 30°C in Sistrom's succinate medium. Cells attached to the surface were imaged at 1 h after inoculation. Images were acquired using phase contrast brightfield microscopy. Scale bar, 2 μm.

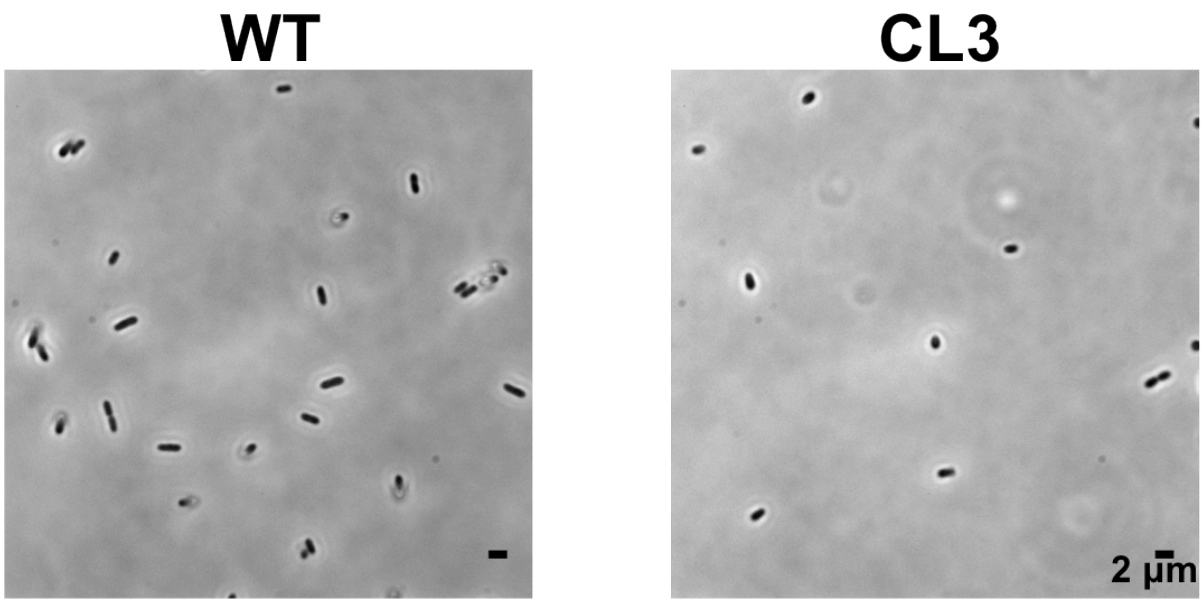

Figure S3

Figure S4. Quantification of *R. sphaeroides* WT and CL3 biofilms grown in wells of a glass bottom microtiter plate. Biofilms were grown on a glass bottom microtiter plate (MatTek, Ashland, MA) for 72 h at 30°C in Sistrom's succinate medium, followed by staining with crystal violet (CV). The extent of biofilm formation was determined by the absorbance of CV at λ, 550 nm. The values represent mean values ± standard deviations obtained from three independent experiments, each performed in 8 replicates. *R. sphaeroides* strain CL3 displayed a 50% reduction in biofilm formation compared with the WT strain after 72 h of incubation.

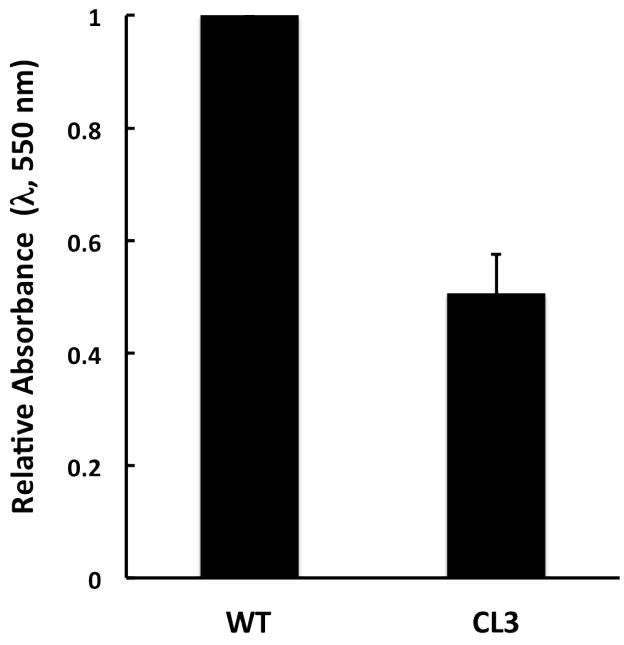

Figure S4

Figure S5. Exopolysaccharides (EPS) extracted from planktonic cultures of *R. sphaeroides* WT and CL3 cells. Cells were grown with shaking in glass test tubes for 72 h at 30°C in Sistrom's succinate medium. The EPS in growth media was precipitated by adding absolute ethanol to a final concentration of 75%, separated on an SDS-polyacrylamide gel using electrophoresis, and visualized by silver staining. EPS from both *R. sphaeroides* strains displayed similar banding patterns and was present in similar amounts.

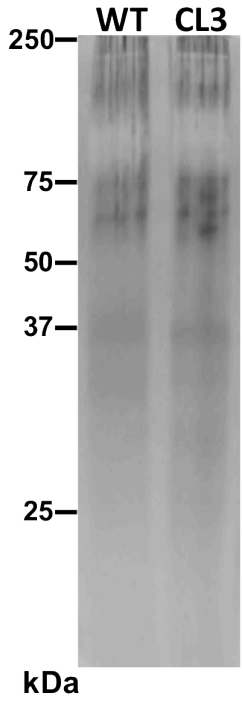

Figure S5