Crystallizing *Staphylococcus aureus* MurG

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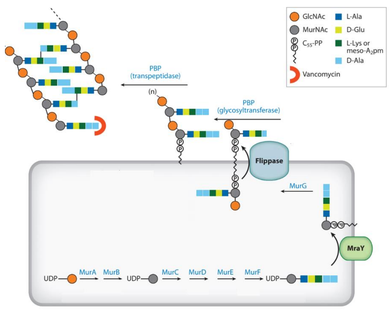
Abstract

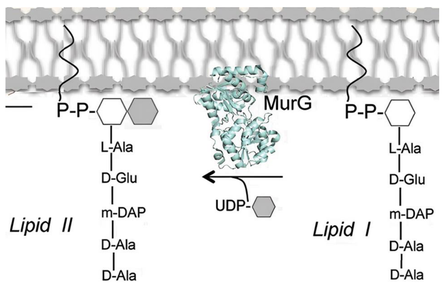
Antibiotic-resistant bacteria are one of the biggest problems the human and veterinary healthcare industries face. As resistance to common-use antibiotics grows, so does the need to discover novel targets and develop new inhibitory drugs against them. A potential new target is the MurG enzyme; a glycotransferase that is involved in the synthesis of the bacterial cell wall. It is proposed that an inhibitor of MurG, the rate-limiting enzyme in the peptidoglycan biosynthesis pathway, could work as a novel antibiotic. To design a novel inhibitor, structural data is needed. While structural data for two MurG enzymes have been solved using x-ray crystallography, no structural data exist for MurG in *Staphylococcus aureus*. Sequence similarity between MurG isoforms is generally low and *S. aureus* MurG is one of 20% of MurG isoforms that have a phenylalanine variation in the active site, a variant yet to be crystallized. This work details an optimized method of expression and purification in addition to describing a range of reproducible crystallization conditions of *S. aureus* MurG. Additionally, this work attempts to further optimize human involvement in the crystal screening process through the use of an automated pipetting robot. Further experimentation is required to obtain large enough high-quality crystals suitable for x-ray diffraction. Structural data gained from x-ray diffraction will allow for the future development of specific inhibitors based on a quinolinone-6-sulfonamide (Q6S) moiety. Previous literature has shown a Q6S moiety to mimic the binding of UDP-GlcNAc in other glycotransferases.

Background and Significance

MurG is proposed to be an excellent target for inhibition due to its close proximity to the cell membrane. MurG is also a rate limiting enzyme in the peptidoglycan biosynthesis pathway. Inhibition of this enzyme can disrupt production of peptidoglycan (PG); a necessary component of the bacterial cell wall. Disruption of PG biosynthesis can lead to cell lysis and death.

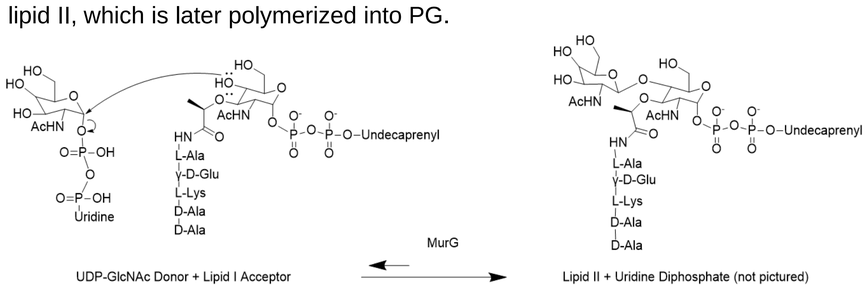


Lovering et al. *Annu. Rev. Biochem.* **2012**, 81 (1), 451–478.

