Development of Immunosuppressed Drosophila for a Novel Screen to Identify Essential Proteins for Host-Pathogen Interaction

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Abstract

Diarrheal diseases are a major cause of death in children under the age of five around the world. A rise of antibiotic resistance has also been detected in bacteria that cause diarrheal diseases, and so to combat these diseases new drugs must be developed. We have made an immunosuppressed *Drosophila* strain to conduct a genetic screen to identify proteins in the intestinal epithelium that are necessary for host-pathogen interactions. This fly line contains less dual oxidase (DUOX) to reduce the production of reactive oxygen species, which ordinarily combats bacterial infections in the gut. In the screen we crossed the immunosuppressed line with flies containing various RNAi knockdown constructs targeting proteins found at the apical surface of epithelial cells. The survival of immunosuppressed flies containing the RNAi constructs will indicate that the knocked-down protein is necessary for infection. Future work can then be done to develop new drugs against these targets.

Introduction

Diarrheal diseases caused by bacteria are a major cause of illness and death globally. In 2010 an average of over 2,000 children under the age of five died from diarrheal diseases every day (Liu *et al.,* 2012). The developing world suffers more from these diseases due to lack of sanitation, malnutrition, and lack of proper medical care. Some diarrheal diseases are easily treated with proper hydration, but more serious cases need antibiotics (Guerrant *et al*., 2001). Lack of proper medical training has led to the over-use of antibiotics in the developing world, which has led to the development of antibiotic resistance (Okeke *et al.*, 1999). It imperative that new non-antimicrobial drugs are developed to combat bacterial infections and limit the spread of antibiotic resistance. Our research searches for possible targets for non-antimicrobial drugs through the use of a genetic screen using the fruit fly *Drosophila melanogaster*.

Much is known about the interactions between bacteria that cause disease and the intestinal cells they infect. Some bacteria rely on similar cell functions to establish

infection, but each of them will infect different parts of the epithelial cells. *Escherichia coli (E. Coli), Salmonella*, and *Shigella* all rely on actin modulation to establish infection, but *E. coli* infects the microvilli, while *Salmonella* enters epithelial cells to establish infection (Dean, 2011). Knockdown of bacterial virulence factors have been done to determine their necessity for infection, but knockdown of epithelial cell proteins has not been tested to determine their necessity during infection.

The first interaction between bacteria and the cells they infect occurs at the microvilli on the apical surface of epithelial cells. These cells contain different proteins that determine cell polarity, shape, and aid in different cell processes. One of those proteins is β_{Heavy}-spectrin, which localizes to the apical surface of epithelial cells (Zarnescu and Thomas, 1999). β_{Heavy}-spectrin is found at the brush border where it crosslinks F-actin which in turn helps stabilize the microvilli (Bement and Mooseker, 1996).

Drosophila melanogaster is used as a model system to study intestinal infections because its host defenses are well known and can be easily manipulated. These defenses typically have analogs in mammals. For example, reactive oxygen species are produced as a defense against bacterial infections in mammals and *Drosophila (*Bae et al., 2010; Ha et al., 2005*)*. Lack of the enzyme dual oxidase (Duox), which produces reactive oxygen species in *Drosophila*, causes flies to be become more susceptible to bacterial infection since they lack the ability to effectively eliminate bacteria (Ha et al., 2005). Flies subjected to RNA interference (RNAi) towards Duox die within 5 days after oral infection with the plant pathogen *Pectobacterium carotovara carotovara* 15 (*PCC*15), while wild-type flies survive (Ha et al., 2005).

Pectobacterium carotovara carotovara 15 is a gram-negative bacterium that causes soft rotting in potatoes and other fruits by the production of pectinases (Barras et al., 1994). While most bacteria seem to pass through the fly without eliciting a response, PCC15 has been shown to induce an immune response and persist in the gut of larvae (Basset et al., 2000). Two bacterial genes have been found to allow for the persistence of PCC15 in the gut of flies: one is a regulator, Hor, and the other is the Erwinia Virulence Factor (Evf) (Basset et al., 2003). Evf is a transmembrane protein that if over expressed causes strong lethality in fly larvae (Muniz et al. 2007). Evf contains a palmitic acid moiety on the C-terminal end of the protein that allows it to bind to membranes of the gut cells and is necessary for infection (Quevillon-Cheruel et al., 2009)

To examine which proteins in the gut are necessary for bacterial infection we will be using RNAi knockdown of various proteins in the gut epithelium of *Drosophila*. A fly line containing an RNAi for dual oxidase will be crossed with flies containing homozygous copies of RNAi for proteins in epithelial cells. These flies will then be orally infected with PCC15 and monitored for survival. If more than 50% of the infected flies survive then the missing protein is essential for bacterial infection.

