Characterizing Legionaminic Acid Biosynthesis from Diverse Species

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I. Background

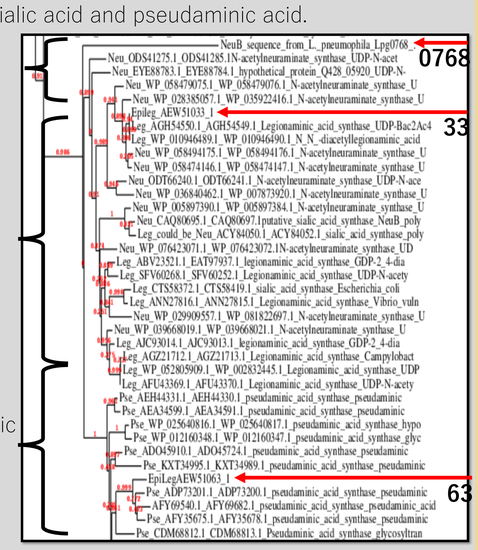
Legionaminic acid belongs to a family of carbohydrates called nonulosonic acids (nine carbon alpha keto acids) and are 5,7-diamino derivatives of the more commonly known sialic acid, Neu5Ac. These carbohydrates are found in cell surface glycoconjugates of gram-negative bacteria such as *H. pylori, C. jejuni,* and *L. pneumophila,* and contribute to their pathogenicity. The physiological role of legionaminic acid in bacterial pathogenicity is not yet well understood since there is no reliable source of it to support its study.[1] This project helps examine the role of synthase genes in a biosynthetic pathway initially characterized in *C. jejuni* in 2009.

The biosynthetic pathway for LA contains five genes: three bacterial protein glycosylation (PglE, PglF, PglD) genes from *C. jejuni*, and two LA synthase genes (LegF, LegI) from *L. pneumophila*. Bioinformatic analysis revealed three closely related LegI genes that might generate a modified 4-epi LA stereoisomer, which has been isolated in previous studies but no dedicated biosynthetic pathway exists. Natively these are synthase genes for sialic acid and pseudaminic acid.

