Supplementary Experimental Procedures

Large Scale Isolation of Hp0579-80 Reaction Product. A 10-ml reaction mixture containing 45 μM of Kdo_2-lipid A substrate (Avanti Polar Lipids), 0.5 mg/ml HMS174 pET21a or HMS174 pHp0579-80 membranes, 50 mM Hepes pH 8.0, and 0.1% Triton TX-100 was incubated at 30 °C for 3 hours. The reaction was then converted into a two-phase Bligh-Dyer consisting of chloroform/methanol/0.01 M HCl (2:2:1.8 v/v). Phases were separated in a clinical centrifuge and the lower phase removed to a separate tube. A second extraction of the resulting upper phase was achieved by addition of fresh lower phase. The lower phases were pooled and dried under a stream of N_2. Lipids were separated by DEAE anion-exchange chromatography as previously described (Tran *et al.*, 2004). Fractions containing the Kdo_2-lipid A substrate and Kdo-lipid A reaction product were converted to a two-phase Bligh/Dyer, as described above, and the lipid recovered from the lower phase.

Mild Acid Hydrolysis of Whole Cell Lysates. Whole cell lysates were heated at 80°C for 30 minutes in 1% acetic acid as described previously (Hug *et al.*, 2010).

Supplementary Figure Legends

Fig. S1. Genomic context of *F. tularensis* and *H. pylori* Kdo hydrolase genes. MacVector was used to generate the image.

Fig. S2. Membrane topology prediction of *H. pylori* Kdo hydrolase machinery. Predicted topology and transmembrane segments of Hp0579 **(A)** and Hp0580 **(B)** based on the TMHMM algorithm (Krogh *et al.*, 2001).

Fig. S3. Mass spectrometry of the Kdo hydrolase reaction products. A large scale reaction using Kdo_2-lipid A substrate was performed using membranes isolated from either HMS174 pET21a or HMS174 pHp0579-80. Reaction products were purified on a DEAE column as previously described (Tran et al., 2004) and analyzed by MALDI-TOF mass spectrometry in the negative-ion mode. The HMS174 pET21a reaction produced a predominant peak at m/z 2237.5, consistent with the Kdo_2-lipid A starting material **(A)**. The HMS174 pHp0579-80 reaction produced a predominant peak at m/z 2017.8, consistent with a Kdo-lipid A species **(B)**.

Fig. S4. Mass spectrometry of the 7.13 Kdo hydrolase mutant. Lipid A was isolated from wild type strain 7.13, 7.13 *hp0579-80::cam* and 7.13 *hp0579-80::cam*, *hp0579-80*$^+$ and analyzed by MALDI-TOF mass spectrometry in the negative-ion mode. Strain 7.13 produced a peak at m/z 1548.9 corresponding to the published wild type *H. pylori* lipid A mass **(A)**. In addition to the wild type peak at m/z 1548.0, 7.13 *hp0579-80::cam* also displayed a peak at m/z 2091.2 **(B)**. 2091.2 corresponds to a hexa-acylated lipid A species, with the 4′-phosphate still present. Strain 7.13 *hp0579-80::cam*, *hp0579-80*$^+$ showed complete reversion to the wild type phenotype, with a single peak present at a m/z of 1548.9 **(C).**

Fig. S5. Mild acid hydrolysis of the G27 Kdo hydrolase mutant whole cell lysates. *H. pylori* strain G27 Kdo hydrolase mutants and complements were subjected to mild acid hydrolysis treatment followed by SDS-PAGE **(A)** and immunoblot analysis **(B, C)**, as described in materials and methods. U = untreated, T = mild acid hydrolysis treated.

References

Hug, I., M. R. Couturier, M. M. Rooker, D. E. Taylor, M. Stein & M. F. Feldman, (2010) Helicobacter pylori lipopolysaccharide is synthesized via a novel pathway with an evolutionary connection to protein N-glycosylation. *PLoS pathogens* **6**: e1000819.