Methods

Fly Stocks

Flies were grown at 25°C on fly media composed of 0.82% agar, 12.94% sugar, 3.22% yeast, 6.12% cornmeal, 0.82% tegosept, and 0.18% propionic acid. An Immunosuppressed fly line was created by combining the dual oxidase RNAi construct (CG3131) along with the driver da-Gal4, the Gal4 inhibitor Gal80, and the fluorescent marker tdTomato, and the marker balancer TM6 (daGal4, tdTomato, $Duox^{RNAi}$/ Gal80, TM6). To improve the immunosuppressed fly line a second daGal4 was added to the line by crossing it to BL55851.

Bacterial Strains

Pectobacterium (formerly Erwinia) carotovora carotovora 15 (PCC) was obtained from Dr. Timothy McNeillis (Plant Pathology, Penn State), and used in the infection experiments. The bacterium was grown on LB agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% 1M NaOH, and 1.5% agar) and broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% 1M NaOH) at 28° C.

Infection Experiment

Adult flies were dehydrated for two hours before infection. After dehydration the flies were exposed to PCC15 at $O.D._{600}$ 200, $O.D._{600}$ 100, and $O.D._{600}$ 50 re-suspended in 5% sterile sucrose on filter paper. The flies were exposed constantly to the bacteria. The filter paper containing the sucrose and bacteria were replaced every 24 hours with fresh bacteria at which point the viable count was determined each time.

Tail PCR

Tail PCR was used to identify the location of the daGal4 on the second chromosome and was performed, as described in Singer and Burke (2003). Tail PCR uses nested primers for the known region of the insert and arbitrary primers (AD primers) that recognize sections of DNA within the genome. The AD primers are a mix of primers that have different annealing temperatures, but will anneal to the same area producing one size band. The nested primers used provide specificity for the P-element and not any other sequence in the genome. The nested primers for the 5' end of the P-elements were 5'-CACCCAAGGCTCTGCTCCCACAAT-3', 5'-TACTCCAGTCACAGCTTTGCAGCA-3', and 5'-ACACAACCTTTCCTCTCAACAA-3'. The nested primers used for the 3' end of the P-element were 5'-ATTCAAACCCCACGGACATGCTAAGG-3', 5'-ACAATCATATCGCTGTCTCACTCAG-3', and 5'-CGACACTCAGAATACTATTCCTTTCAC-3'. The reverse primers used are described in Singer and Burke 2003. DNA was extracted from the BL 55851 line using standard procedures, and was quantified using a Nanodrop spectrophotometer. Samples were also analyzed using a pre-existing lab stock of DNA from the P-element free strain Oregon R

as a negative control. The sequence of this band was determined by the Huck Institute Genomics Core Facility and analyzed using the NCBI (National Center for Biotechnology information) Blast website.

Results

Development of an Immunosuppressed Fly Line

The reactive oxygen immune response of *Drosophila melanogaster* to bacteria was suppressed by the addition of dual oxidase RNAi ($Duox^{RNAi}$) to the third chromosome. Figure 1 illustrates the genetic makeup of the fly stock, which contains the following other genetic elements: (i) da-Gal4used to express the $Duox^{RNAi}$ throughout the fly; (ii) the fluorescent protein tdTomato, to allow easy differentiation between the immunosuppressed flies from the immunocompetent ones; and (iii) a tub-Gal80 construct to inhibit the action of Gal4, and therefore the expression of the $Duox^{RNAi}$, allowing the stock to remain healthy until it is used in a test cross.

