Biocide Resistance in E.coli

The ideal lab in an Evolutionary Biology class would be one where we demonstrate natural selection in response to a selection pressure. This is easy enough to do with bacteria (see Lenski and Travisano 1994, Travisano and Lenski 1995, 1996, or any of the publications listed on Richard Lenski’s website: http://myxo.css.msu.edu/Publications.html). However, what are less well studied in the classroom are the costs of evolution. In the case of bacteria, there is a demonstrated cost of antibiotic resistance (Lenski 1998).

The selective agent for this lab will be 2,4,4’-Trichloro-2’-hydroxydiphenyl ether, better known as Triclosan (and marketed as Irgasan DP300). Triclosan is classed as a biocide (a substance toxic to cells in general) or antimicrobial agent (a substance toxic to bacteria, fungi, and protistans) because it kills or inhibits the growth of a wide range spectrum of microbes. It is not considered an antibiotic (a microbial product that kills or inhibits the growth of susceptible microbes) because of its origin and its broad spectrum of toxicity.

Bacteria and fungi are well-known for evolving resistance to antibiotics (Levy 1998), but are thought to be less likely to evolve resistance to biocides because these compounds often act by different mechanisms. Indeed, the multiple mechanisms by which Triclosan could or did kill bacteria suggested little risk for the evolution of resistance. However, targets of Triclosan activity were discovered which are associated with mechanisms of antibiotic resistance in bacteria (Schweizer 2001) indicating that Triclosan may act more like an antibiotic than a true biocide (McMurray, et al. 1998; Levy et al. 1998).

The goal of this lab is to conduct a series of selection events on a population of E. coli for 11 generations, then challenge the selected population to competition with its near ancestral line. The selective agent will be a solution of Irgasan applied to a population of Dr. Thompson’s E. coli that express green fluorescent protein (GFP). After 11 rounds of selection, you will test the competitiveness of the selected GFP expressing E. coli against a near ancestral line – an E. coli culture expressing cyan fluorescent protein (CFP). The structure of the fluorophore (three amino acids) inside the fluorescent protein differs by one amino between GFP (tyrosine) and CYP (tryptophan). We will assume that the metabolic cost difference between the two amino acids is relatively small.

Tyrosine (GFP)  Tryptophan (CYP)
Selection for Triclosan resistance

1) Create a lawn of *E. coli* on each of three petri dishes (containing tryptic soy agar).
2) Place one treatment paper disk in each petri dish (1 for Triclosan, 1 for EtOH, 1 for ddH2O)
3) Label the dish with
   - the treatment
   - the date
   - your name
4) Secure the 3 treatment dishes together with lab tape.
5) Place in growth chamber (35°C for 24 hours)

- After 24 hours

6) Measure the width of the zone of inhibition (make and record 2 measurements report the average)
7) Isolate the most resistant *E. coli* by scraping the inner margin of the zone of inhibition with the tip of a clean & sterile pipette tip. If no zone of inhibition is apparent, scrape bacteria from an area adjacent to the paper disk. If isolated bacterial colonies are visible within the zone of inhibition, scrape those.
8) Transfer the bacteria collected from the petri dish to a culture tube containing sterile tryptic soy broth by inserting the pipette tip into the broth and swishing. (Be neat! Don’t muck up the pipette itself.) Cap the tube and label with:
   - the treatment
   - the date
   - your name
9) Place in growth chamber (35°C for 24 hours)
10) Repeat 10 more times (N = 11)
Relaxation of selection

1) Create a lawn of *E. coli* on each of three petri dishes (containing typtic soy agar).
2) Place one paper disk soaked in sterila ddH20
3) Label the dish with
   - the treatment
   - the date
   - your name
4) Secure the 3 treatment dishes together with lab tape.
5) Place in growth chamber (35°C for 24 hours)

❖ After 24 hours

6) Measure the width of the zone of inhibition (make and record 2 measurements report the average)
   No zone was measured because none was expected.
7) Isolate the *E. coli* closest to the paper disk by scraping with the tip of a clean & sterile pipette tip.
8) Transfer the bacteria collected from the petri dish to a culture tube containing sterile tryptic soy broth by inserting the pipette tip into the broth and swishing. (Be neat! Don’t muck up the pipette itself.) Cap the tube and label with:
   - the treatment
   - the date
   - your name
9) Place in growth chamber (35°C for 24 hours)
10) Repeat 5 more times (N = 6)

Measuring the cost of triclosan resistance – competition with non-selected *E. coli*.

Twenty-four hours following the last round of evaluation (round 11 and round 6) and culturing, the turbidity of each culture of GFP *E. coli* was measured at 600 nm using the Nanodrop spectrophotometer (Thermo Scientific). The volume of each culture tube was adjusted with tryptic soy broth so that it’s absorption at 600 nm would be close to that of the stock culture of CFP *E. coli* (the “ancestral line”).

A competition arena was established by adding 1 ul of a GFP *E. coli* culture (the treatment) and 1 ul of BFP *E. coli* (the “ancestral line”) to 1 ml of tryptic soy broth with 1x IPTG fluorescent inducer.

All tryptic soy broth and tryptic soy agar used throughout the experiment contained kanamycin sulfate (50 ug/ml). The *E. coli* used contained a plasmid for both the fluorescent protein and kanamycin resistance. The latter selects for those *E. coli* that have been successfully transformed. The kanamycin was kept in all cultures so that any back mutations back kanamycin susceptibility would be eliminated.

Competition cultures were incubated at 35-37°C for 24 hours and then held at 2.2 °C until the cultures could be measured.
Measuring the outcome of competition

One hundred microliters of each culture were pipetted into a well of a 96-well plate. Eight wells were filled with tryptic soy broth as blanks. Samples were subject to two scans using the Wallac Victor2 microplate reader (Perkin-Elmer) to assess the relative amount of GFP and EFP E. coli.

1) Cyan fluorescent protein E. coli were excited at 430 nm and the absorbance of the fluorescence at 486 nm was recorded.
2) Green fluorescent protein E. coli were excited at 485 nm and the absorbance of the fluorescence at 535 nm was recorded.
3) Eight replicate wells were filled with 100 ul of tryptic soy broth with IPTG fluorescent inducer as control blanks.

The key is not to excite both fluorescent proteins with the same wavelength. I have selected filter sets (based on what is available for our plate reader) that avoid this. The filter sets we have roughly approximate those below.

CFP  
GFP

Excitation region

We should be cautious in using the absolute fluorescence value as an indicator of the abundance of either fluorescent protein expressing bacteria. The filter sets may not be perfect for measuring the abundance of bacteria on the basis of fluorescence alone. Indeed, using fluorescence alone is really a proxy for estimating abundance of bacteria. Instead it would be more prudent to use a ratio of absorbances.

Absorbance of BFP E. coli  
Absorbance of GFP E. coli.

I will leave it to you to determine how this value should be used to determine which bacterial lineage is more successful.
Useful references

The following articles should be freely available through Schmidt Library electronic journal collection or through internet searching. Exceptions are as noted.