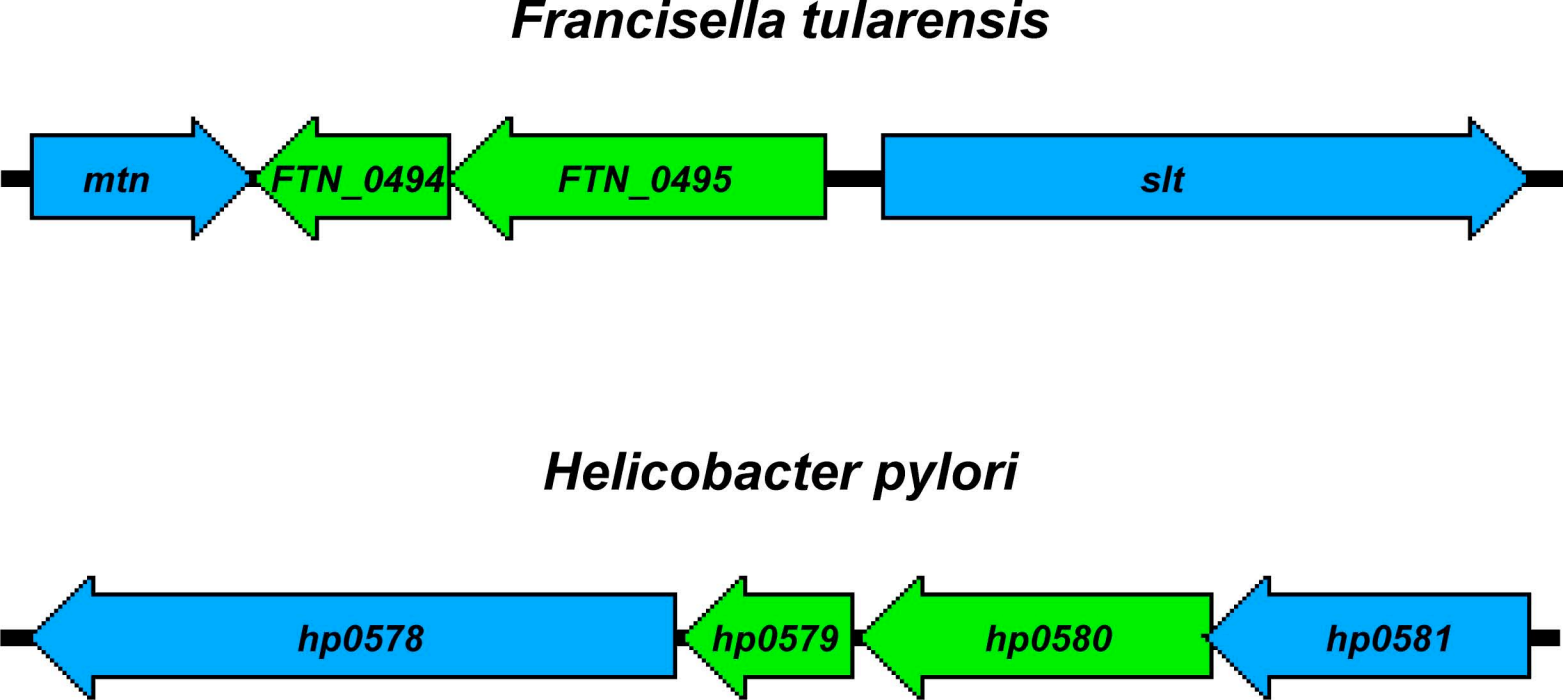
Krogh, A., B. Larsson, G. von Heijne & E. L. Sonnhammer, (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**: 567-580.

Tran, A. X., M. J. Karbarz, X. Wang, C. R. Raetz, S. C. McGrath, R. J. Cotter & M. S. Trent, (2004) Periplasmic cleavage and modification of the 1-phosphate group of Helicobacter pylori lipid A. *J Biol Chem* **279**: 55780-55791.