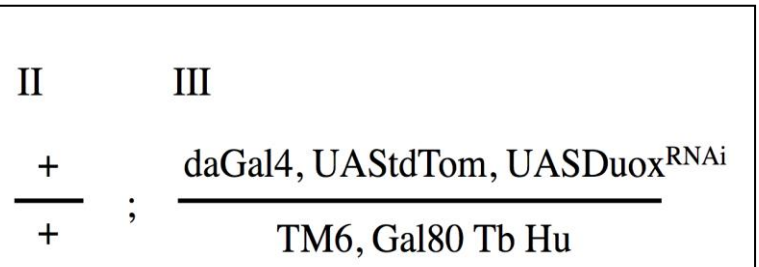

Figure 1. Genotype of the immunosuppressed fly line stock.

*Testing the Immunosuppressed Fly Lin*e

The immunosuppressed fly line was crossed with *yw* wild-type flies to determine the dosage of bacteria that would be lethal to the immunosuppressed flies, but not the immuocompetent siblings. Bacterial dosage was determined by using cultures of different optical densities measured at a wavelength of 600nm (OD_{600}). Tested were cultures with an OD_{600} of 12.5, 100, and 200. Figure 2B illustrates the survival rate following the oral infection by PCC15 with the corresponding OD_{600}. Ha *et al.* (2005) showed that flies lacking Duox died after five days when exposed to PCC15 at an OD_{600} of 12.5. The oral infection with the immunosuppressed flies did not yield the same result. After six days of oral infection only 10% of the flies at OD_{600} 12.5 had died (Figure 2B). In light of this result higher infection with higher OD_{600} concentrations were also tried, with very similar results (Figure 2B). To improve the immunosuppression and obtain the result reported in Ha *et al.* (2005) we have added an extra da-Gal4. Since we have extra Gal4-dependent genetic elements compared to Ha *et al.* (2005), we hypothesized that there is not enough Gal4 to suppress the Duox effectively.

A

Immunosuppressed

II III

$\frac{daGal4}{+}$; $\frac{daGal4, UAStdTom, UASDuox^{RNAi}}{+}$

Immunocompetent

II III

$\frac{+}{cyo}$; $\frac{+}{TM6, Gal80\ Tb\ Hu}$

B

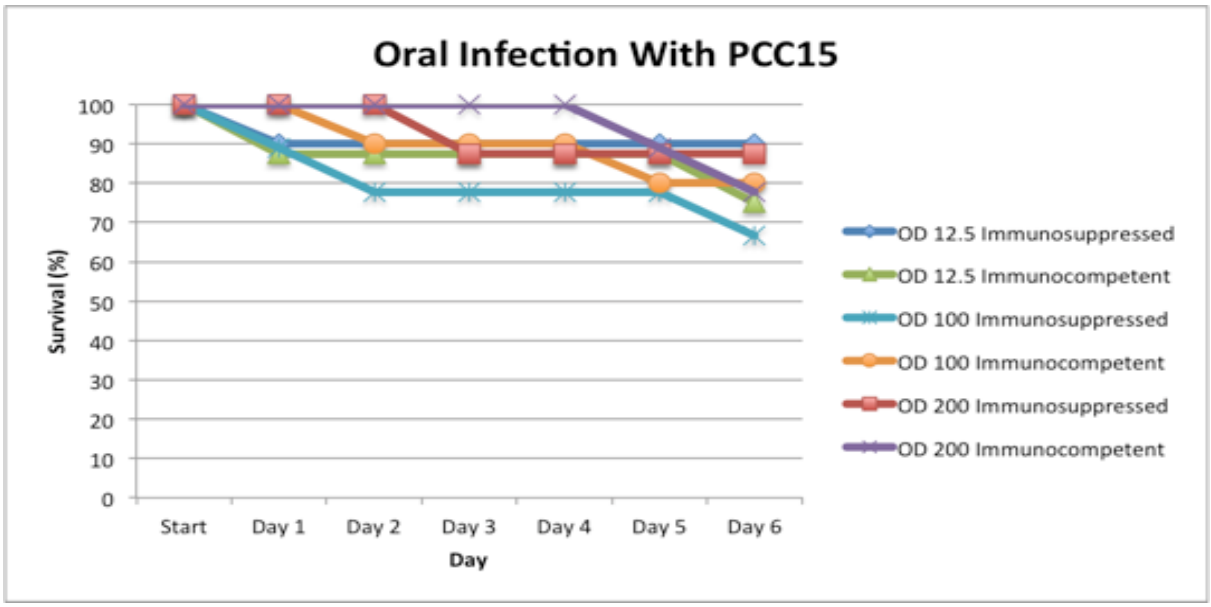

Figure 2. (A) Genotype of immunosuppressed flies and immunocompetent flies used to test lethal does of PCC15. (B) Survival rates of immunosuppressed and immunocompetent flies after oral infection with PCC15 at a range of OD_{600}. Ten immunosuppressed and ten immunocompetent flies were tested in each trial

