Wasp Proteins

Project Journal

Wasp Proteins – Sequencing, Synthesis, & Characterization

- Current Goals:
  - Ordering wasps
  - Investigating generating recombinant proteins
- Possible Leads:
  - The Bug Maniac (emailed)
  - Etsy
  - eBay
- Further Research: KU
  - http://www.newscientist.com/article/dn21755-strange-fat-explains-skins-waterproof-properties.html
  - http://hal.archives-ouvertes.fr/docs/00/89/19/41/PDF/hal-00891941.pdf
    - these brood cells that are in colletidae bees are lined with a waterproof membrane that could potentially be used to gloss or coat the drone and possibly make it waterproof (still researching)
  - Natural Polyesters: Dufour's Gland Macrocyclic Lactones Form Brood Cell Laminesters in Colletes Bees.
    - “Bees in the genus Colletes (cellophane bees) make their brood cells in the ground and coat them with a highly resistant, waterproof, transparent membrane. This membrane is a polyester constructed mainly from 18-hydroxyoctadecanoic acid and 20-hydroxy-eicosanoic acid, which are stored as their corresponding lactones in the Dufour's gland of the bee.” http://www.ncbi.nlm.nih.gov/pubmed/17758016
  - waterproof substance is secreted by the Dufour’s glands of colletidae bees
    - https://www.greatsunflower.org/Colletes

- http://link.springer.com/article/10.1007/BF00987667 (need this paper)
    - The liquid secretion of the Dufour's (definition of Dufour glands: http://en.wikipedia.org/wiki/Dufour's_gland) gland in mated females comprises a mixture of macrocyclic lactones, straight chain mono- and dicarboxylic acids, together with trace amounts of di- and trihydroxy monocarboxylic acids and a series of *n*-alkanes. Polymerization of the liquid to form the membranous nest cell lining, which also contains alanine and glutamic acid as major constituents, may be mediated by an enzyme from the thoracic salivary gland and voided via the mouth. The composition and structure of the cell linings are similar to those of Wigglesworth's "cuticulin."
- https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=0CCUQFjAA&url=http%3A%2F%2Flife.tau.ac.il%2Fdepartments%2Fzoology%2Fgroup_members%2Fkatzav%2Fdocuments%2Ftamy97nat.pdf&ei=5_uQU5e5AceGyATR5IGgCw&usg=AFQjCNEqI5vBmiWOHy7kvXwUvyijA76e3Q&bvm=bv.68445247,d.aWw
  (Chart on page 240 give hydrocarbon composition of waterproofing components in Dufour glands)

- 6/4/14
    - Peptide Mass Fingerprinting approach (thanks Gary!):
        - From wasp nest:
            - Extract protein and run gel (SDS-PAGE) to help determine the number of proteins present in the protein/cellulose material.
            - Perform gel extraction of significant bands, do trypsin digest (cuts after Arg/Lys) and run through LC-MS to find fragment masses (Peptide Mass Fingerprint).
        - From wasps:
            - Find wasps! Immediately preserve with RNAlater or TRIzol solution (phenol/$CHCl_3$/GuSCN)
            - Generate cDNA library from collected mRNA
            - Using cDNA, generate transcriptome
            - Use transcriptome to find proteins which would have the same trypsin digest fingerprint
            - Can also BLAST or protein-BLAST to find homologues

- 6/5/14
    - https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=0CCUQFjAA&url=http%3A%2F%2Fxa.yimg.com%2Fkq%2Fgroups%2F17598545%2F1034483345%2Fname%2FCaneGerdinMandSecJKES83.pdf&ei=RfaVU9mSGY6zyASpsIFo&usg=AF

QjCNF_Nwvqru_7JnKIaE-G9W19cWp_lA&sig2=n8aGNcm5aWHiaVYG6PxHzA&bvm=bv.68445247,d.aWw

- 6/16/14 (finally progress!)
  - Wasps + nest samples acquired!
    - Wasps were in the process of nest-building
    - Stored in RNAlater for preservation
    - Frozen overnight in -80°C

- 6/17/14
  - Two wasps were ground in dry ice
  - RNA extracted from ground wasps with Qiagen RNeasy Mini Kit (http://www.qiagen.com/products/catalog/sample-technologies/rna-sample-technologies/total-rna/rneasy-mini-kit)
  - Ran product on gel and produced accurate RNA bands
  - Researched mechanism for protein extraction from paper wasp nest

- 6/24/14
  - Discussed protein extraction w/ Jesica and ordered Sigma Plant Total Protein Extraction Kit (http://www.sigmaaldrich.com/catalog/product/sigma/pe0230?lang=en&region=US)