Table S1. Oligonucleotides

Name	Sequence
Fhp0579	5′-GCGCGCCATATGCTTATATCTTCTTCT-3′
Rhp0579	5′-GCGCGCGGATCCTTAATACAACCTTTTTTT-3′
Fhp0580	5′-GCGCGCCATATGGAACCTTCAAGAAAT-3′
Rhp0580	5′-GCGCGCGGATCCTCACTTGAGGAGGGATTT-3′
Hp0580P1	5′-GCGCGCCTCGAGCATGACAATCTCTATGCGAC-3′
Hp0580P2	5′-**CTTAGCTCCTGAAAATCTCGG**AAAGCCCCACAAAAAAGGCG-3′
Hp0580P3	5′-**TAATACCTGGAGGGAATAATG**CCGCTCTCATATCAAACACA-3′
Hp0580P4	5′-GCGCGCTCTAGAAGCGAACTGGATAACGCTAC-3′
Hp0579P3	5′-**TAATACCTGGAGGGAATAATG**TCCAGTTCGCTTTTGATGCG-3′
Hp0579P4	5′-GCGCGCTCTAGAATCGTGAGCGTGTCTTCATG-3′
CamF	5′-CCGAGATTTTCAGGAGCTAAG-3′
CamR	5′-*CATTATTCCCTCCAGGTA*TTACGCCCCGCCCTGCCACTC-3′
F580comp	5′-GCGCGCGGATCCCAGTAAAAAAGCGCGTTTGTC-3′
R580comp	5′-GCGCGCGAATTCTCACTTGAGGAGGGATTTTAA-3′
R579comp	5′-GCGCGCGAATTCTTAATACAACCTTTTTTTAAC-3′
FphoA	5′-GCGCGCCATATGCTGTTTACCCCTGTGACA-3′
F580phoA	5′-GCGCGCTCTAGATTGGAACCTTCAAGAAAT-3′
R580phoA	5′-GCGCGCCTGCAGCCTAGGCTTGAGGAGGGATTTTAA-3′
FphoAfus	5′-GCGCGCCCTAGGCTGTTTACCCCTGTGACA-3′
RphoAfus	5′-GCGCGCAAGCTTTTATTTCAGCCCCAGAGC-3′
F580phoApet	5′-GCGCGCCATATGGAACCTTCAAGAAAT-3′
Fgfp	5′-GCGCGCGCTAGCAGGTCGACTCTAGAGGAT-3′
Rgfp	5′-GCGCTCAGTTGGAATTCA-3′
F580gfp	5′-GCGCGCAAGCTTGCTAGCTTGGAACCTTCAAGAAAT-3′
R580gfp	5′-GCGCGCTCTAGACTTGAGGAGGGATTTTAA-3′

Restriction enzyme sequences are underlined
Cam cassette complimentary sequence is in **bold**
RBS and start codon sequences are in *italics*