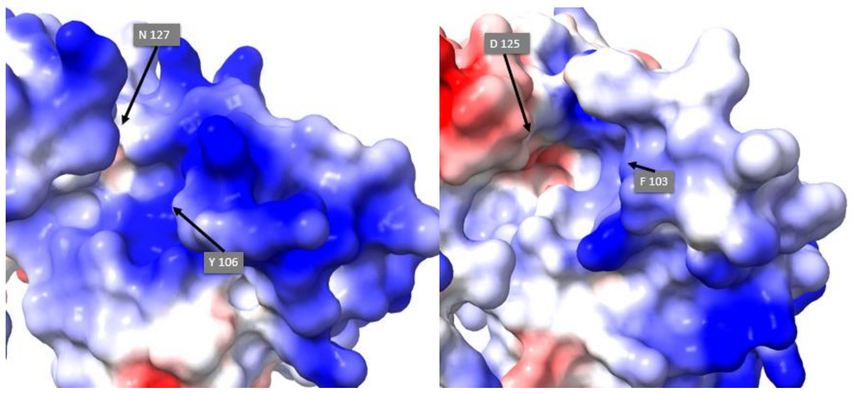


Laddomada et al. *Sci Rep* **2019**, 9 (1), 4656

MurG catalyzes the transfer of a GlcNAc monosaccharide from UDP-GlcNAc to a substrate known as Lipid I, to form the disaccharide pentapeptide known as lipid II, which is later polymerized into PG.



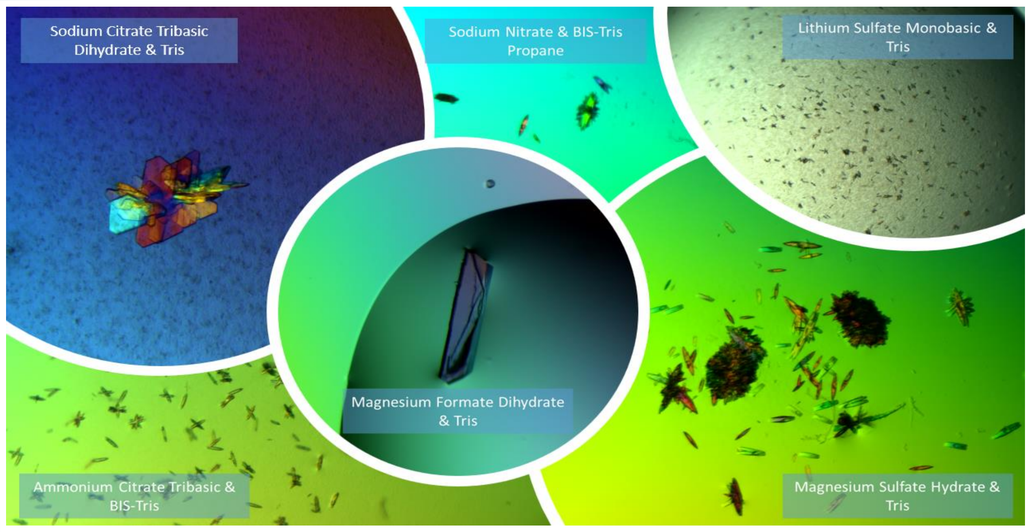
There are 73 isoforms of MurG, but only 2 isoforms have solved crystal structures: *E.coli* MurG (1F0K) and *Pseudomonas aeruginosa* MurG (3S2U). All isoforms have less than 20% sequence homology however, the active site remains highly conserved in most forms.[3] *Staphylococcus aureus* MurG has 2 rare active site variants and is part of ~20% of MurG isoforms that have a phenylalanine substitution in active site.[3] These active site variations make *S. aureus* MurG unique. *S. aureus* MurG has a positively charged active site at physiologically relevant pH, as compared to *E.coli* MurG that has a negatively charged active site at physiologically relevant pH.



Electrostatic prediction of acceptor binding site in Molecular models of E. coli MurG (A) and S.A MurG (B). Putative acceptor residues are labeled based on sequencing Data. Molecular graphics and analyses performed with UCSF ChimeraX.[4]

I hypothesize that by crystalizing *Staphylococcus MurG*, we can better understand the active site and develop inhibitors that are specific to *S.Aureus*