- 6/25/14
  - Extracted mRNA from total RNA; ran gel but didn't see an RNA smear (likely due to low concentration)
  - Will extract from good wasp sample tomorrow

- 6/26/14
  - Extracted total RNA from nest-building wasp sample with RNeasy kit

- 6/27/14
  - Asked Michael Sheehan about wasp species and determined they are *Polistes dominula*, an invasive species from Europe
  - Female wasps have straight antennae; male wasps have curved antennae
  - Learned that only female wasps actively work on the nests
  - Wasp ID Page
  - Trapped wasps from the roof
  - Species with skinny abdomen likely ***Mischocyttarus flavitarsis***
  - Extracted RNA from two female *P. dominula* collected in the morning

- 7/3/14
  - Performed protein extraction (http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/pe0230bul.pdf) from dried, frozen nest sample

- 7/7/14
  - Ran protein on SDS-PAGE gel
    - First gel failed (may have run off)
    - Second gel stained, but only partially
  - Unclear as to whether we have bands or not (several appeared!)

- 7/8/14
  - Prepared cDNA from RNA previously extracted
  - Prepared cDNA according to Jesica's protocol

- 7/9/14
  - Ran Nano-drop on cDNA samples
    - best concentration was 13 ng/uL
    - this is a pretty bad concentration, we need to recreate cDNA library

- 7/10/14
  - re-ran SDS-PAGE got a cleaner result
    - 4-5 bands were found that should be sequenced
  - got correspondence from Illumina that preparation kit would be $4000- it's a no-go

- 7/11/14
  - Spoke to Stanford Functional Genomics Facility- they will do transcriptome sequencing for $2700
  - Alphalyse quote stands at $175 per protein

- 7/14/14
  - spoke to Lynn about sequencing: we got a go ahead
  - hydrophobicity screen for protein?
    - put some amount of cellulose in agar if it does not compromise agar texture

- 7/15/14
  - Prepared timeline for wasp project
  - Tested consistencies of various % agar plates
    - 1% and 0.5% fairly solid
    - 0.25% barely solid, very leaky
    - 0.1% doesn't solidify

- 7/16/14
  - Reattempted cDNA generation
  - Met with Joe to discuss hydrophobicity screens
    - suggested using normal agar plate and hydrophilic dye
    - grow colonies, pour dye onto plate
    - watch for areas with low dye absorption
    - agreed that we should try to put cellulose in agar plates

    - Joe said primer attempt is long shot but worth a try

- 7/21/14
    - Discovered that the genome of *Polistes dominula* was recently sequenced
    - http://www.ncbi.nlm.nih.gov/bioproject/234105
    - http://goblinx.soic.indiana.edu/PdomGDB
    - Communicated with Gary Wessel and confirmed in-gel trypsin digest and MS/MS protein technique
    - Address for Gary at Brown:
        - Gary M. Wessel
        - Department of Molecular and Cellular Biology & Biochemistry
        - 185 Meeting Street
        - Brown University
        - Providence, RI 02912
        -
        - FedEx number 2208-9230-1 (internal number 9-92956)
        -
        - http://www.brown.edu/Research/Wessel_Lab/
        -
        - T: 401 863-1051
        - F: 401 863-2421
        - e: rhet@brown.edu
    - Goal to run protein gels

- 8/18/14
    - Attempted PCR clean-up of old cDNA samples, but NanoDrop indicated that they had degraded

- 8/19/14
    - Nanodropped RNA samples from 6/30 – samples are miraculously still in good condition (concentrations ~200-500 ng/µL, 260/280s ~2.15)
    - Re-attempted cDNA generation from 6/30 RNA samples 5, 1, and 4
    - cDNA generation protocol:
        - Reverse transcription kit: Thermo Sci – Dynamo Cyber Green 2 Step GNTPCR
            - 1µL reverse transcriptase
            - 1µL OligoT primer
            - 1µL RNA
            - 7µL Milli-Q water
            - 10µL 2x buffer
            - Cycle:
                - Primer extension (25°C for 10 min)
                - cDNA synthesis (37°C for 30 min)
                - Reaction termination (85°C for 5 min)
                - Cooling sample (4°C forever)

        - Add 1µL RNase A (1:25 dilution w/ TBS buffer, room temperature for 1 hour)
        - Ligate tag to cDNA ends: T4 RNA Ligase I (NEB) for SS RNA/DNA
            - 2µL T4 reaction buffer
            - 2µL 1mM ATP
            - 2µL enzyme
            - 2µL tag
            - 12µL cDNA
            - Cycle:
                - Incubate (37°C for 30 min)
                - Heat inactivation (65°C for 15 min)
    - Stopped after tag ligation step and placed in -20°C
    - Need to analyze protein data for candidate sequences and design primers to amplify specific sequences for expression in yeast