Improving the Immunosuppressed Fly Line

To improve the immunosuppressed fly line a second daGal4 was added on the second chromosome by crossing the first immunosuppressed fly line with the fly line BL55851, which contains a homozygous copy of da-Gal4 on the second chromosome. This addition would result in an increase in expression of $Duox^{RNAi}$ and therefore more effectively reduce the expression of the Duox protein. Figure 3A illustrates the genotype of the new immunosuppressed fly line. Since da-Gal4 does not have a phenotype and the fly line contains many genetic elements an assay must be developed to determine whether the second da-Gal4 was successfully added. To do this we must first determine the exact molecular location of the da-Gal4 in the BL55851 line, and we decided to do this by using Tail PCR (Singer *et al.* 2003). Once the location is known we can easily create a specific PCR assay to screen the new line for the addition of the second daGal4.

Tail PCR works to capture the DNA adjacent to the da-Gal4 construct by using nested primers for the known region of the P-element and arbitrary primers (AD primers)

that recognize sections of DNA within the genome. The nested primers for the P-element used in Tail PCR will produce sequentially smaller products in each of the three PCR reactions, which make them easy to identify. The nested PCR primer also adds specificity to the reaction. The DNA gel shown in Figure 3B shows two likely positive results. The sequence of the band labeled 3' AD3 Mix (Figure 3B, lane 3' AD Mix 3) should correspond to the 3' end of the P-elements plus the flanking DNA in chromosome II. NCBI Blast analysis of the sequence indicated that, the P-element was located on the third chromosome, whereas the location of the da-Gal4 in the line BL55851 was reported to be in the second chromosome. It is not unheard of for P-element containing stocks to contain cryptic P-elements (Dr. Graham Thomas, personal communication), and it would appear that this is what has been uncovered. An initial attempt to analyze the bands in the AD3 samples (Figure 3., Lane 3' AD3 3) was unsuccessful, and so further experiments will need to be done to confirm the location of the da-Gal4 construct in the BL55851 line.