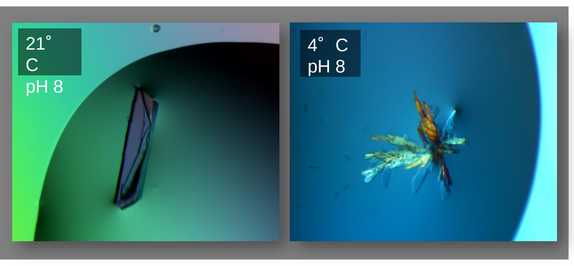
Crystal Hits



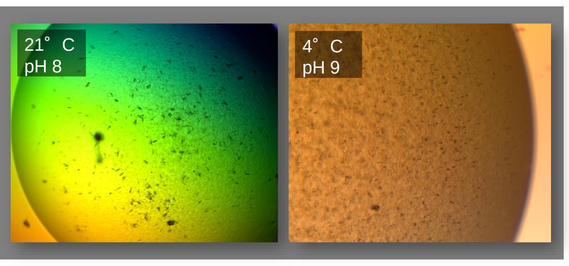
296 individual crystallization reagents were screened and 23 produced crystals that contained protein. Reagents conducive to crystallization contained a Tris, Bis-Tris, or Bis-Tris Propane buffer at a pH of 7-9.

Crystal Optimization

Magnesium Formate Dihydrate & Tris
- pH dependent
- Temperature dependent
- Concentration dependent
- Changing conditions effects size and morphology

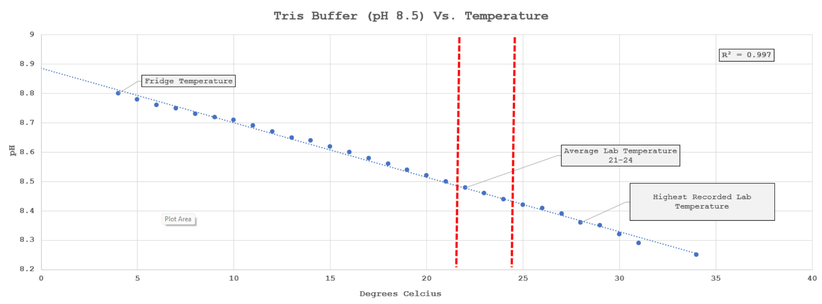


Lithium Sulfate Monohydrate & Tris
- Not pH dependent
- Not temperature dependent
- Not concentration dependent
- Changing conditions does not affect size or morphology



Roughly 200 additional tests focused on optimizing buffers conducive to crystal growth. These tests varied buffers, pH, protein concentration, and precipitate concentration. Magnesium formate dihydrate and lithium sulfate monohydrate reagents created the most consistent crystal hits.

Temperature Effects pH of Buffers



Regulation of laboratory temperature added an unexpected variable to this experiment. Due to inconsistencies in lab climate, consistency in results was poor. Tris buffers are temperature sensitive, meaning that as temperature increases or decreases, so does the pH of the solution. In some cases, temperature fluctuations in the lab prevented nucleation and formation of crystals.

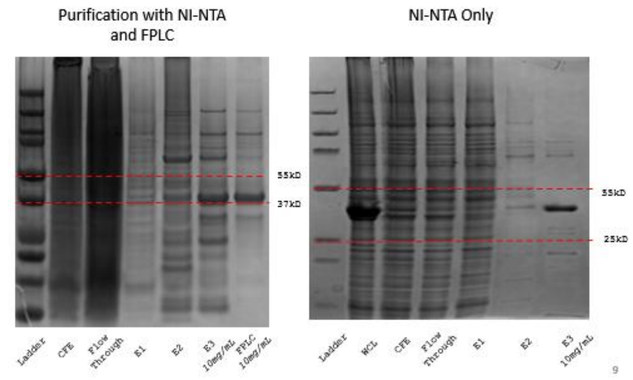
Summary

- Found several conditions conducive to crystallization of *Staphylococcus Aureus* MurG
- Optimization of select reagents shows promising results for future crystallization experiments
- Developed a better understanding for physical chemical properties and how they effect protein crystallization
- Developed novel automation techniques that can increase productivity of future experimentation in crystallography and beyond

Future Directions

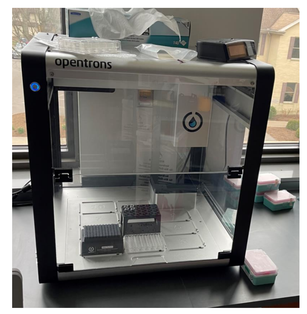
- Precise temperature control for more consistency in results
- Try out other buffers like Bis-Tris Propane and Bis-Tris
- Streak Seeding with lithium sulfate monohydrate to form larger crystals
- Removal of EK site to allow for more variability in optimization tests