- 8/29/14
    - Analyzed peptide mass fingerprinting data for mascot scores and number of fragment hits, and ran position-specific iterated BLAST on all thirty proteins found in our gel extracts.
    - Decided to proceed with six candidate genes: (protein ID, our informal name for the protein)
        - >PdomMRNAr1.2-03231.1 (chitin-binding 1)
        - >PdomMRNAr1.2-08705.1 (chitin-binding 2)
        - >PdomMRNAr1.2-10508.1 (glucose dehydrogenase 1)
        - >PdomMRNAr1.2-04156.1 (glucose dehydrogenase 2)
        - >PdomMRNAr1.2-02758.1 (uncharacterized 1)
        - >PdomMRNAr1.2-10259.1 (uncharacterized 2)
    - Chitin-binding proteins have chitin-binding domains and hydrophobic regions
    - Glucose dehydrogenases have glucose-binding domains
    - Uncharacterized proteins did not have significant hits when BLASTed
    - Designing primers for glucose dehydrogenases and uncharacterized sequences
    - Codon-optimizing for *E. coli* and synthesizing both chitin-binding sequences and uncharacterized 1
    - Will purify chitin-binding proteins with chitin-coated magnetic beads
    - Will purify other proteins with hist-tags
    - Planning a waterproofing assay in which we'll blot protein solutions onto paper and test for water absorption via hydrophilic dyes

IMPORTANT NOTE: for the remainder of this lab notebook, the candidate genes will be referred to with the following shorthand:

- C1 for >PdomMRNAr1.2-03231.1 (chitin-binding 1)
- C2 for >PdomMRNAr1.2-08705.1 (chitin-binding 2)
- G1 for >PdomMRNAr1.2-10508.1 (glucose dehydrogenase 1)
- G2 for >PdomMRNAr1.2-04156.1 (glucose dehydrogenase 2)
- U1 for >PdomMRNAr1.2-02758.1 (uncharacterized 1)

- U2 for >PdomMRNAr1.2-10259.1 (uncharacterized 2)

Specific bacterial clones of the genes will also be shortened – e.g. C1-1.

- 9/3/14
  - Obtained primers for glucose dehydrogenases and uncharacterized proteins
  - Attempted to PCR wasp genes above from cDNA generated on 8/19
    - 50µL reactions
      - 1µL primer mix
      - 2µL template cDNA
      - 22µL Milli-Q H2O
      - 25µL 2x master mix
    - 55°C annealing temperature, 2 minute elongation step
  - cDNA PCR failed. Will attempt RT-PCR from 6/30 RNA extracts tomorrow

- 9/4/14
  - Ran RT-PCR on RNA sample 5 from 6/30. Used Qiagen OneStep RT-PCR kit (http://www.qiagen.com/products/catalog/assay-technologies/end-point-pcr-and-rt-pcr-reagents/qiagen-onestep-rt-pcr-kit)
    - 50µL reactions
      - 33µL RNase-free water
      - 10µL 5x QIAGEN OneStep RT-PCR Buffer
      - 2µL dNTP mix (10mM each dNTP)
      - 1.5µL primer mix (20µM)
      - 0.5µL RNase inhibitor
      - 1µL template RNA (extract #5 from 6/30, approx. 500ng/µL)
      - 2µL QIAGEN OneStep RT-PCR Enzyme Mix
    - Program (lid 95°C):
      - Reverse transcription: 30 min at 50°C
      - Initial PCR activation step: 15 min at 95°C
      - Denaturation: 30 sec at 94°C
      - Annealing: 30 sec at 50°C
      - Extension: 2 min at 72°C
      - Cycle denaturation, annealing, and extension steps 32 times
      - Final extension: 10 min at 72°C
  - RT-PCR was successful for both glucose dehydrogenases and uncharacterized #2, but failed for uncharacterized #1. Cleaned up samples that succeeded. U1, C1, and C2 will instead be

- 9/5/14
  - Aryo ligated G1, G2, and U2 into pYES2.1/V5-His-TOPO® yeast expression vector
    - Followed protocol for Life Technologies' pYES2.1 TOPO® TA Expression Kit

(http://tools.lifetechnologies.com/content/sfs/manuals/pyes2.1topo_man.pdf)
    - Transformed into 5-alpha *E. coli* and plated onto LB+amp plates