Francisella tularensis
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slt
Helicobacter pylori
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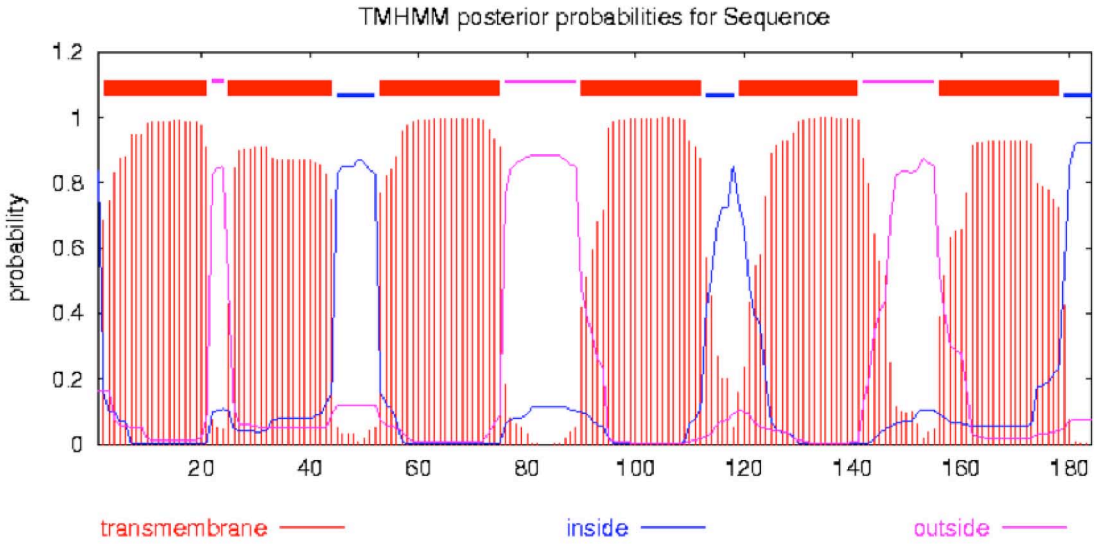
hp0579
hp0580
hp0581

A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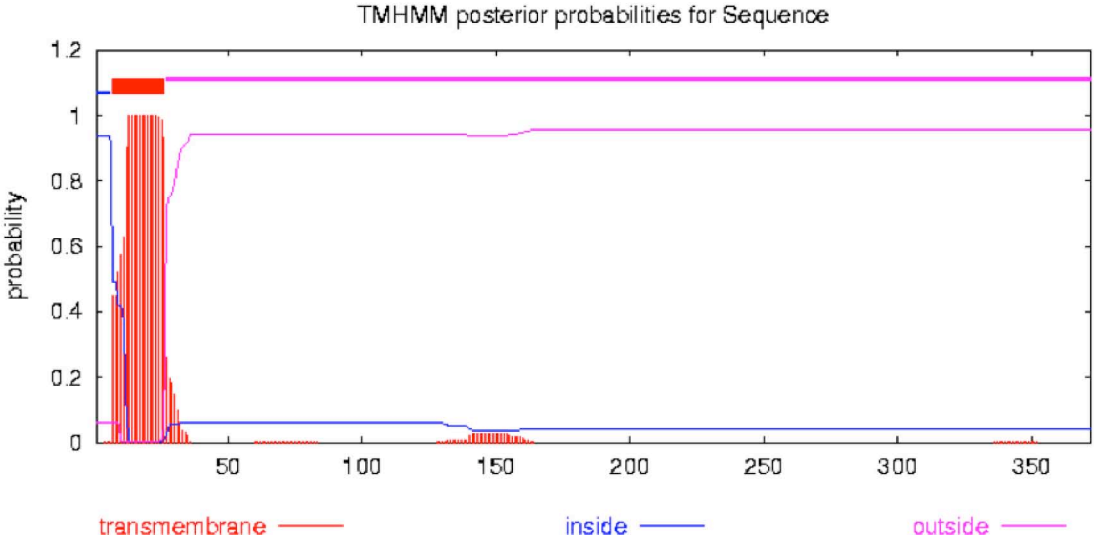
120
140
160
180
transmembrane
inside
outside