Purification



- S.A MurG can be purified using NI-NTA and size exclusion FPLC.
- Optimized Ni-NTA chromatography shows highly pure protein concentrated to 10mg/mL.
- By optimizing Ni-NTA purification, no additional step of size exclusion via FPLC is required.
  - This saves time and resources during purification

Automation



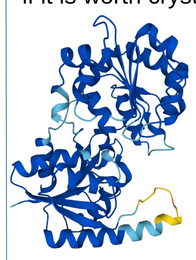

```
from opentrons import protocol_api

metadata = {
    'protocolName': 'Protein crystallization screens',
    'author': 'Patrick Wood',
    'source': 'Python'
    }

def run(protocol: protocol_api.ProtocolContext):
    block_name = protocol.load_labware('Maxiwell-48-plate-xtal'
    if block_name not in labware.list():
        labware.create(
            block_name,
            grid=(8, 8),
            spacing=(9, 9),
            diameter=8.4,
            depth=42.2,
            volume=2200
        )
```

```
for tube, dests in zip(tubes, tube_dests):
    p1000.pick_up_tip()
    for dest in dests:
        for _ in range(2):
            p1000.set_flow_rate(aspirate=500, dispense=500)
            p1000.aspirate(750, tube)
            p1000.delay(seconds=0.5)
            p1000.touch_tip()
            p1000.dispense(750, dest)
            p1000.delay(seconds=0.5)
            p1000.set_flow_rate(dispense=1500)
            p1000.blow_out(dest.top())
    p1000.drop_tip()
```

- Automated workflow reduces amount of in-lab hours
- Reduces repetitive tasks like pipetting
- Consistent and accurate
- Easy to operate, coding is not always necessary

AI Crystal Structure Prediction

Using AI programs like Deepmind's AlphaFold Program, we can generate predicted structures of uncrystallized enzymes.[5] We can compare the predicted structure to solved structures to see if there are any differences and if it is worth crystallizing *S. aureus* MurG



Jumper et al. *Nature* **2021**, *596* (7873), 583–589

Pros to AI Modeling:
- Fast, structures can be predicted in hours instead of years
- Inexpensive, no need for protein production, crystallization reagents, only need sequence

Cons to AI Modeling:
- Not 100% accurate. Cannot fully trust electrostatic effects
- "Guesses" how a protein folds based on previously crystalized proteins

AI modeling is a useful tool but cannot not replace traditional crystallography methods yet.

References

(1)Lovering et al. *Annu. Rev. Biochem.* **2012**, *81* (1), 451–478.

(2)Laddomada et al. *Sci Rep* **2019**, *9* (1), 4656. https://doi.org/10.1038/s41598-019-40966-z.

(3)Crouvoisier et al. *J.Biochimie* **2007**, 11.

(4)Pettersen et al. *Protein Sci* **2021**, *30* (1), 70–82.

(5)Jumper, et al. *Nature* **2021**, *596* (7873), 583–589.

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Program in Biochemistry and Molecular Biology

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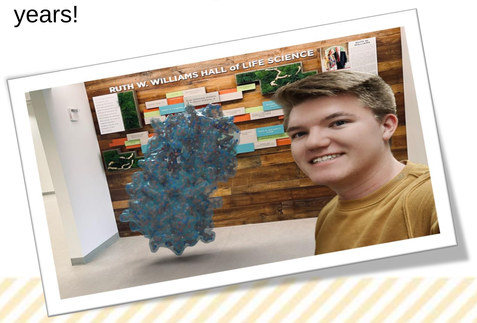

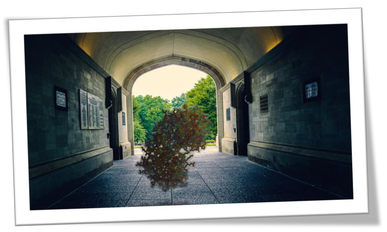

Please direct additional comments and questions to:
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