- 9/8/14
    - All three G1, G2, U2 plates showed growth
    - Inoculated liquid cultures for three colonies from each plate
    - After four hours of growth, started colony PCR reactions with 1µL from each culture. Left PCR samples in the freezer to run a gel tomorrow morning
        - Annealing temperature 52°C, elongation step 2 min, 28 cycles
            - 15µL OneTaq master mix
            - 13µL Milli-Q H2O
            - 1µL primer mix
            - 1µL culture
    - Ran a PCR on the two cuticle/chitin-binding proteins Kosuke codon-optimized and synthesized via IDT
        - Annealing temperature 55°, elongation step 40 sec, 30 cycles
            - 25µL OneTaq master mix
            - 23µL Milli-Q H2O
            - 1µL primer mix
            - 1µL template DNA
        - Running a gel tomorrow morning
        - Cleaned up and NanoDropped
            - C1: 69.3 ng/µL, 1.93 260/280
            - C2: 55.3 ng/µL, 1.96 260/280

- 9/9/14
    - Ran gels on colony PCR reactions and on cuticle protein PCR reactions
        - Colony PCR showed bands of right size for every culture. Miniprepped G1-1, G1-2, G2-1, G2-2, U2-2, and U2-3 and sent for sequencing
            - Miniprep concentrations were very high (~700 ng/µL), so these were also run on a gel and appeared free of genomic DNA contamination. Probably very high plasmid DNA concentration because cells were given 20 hours to grow
        - Cuticle protein PCR reactions showed bands of right size. Digested and put in -20°C freezer for ligation tomorrow
            - 10µL reaction volumes
                - 1.4µL C1, 1µL Flexi enzyme blend, 2µL 5x Flexi-Buffer, 5.6µL Milli-Q H2O
                - 2µL C2, 1µL Flexi enzyme blend, 2µL 5x Flexi-Buffer, 5µL Milli-Q H2O
                - 30 min at 37°C, 20 min at 65°C

- 9/10/14

    - G1-1 and G1-2 had several mutations, but in the same places, pointing to genomic differences between our wasps and those whose genomes were sequenced. G1-2 looks good
    - Both G2 inserts went in backwards
    - U2 has extra exon, mutations
    - Miniprepped U2-1, G2-3, and G1-3, sent for forward sequencing. Inoculated four more cultures from each plate
    - Ligated C1 and C2 into Flexi-vector, transformed into *E. coli*
    - Submitted G1-2R, U2-2R for sequencing

- 9/11/14
    - Miniprepped culture #s 4-7 each for G1, G2, and U2
    - Plates with C1 and C2 grew. Inoculated three LB+amp cultures per plate

- 9/12/14
    - Miniprepped C1 and C2 cultures
    - Sent forward and reverse sequencing for G1 cultures 4-7, G2 cultures 4-7, and U2 cultures 4-7
    - Sent forward sequencing for C1 cultures 1-3 and C2 cultures 1-3

- 9/15/14
    - Transformed G1-2, G2-5, and U2-5 into uracil-auxotrophic yeast and plated on uracil-deficient SC minimal medium. Protocol for transformation and medium recipe: http://tools.lifetechnologies.com/content/sfs/manuals/pyes2.1topo_man.pdf. Left in 30°C incubator.
    - C1 sequences all came back bad, but C2-1 was good with no mutations.
    - Ran colony PCR on eight more C1 colonies. Inoculated cultures of C1-7, C1-8, C1-10, and C1-11, which looked good on the gel.

- 9/16/14
    - Miniprepped C1-7, C1-8, C1-10, and C1-11 and sent for sequencing
    - Transformed C2-1 into T7 cells and plated onto LB+amp.

- 9/17/14
    - Yeast transformants did not appear to be growing, possibly because the uracil selection process impedes growth. Plated more G1-2, G2-5, and U2-5, each with four times as many cells, onto uracil-deficient minimal media and plated a positive control onto uracil-positive YPD media plate
    - Only the C1-11 sequencing read came back clean. The alignment was good except for one point mutation from glutamine to arginine. Decided to continue with C1-11 due to shortage of time
    - C2-1 plate from yesterday showed no growth. Re-transformed C2-1 into T7 cells, as well as C1-11, and plated onto LB+amp

    - Codon-optimized U1 arrived from IDT. PCRed with ampli-wasp primers and Q5 polymerase