B


TMHMM posterior probabilities for Sequence
probability
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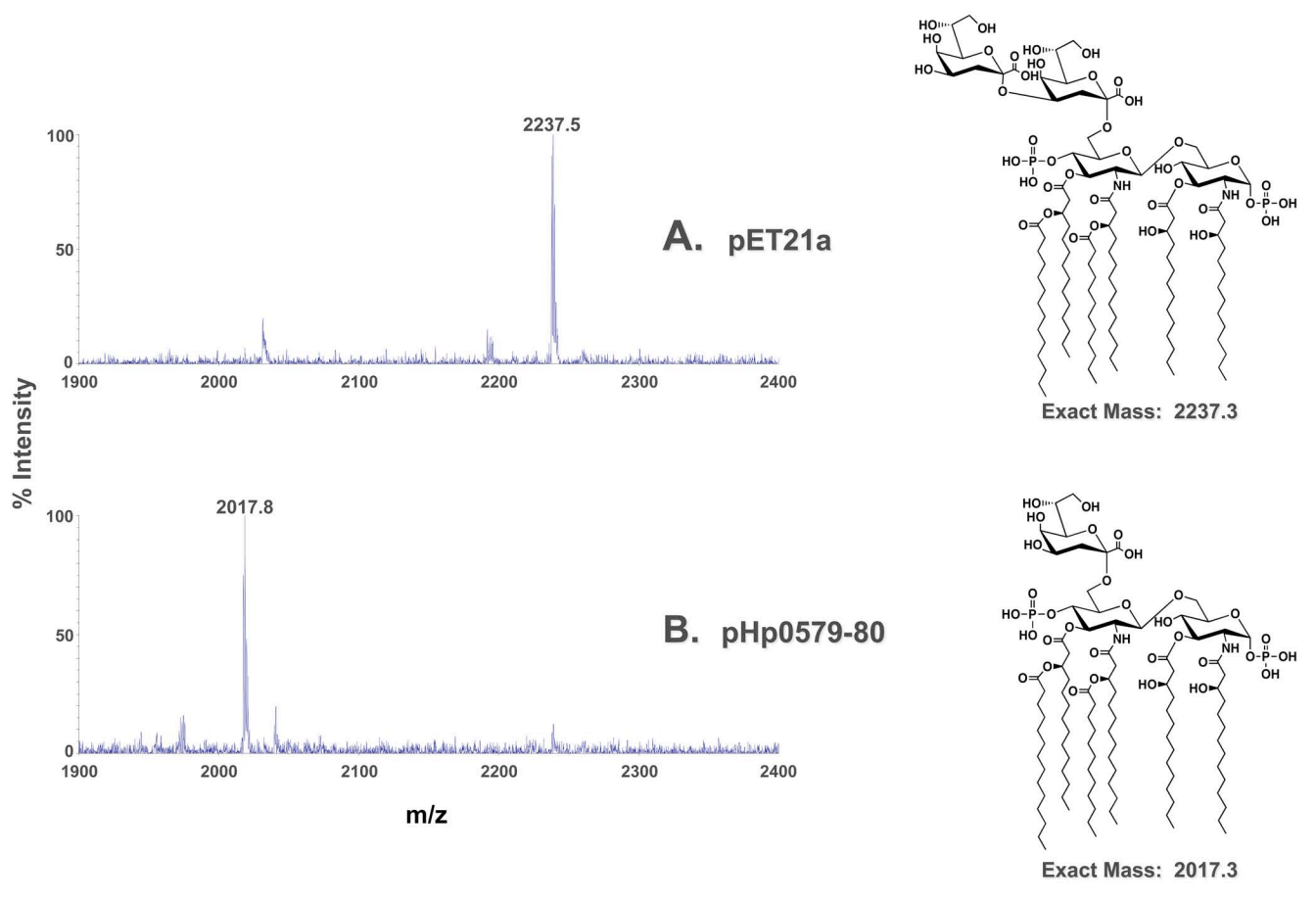
transmembrane
inside
outside