Sialic Acid Synthases

Leg. Acid Synthases

Pseudaminic Acid Synthases



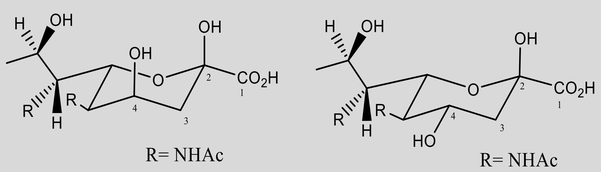
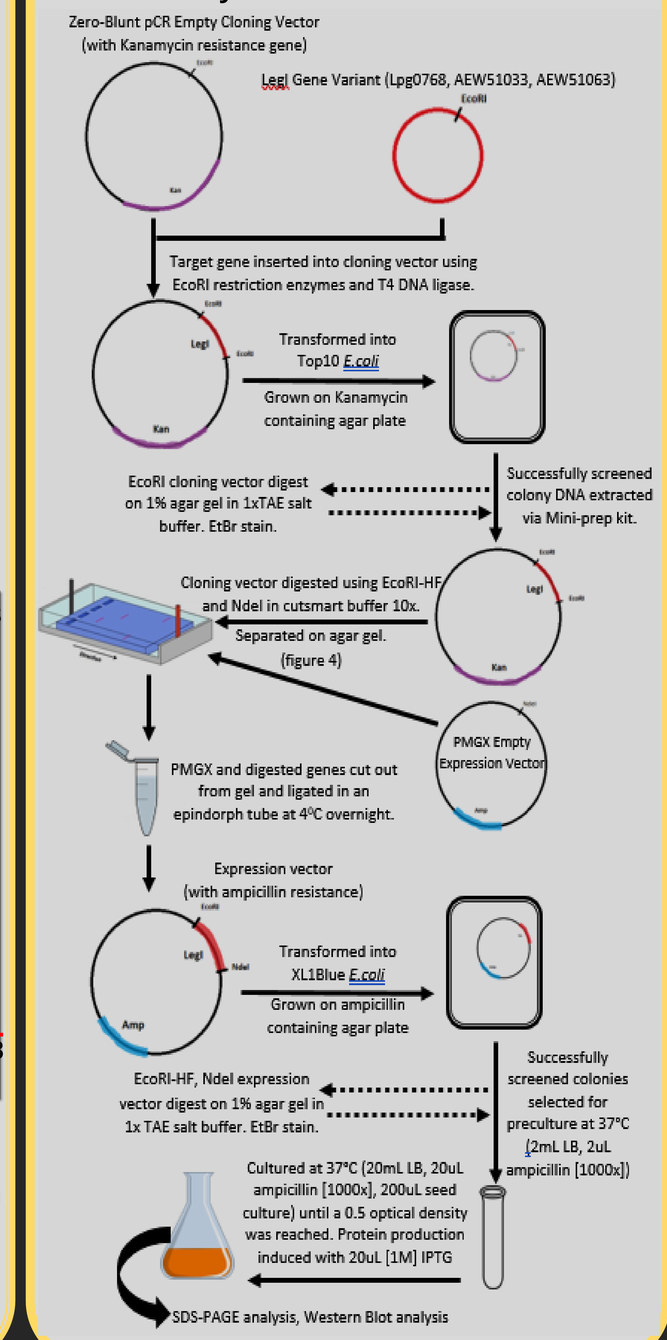
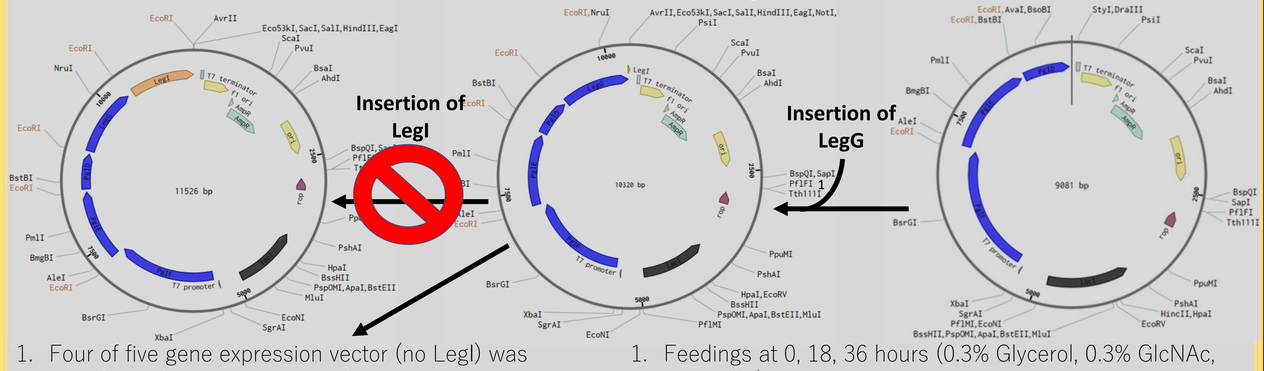


Figure 1. Comparison of 4-epi-legionaminic acid (left) and native legionaminic acid (Leg5,7,Ac2) structures.

II. Methods – Confirming LegI Synthase Production



II. Methods – Production of 2-epibacillosamine



1. Four of five gene expression vector (no LegI) was transformed into a unique production strain.
2. Production: 20mL minimal media at 37°C, 200 rpm until OD600 of 0.5
   - 0.2mL starter culture
   - 0.1% GlcNAc
   - 10.25% casitone
   - 0.5% (v/v) glycerol
   - Ampicillin
3. Induction: 0.2 mM IPTG at 30°C, 200rpm, for 168 hours.

1. Feedings at 0, 18, 36 hours (0.3% Glycerol, 0.3% GlcNAc, Ampicillin)
2. Workup and NMR analysis of monosaccharide product in optimized production conditions.

