Exploring potential cross feeding and symbiosis factors necessary for growth of a novel *Rhizobiales* from *Cephalotes* ants

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Introduction

The overarching question of our work is "how has the microbial component of the *Cephalotes* ant holobiont system contributed to ant evolution?" The *Cehpalotes* (turtle) ant genus is comprised of 117 species that stretch across neotropical areas such as the Caribbean, American Southwest, and South America. *Cephalotes* diet is comprised mainly of pollen and nectar; it is carbohydrate-rich but nitrogen-poor. Further, different species of *Cephatotes* share an exceptionally similar microbial gut-biome community. Recent metagenomic studies by Russell *et. al.* (1) show an abundance of a *Rhizobiales* in the microbial gut-biome of every species of *Cephalotes.* Ongoing metagenomic analysis suggests that the *Rhizobiales* may break down urea, a critical nitrogen source for the ants (2). However, this cannot be tested until a member of this group is isolated and cultured. Here, we describe the efforts to cultivate *in vitro* a member of the *Rhizobiales* from *Cephalotes texanus*, dubbed POW232. Initial studies showed that POW232 only grows in coculture with specific bacteria on blood agar plates (Figure 1). Thus, our objective of this study is to determine the factors provided by other bacteria and the blood agar that, in combination, POW232 needs to grow. In doing so, we may learn of potential cross feeding and symbiosis factors supplied within the gut microbial environment or by the host ant itself.

Figure 1. A *Cephalotes texanus* turtle ant. Public domain image by Alejandro Santillana. Produced as part of the "Insects Unlocked" project. May 24, 2016

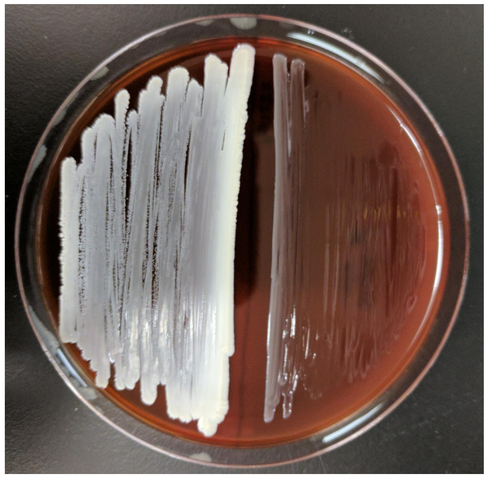

Figure 2. POW232 (right) cocultured with JDR-1, an *Enterococcus* isolated from ant larvae (left) seven days post plating. POW232 grows only along border shared with JDR-1.

Methods

A series of qualitative experiments using various types of agar, agar additives, and bacteria were performed, each informing the next. In each experiment, we recorded whether or not POW232 grew in the experimental culture based on qualitative analysis. Each experiment was done in triplicate, with POW232 incubated in a 1% CO_2 incubator on blood agar at 30°C unless otherwise indicated. Tests for growth-supporting bacteria were done by streaking alongside POW232 on blood agar. Positive controls of POW232 and supporting bacteria (JDR-1 or RFC-10, an *Enterobacteria)* were made for each experiment.

Supplemented Media Experiments

A variety of plate variables were tested on different plates: TSA plates supplemented with vitamins, minerals, or hemin; brain and heart infusion agar; Schaedler agar; TSA plates with fetal and adult bovine serum; pH plates ranging from pH 4-8. No POW232 growth was found on any plates, suggesting that POW232 requires an uncommon growth factor in or on red blood cells.

Separation of Blood Agar Components

Bacteria that supported POW232 growth showed signs of hemin catabolism on blood plates. Thus, we tested products of red blood cell (RBC) catabolism: iron, hemin, and bilirubin- the primary product of hemin catabolism. We centrifuged the blood to separate red blood cells and plated POW232 on agar containing the red blood cell pellet or blood plasma. Further, we plated POW232 on both heat and syringe-lysed blood agar plates. Only the plates with the blood cell pellet and the supporting bacteria JDR-1 showed growth of POW232. This suggests that the presence of whole RBC's is required for POW232 growth. It may also indicate that POW232 growth requires a cocultured bacteria that plays a dynamic role in producing or modifying products of RBC catabolism.

Coculture with Variable Bacteria

To determine the cocultured bacteria's role in POW232 growth, we sought to find bacteria that did *not* support POW232 growth. To do this, samples of known bacteria with sequenced genomes were spotted on a lawn of POW232 on blood plates (Figure 3).

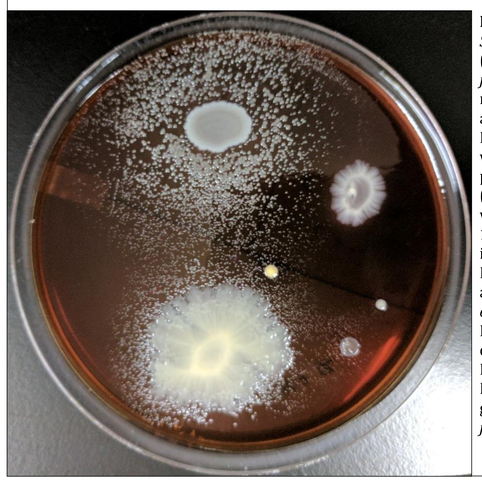

Figure 3. *S. Epidermidis* (top left) and *E. faecalis* (top right) spotted on a lawn of POW232. RFC-10 was used as a positive control (bottom). Picture was taken after 14 days of incubation. Distinct colonies around *S. epidermidis and* RFC-10 demonstrate POW232 growth. Note lack of growth around *E. faecalis.*