2237.5
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50
0
1900
2000
2100
2200
2300
2400
A. pET21a
% Intensity
2017.8
100
50
0
1900
2000
2100
2200
2300
2400
B. pHp0579-80
m/z
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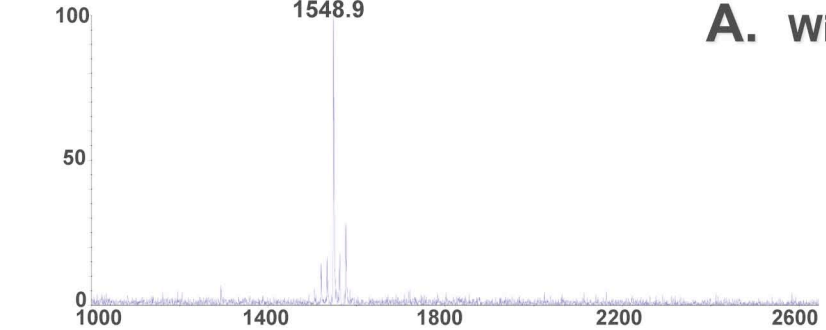
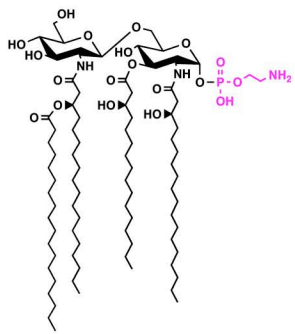
Exact Mass: 2017.3



A. Wild type (B128)



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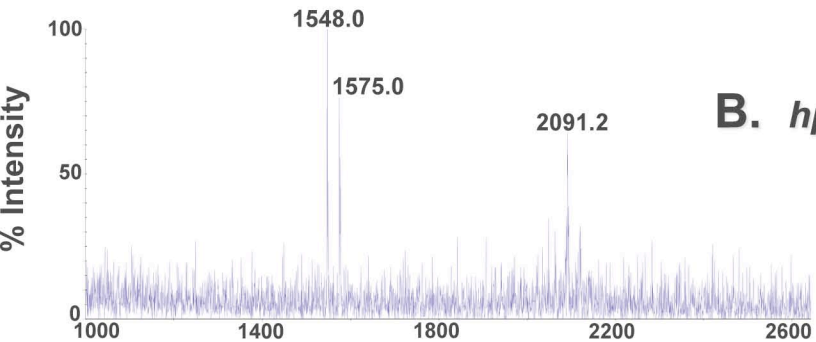
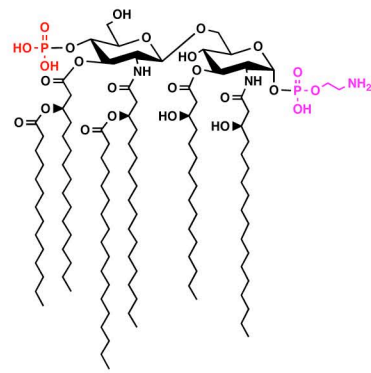