- 9/18/14
    - None of the yeast plates appeared to show growth
    - C1-11 and C2-1 T7 cells both grew on LB+amp plates. Inoculated two 3mL LB+amp liquid cultures with 5 colonies from each plate, and one 3mL LB+chlor liquid culture with control T7 cells and left in 37°C shaking incubator
    - Ran U1 on a gel and it looked good, though with some non-specific amplification. Cleaned up and digested with 2µL cleaned U1, 1µL Flexi-blend, 2µL Flexi-buffer, and 5µL Milli-Q H2O – 30 min at 37°C, 20 min at 65°C. Then ligated with 1µL insert, 1µL digested Flexi backbone, 2µL sticky-end ligase – left at room temperature for an hour and then 4°C overnight

- 9/19/14
    - YPD yeast control plate showed growth. The minimal media plates are probably not growing because the transformation failed, but possibly because the uracil selection greatly impedes growth. Left minimal media yeast plates in 30°C incubator over weekend
    - Inoculated two LB+amp 100mL cultures with the 3mL C1-11 and C2-1 cultures from yesterday, as well as an LB+chlor 100mL culture with the 3mL T7 control culture. Added IPTG after four hours and left on shaker at room temperature over weekend
    - Transformed NEB 5-alpha *E. coli* with digested and ligated U1 in Flexi-backbone and plated onto LB+amp plate. Left at room temperature over the weekend

- 9/22/14
    - Pelleted the C1-11, C2-1, and T7 control cultures, removed supernatant, washed with 10mL protein wash buffer, removed buffer and pelleted again. Placed in -80°C freezer.
    - The U1-transformed 5-alpha LB+amp plate showed growth. Inoculated three 2mL LB+amp liquid cultures, labeled U1-1, U1-2, and U1-3. Left in 37°C shaking incubator overnight.

- 9/23/14
    - Miniprepped U1-1, U1-2, and U1-3 and sent for sequencing using Flexi-vector sequencing primers.

- 9/26/14
    - The G1, G2, and U2 plates never showed any growth. Placed in 4°C fridge.
- 9/29/14
    - Analyzed U1 sequencing data. U1-3 was ligated correctly into the Flexi-vector, though with a point mutation from serine to arginine.

Decided to continue with U1-3 despite mutation due to shortage of time.

- 10/2/14
  - Transformed U1-3 into T7 *E. coli* and plated onto LB+amp. Incubated overnight at 37°C.

- 10/3/14
  - U1-3 T7 transformants grew on LB+amp.
  - Colony PCR

- 10/11/14
  - Attempted to purify C1 and C2 from 9/22/14 pellets in -80°C freezer.
  - Used New England Biolabs "Chitin Magnetic Beads" kit (https://www.neb.com/products/e8036-chitin-magnetic-beads)
  - Freeze-thaw cycle protocol (to lyse cells):
    - 1. Transfer pellet into 2mL tube
    - 2. Freeze-thaw cycle 3 times (liquid nitrogen, ice)
      - 2 minutes in liquid N2 tank
      - 2 minutes in room temp water
      - 6 minutes in ice
    - 3. Fill with chitin-binding/wash buffer up to 2mL, vortex to resuspend
    - 4. Transfer 0.5 mL to a 1.5mL tube, store remaining 1.5mL in -80°C as backup
    - 5. Spin down the transferred 1mL of buffer at 14000rpm, 4°C, 5 minutes
    - 6. Transfer supernatant to a new 1.5mL tube (dispose of pellet)
    - 7. Proceed with NEB protocol, elute with 100µL of 20mM NaOH unbuffered. Store in -80°C.

- 10/14/14
  - reattempted yeast transformation with better competent cells: G1-2, G2-5, U2-5, RFP control (also in pYES2.1/V5-His-TOPO® vector). Used same transformation protocol as before: (http://tools.lifetechnologies.com/content/sfs/manuals/pyes2.1topo_man.pdf)
    - used 4µL G1-2 (280.0ng/µL), 2µL G2-5 (646.4ng/µL), 3µL U2-5 (370.0ng/µL), 5µL RFP (25.5ng/µL) for transformations
    - plated on uracil-deficient minimal media with glucose carbon source, as glucose represses pYES2.1/V5-His-TOPO® vector expression. Left in 30°C incubator
  - Ran SDS-PAGE gel on purified protein samples from chitin magnetic bead protocol on 10/11/14. C1 did not appear to be present, but the dominant band in the C2 lane was roughly the size of C2 (~11kDa), indicating that C2 may have chitin-binding activity!

- 10/16/14
    - yeast transformants from 10/14/14 all grew
    - next step is to inoculate yeast cultures with uracil-deficient minimal media with galactose carbon source, as galactose induces pYES2.1/V5-His-TOPO® vector expression