Note: 20mL F2 minimal media contains (per Litre):
- 412.24g of K2HPO4
- 6.0g of KH2PO4
- 4.0g (NH4)2SO4
- 175.5mg of MgSO

III. Results – Biosynthetic Pathway Expression without LegI

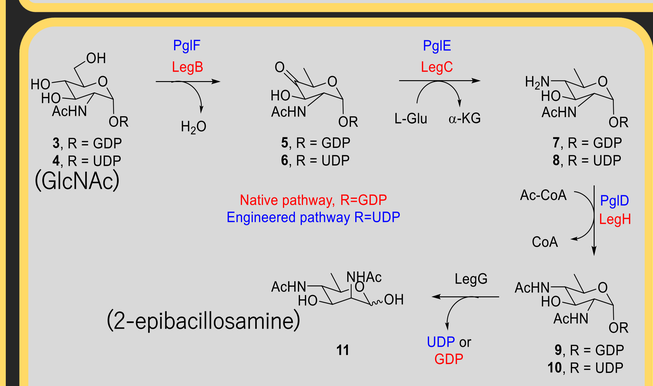


Figure 6. Biosynthetic pathway containing first four genes of five, leading to the production of 2-epibacillosamine.[1]

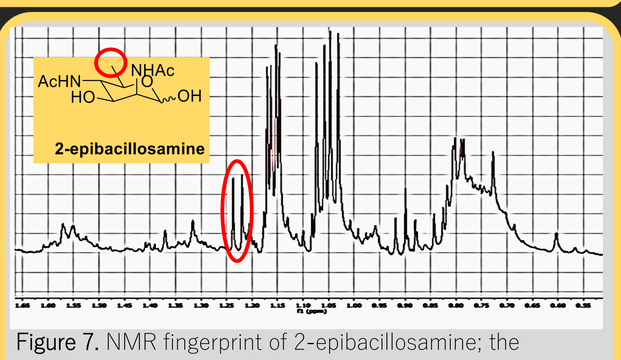


Figure 7. NMR fingerprint of 2-epibacillosamine; the resulting monosaccharide from the 4 gene expression vector. The doublet at 1.22 ppm corresponds to the methyl group shown.

III. Results – LegI Expression

Lanes 1-4: EpiLegI-33

Lane 5: Blank

Lane 6-9: EpiLegI-63

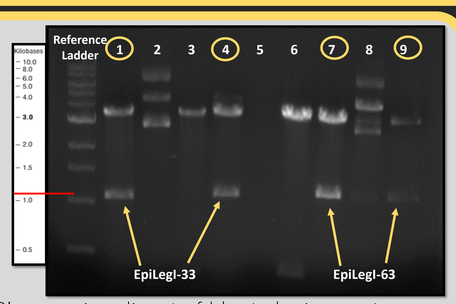


Figure 2. EcoRI screening digest of blunt cloning vector containing EpiLegI-33 and 63 via 1% agarose gel electrophoresis in 1x TAE buffer (stain: EtBr).

Lane 1: PMGX backbone control

Lanes 2-5: EpiLeg-51033

Lane 6-9: EpiLeg-51063

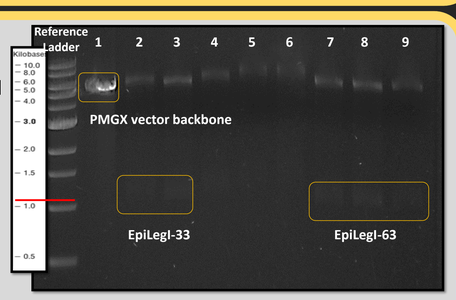


Figure 3. EcoRI-HF, NdeI screening digest of the expression vector containing EpiLegI-33 and 63 via 1% agarose gel electrophoresis in 1x TAE buffer (stain: EtBr).