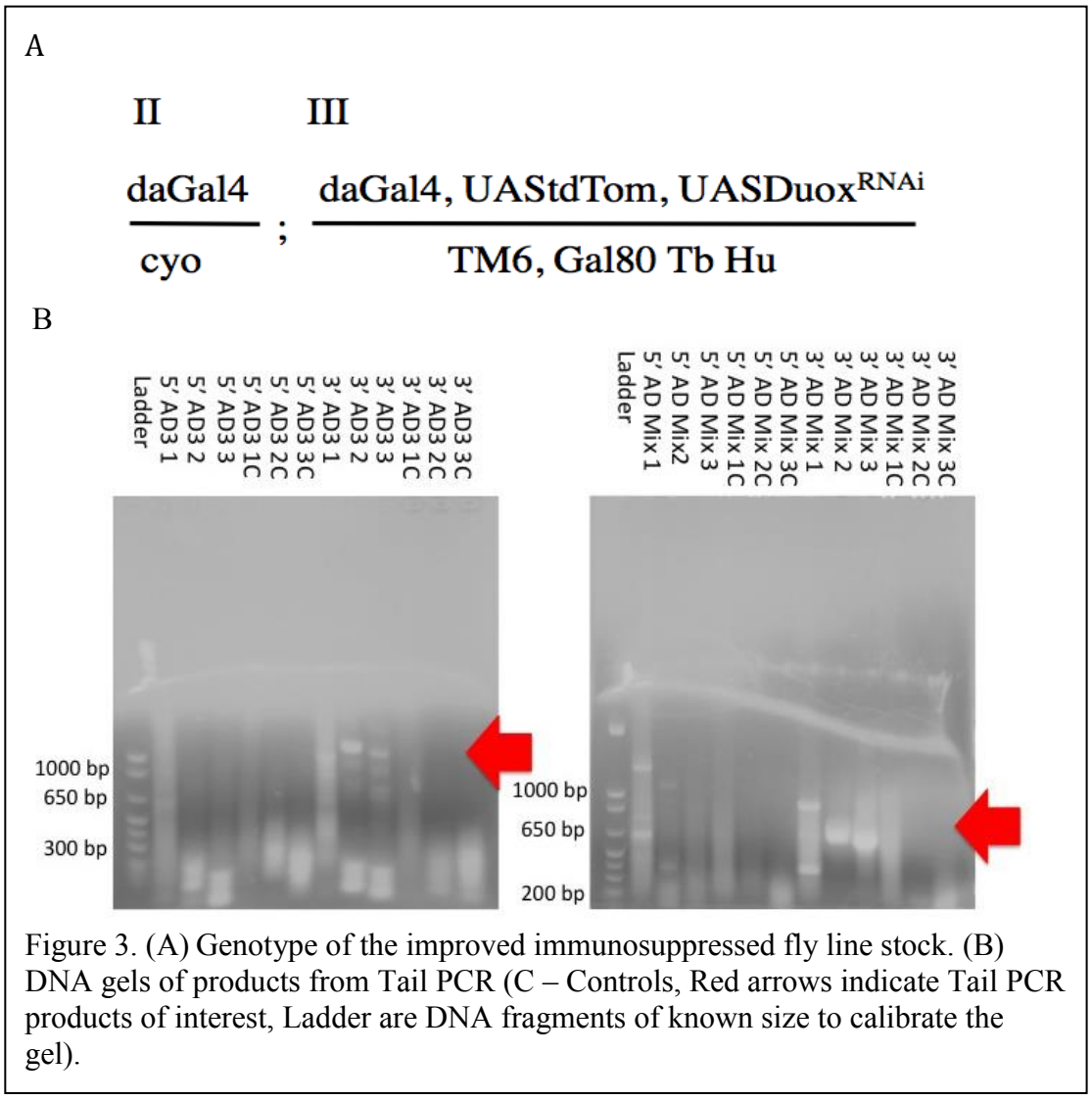

Figure 3. (A) Genotype of the improved immunosuppressed fly line stock. (B) DNA gels of products from Tail PCR (C – Controls, Red arrows indicate Tail PCR products of interest, Ladder are DNA fragments of known size to calibrate the gel).

Discussion

Knockdown of Duox has been shown to be lethal in fruit flies when they are exposed to PCC15 (Ha *et al.* 2005). We developed an immunosuppressed fly line incorporating $Duox^{RNAi}$ to examine which proteins in the gut are necessary for bacterial infection. While testing the immunosuppressed fly line with an oral infection of PCC15 we did not see the same lethality seen in that paper. To improve our line we added a second da-Gal4 driver by crossing the immunosuppressed fly line stock with a fly line containing a homozygous copy of a second da-Gal4 element on the second chromosome. Tail PCR was used to try and determine the location of the second da-Gal4, but sequencing indicated that the da-Gal4 line probably has a second P-element on the third chromosome that is confounding our analysis.

Once the location of the second da-Gal4 is confirmed we will use this knowledge to design a specific primer flanking the P-element in the genomic DNA of chromosome II. The combination of this primer with one in the da-Gal4 P-element will be unique, even in the context of all the other genetic elements in the new immunosuppressed line, and will provide definitive proof that it is indeed present. Following this determination, a genetic screen will be performed to examine the host-pathogen interaction (i.e. fly<-> PCC15). In this screen we plan to cross our newly developed fly line with other fly lines containing RNAi knockdown constructs for proteins at the apical surface of epithelial cells as well as for other proteins involved in signaling in this region. A positive result in the screen would be indicated by at least a 50% survival of the immunosuppressed flies containing the RNAi knockdown for the apical or signaling protein following bacterial infection. This result would indicate that the knockdown of that particular protein is essential for the bacteria to effectively infect the cells. A negative result would be indicated by near 100% death of the immunosuppressed flies containing the RNAi knockdown of the apical or signaling protein. This screen will provide insight into host-pathogen interaction and the development of new drugs targeting the identified proteins.

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