B. *hp0579-80::cam*



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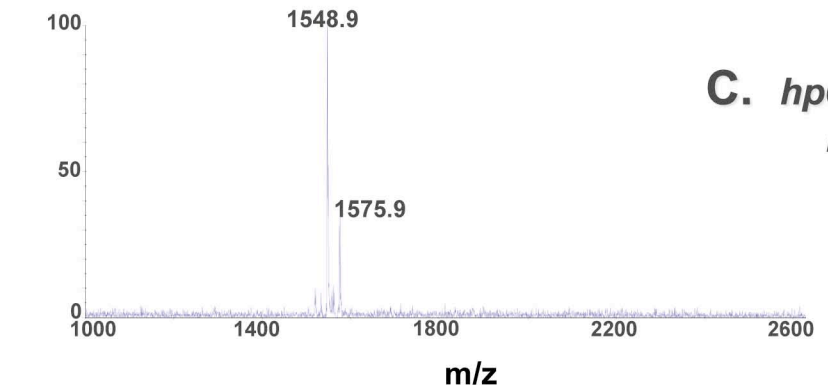
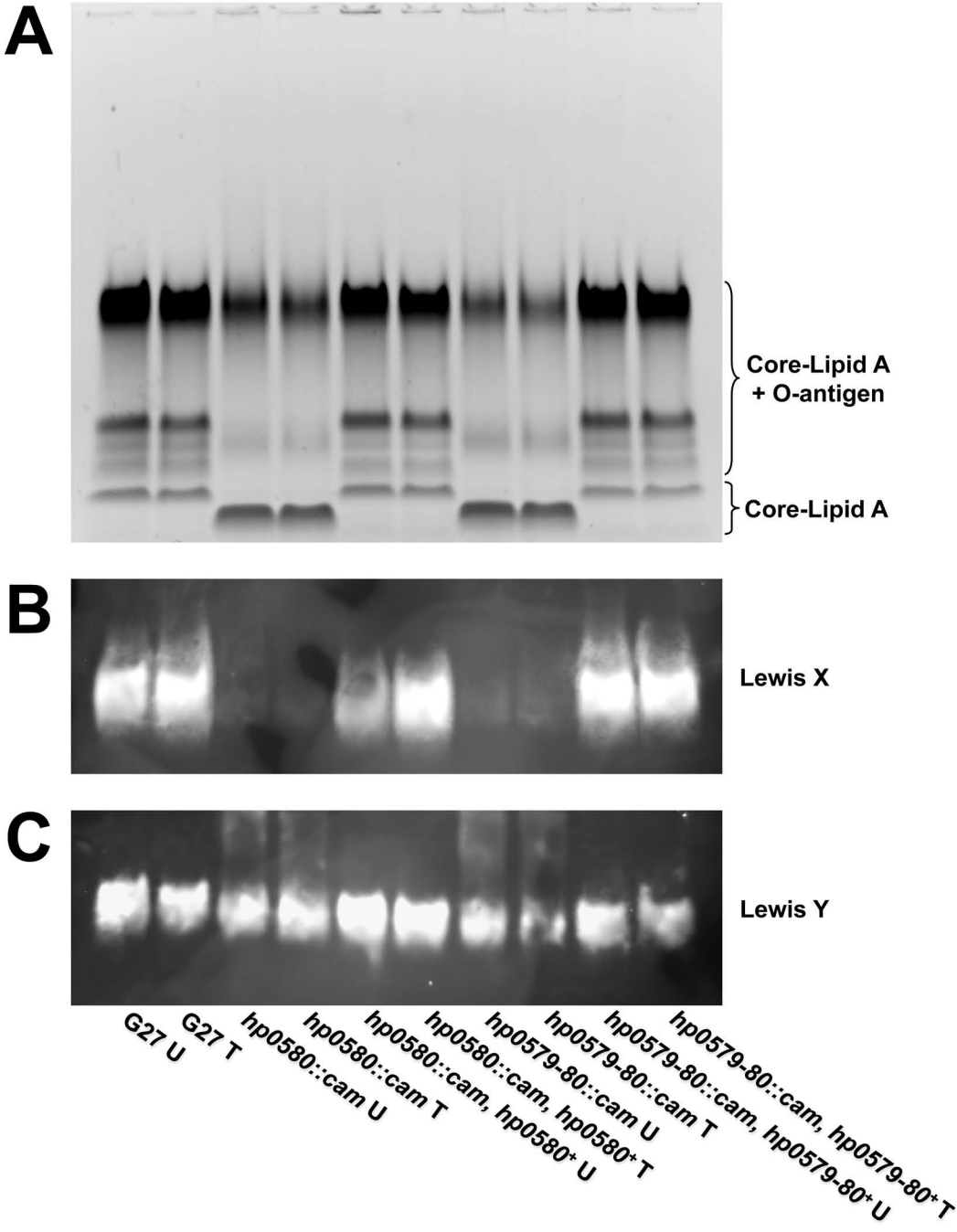


C. *hp0579-80::cam, hp0579-80^{+}*


A
Core-Lipid A
+ O-antigen
Core-Lipid A
B
Lewis X
C
Lewis Y
G27 U
G27 T
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hp0580::cam, hp0580+ U
hp0580::cam, hp0580+ T
hp0579-80::cam U
hp0579-80::cam T
hp0579-80::cam, hp0579-80+ U
hp0579-80::cam, hp0579-80+ T