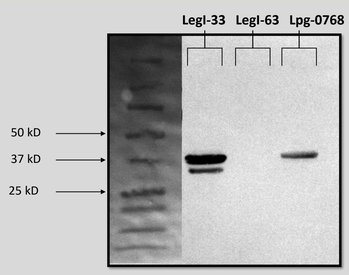


Figure 4. Western blot to confirm expression of synthase enzymes using Anti-6x-His tag antibodies. Imaged using enhance chemiluminescent kit.

Lane 0,8: 10kBladder

Lanes 1-7: EpiLeg-33

Lane 9-15: EpiLeg-63

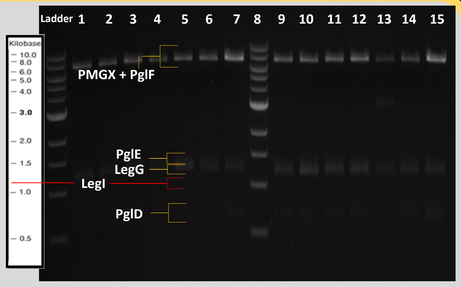


Figure 5. EcoRI screening digest of the pentacistronic vector, pglFED-LegI, containing EpiLegI-33 and 63 via 1% agarose gel electrophoresis in 1x TAE buffer (stain: EtBr).

V. Conclusions

- 2-epibacillosamine, like legionaminic acid, is another difficult to isolate pathogenic factor in gram negative bacteria. The tetracistronic, pglFED-LegG, offers the first developed biosynthetic pathway for its production.
- Sub-cloning of the LegI gene in with the pgl operons proved unsuccessful using in house golden gate assembly. The figure 4 digest is missing a DNA fragment at 1.2kB corresponding to the expected LegI insertion (fifth component).
- Monosaccharide production was induced anyway in the tetracistronic vector lacking LegI. NMR analysis revealed a doublet peak at 1.22ppm, corresponding to the methyl group seen in 2-epibacillosamine confirming its production.
- The tetracistronic vector supports the hypothesized 2-epibacillosamine pathway in figure 6.
- Glycerol; an energy dense, metabolically significant molecule; provided the greatest potential for cultures to grow. The glycerol rich medium optimized production of glycoconjugates, in this case 2-epibbacillosamine,
- Figure 2 illustrates successful cloning vector recombination for EpiLegI-33 and EpiLegI-63. The LegI gene is around 1.2kB in length, and the digested sample shows a fragment at 1.2kB and a cloning vector backbone at 3.5kB.
- Figure 3 illustrates successful recombination of the expression vector in lanes 2, 3 (EpiLegI-33) and 7, 8, 9 (EpiLegI-63). An over dilution of the extracted DNA led to very dim images, but DNA fragments are still marginally visible at 1.2kB.
- Western blot using Anti-6x-His tag antibodies confirmed synthase production for EpiLegI-33 and Lpg-0768, but not for EpiLegI-63. This indicates EpiLegI-63 is not feasible for the full synthetic pathway for Legionaminic acid.
- Initially the third gene, Lpg-0768, was not successfully transformed as the growth media did not produce colonies. Later on in the project it was successfully cloned and expressed but images are not provided on the space limited poster.

VI. Future Directions

- Sub cloning LegI into the full legionaminic acid biosynthetic pathway to express legionaminic acid.
- Bacillosamine synthase targeting antibiotics can be designed and tested to investigate the physiological significance of bacillosamine glycoconjugates and their effects on gram negative prokaryotic pathogenicity.
- Optimization of growth mediums for 2-epibacillosamine production to achieve a more concentrated final product.

VI. Works Cited

1. Hassan, M. I.; Lundgren, B. L.; Chaumun, M.; Whitfield, D. M.; Clark, B.; Schoenhofen, I. C.; Boddy, C. N. Angew. *Total Biosynthesis of Legionaminic Acid, a Bacterial Sialic Acid Analog.* Chem. Int. Ed. 2016, 55, 12018-12021.

VII. Acknowledgements

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