Genomic Analysis

Of the 12 bacteria cocultured with POW232, only three strains failed to support growth of POW232 (Table 1). Using BLAST genomic analysis and RAST metabolic pathway prediction, we compared potential proteins and metabolic pathways of all 12 bacteria. Only one gene was found to be present in all bacteria that supported POW232 and absent in those that failed to support POW232 growth: a glycerol 3-phosphate transporter. (Table 1)

Bacteria	Support growth of POW232	Possess G3P transporter
S. epidermidis	+	+
E. faecalis	-	-
S. enteritidis	+	+
S. pneumonia	-	-
S. agalactiae	+	+
S. aureus	+	+
S. pyogenes	+	+
JDR-1	+	+
RFC-10	+	+
CV58 , a *Ventosmonas* within *Pseudomonadaceae*	+	+
CV33, a *Burkholderies*	-	-
*P. aeruginosa**	-*	+

Table 1. Identification of bacteria that supported POW232 growth and those that did not (highlighted in red). All bacteria that supported POW232 growth possess the G3P transporter gene. Bacteria that did not support POW232 growth lack the gene. **P. aeruginosa* is the exception; although it possesses the G3P transporter gene, it also secreted chemicals that were cytotoxic to POW232.

Investigation of the Glycerol 3-Phosphate Transporter

The glycerol 3-phosphate transporter uses a shuttle mechanism to pump G3P into the cell while expelling inorganic phosphates into the extracellular matrix. To begin exploring the role of the G3P transporter in relation to POW232, we plated POW232 onto TSA plates containing a range of phosphate concentrations. POW232 failed to grow on any plates, demonstrating that the inorganic phosphate supplied by the G3P transporter is not the necessary growth factor. Additionally, we plated POW232 with G3P transporter knockout mutant *E. coli* (3) on blood agar. POW232 grew on these plates, suggesting that the presence of a glycerol 3-phosphate transporter is not required for POW232 growth.

Construction of an *E. coli* Transposon Library

With the knowledge that wild-type *E. coli* can stimulate POW232 growth on blood agar, our current efforts are focused on constructing an *E. coli* transposon knockout library. Using electroporation, we randomly insert an EZ Tn5 transposome into the genome of *E. coli*. These mutants are antibiotically selected for and spotted on a lawn of POW232 on blood agar plates. (Figure 4). Any *E. coli* mutant that fails to support growth of POW232 will have the transposon and surrounding gene(s) sequenced to determine what gene(s) are knocked-out.

Figure 5. Two *Cephalotes varians* ants. Photo found and purchased from alexanderwild.com

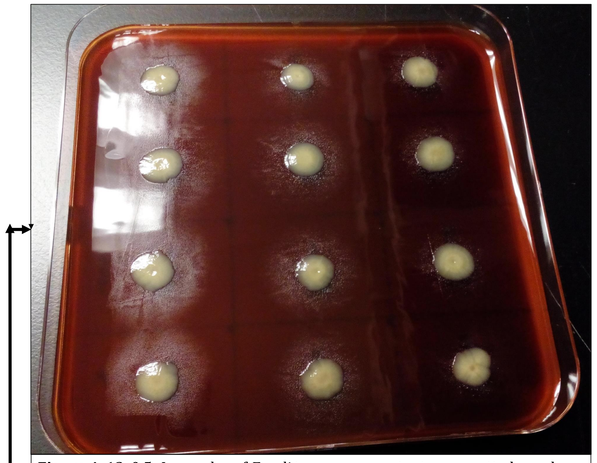

Figure 4. 12, 0.5µL samples of *E. coli* transposon mutants are spotted on a lawn of POW232. Picture taken 5 days post spotting. Halo of POW232 around *E. coli* mutants demonstrates growth of POW232

Future Plans

As of writing this poster, the entire POW232 genome is being sequenced. Genomic data will allow us to pinpoint any metabolic pathway deficiencies so we may more accurately run *in vitro* tests. Additionally, we are cultivating human epithelial and T-cell lines. These will allow us to examine if growth POW232 growth is dependent on just red blood cells if or other cells contain the growth factors.

Knowing what nutrients or signals are supplied by the cocultured bacteria will elucidate possible cross feeding factors present in the *Cephalotes* gut microbial environment. Further, discovering what growth factors are uniquely supplied by the RBC's or other eukaryotic cells will illuminate potential host/microbe relationships between the *Cephalotes* ant and POW232. These data will further our understanding of how the microbial components of the *Cephalotes* ant gut interact with each other and the host to contribute nutrients and survive in new or altered niches.

References and Acknowledgements

(1) Russell, J. (2014) *et. al*. Correlates of gut community composition across an ant species (Cephalotes varians) elucidate causes and consequences of symbiotic variability. *Molecular Ecology*.

(2)Hu *et. al.* Nitrogen conservation, conserved: 46 million years of N-recycling by the core symbionts of turtle ants. Submitted to *Nature Communications*

(3) Overbeek, R. *et. al*. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Research*

(4)*E. coli* mutants were supplied by The Coli Genetic Stock Center, Wertz et. al.

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