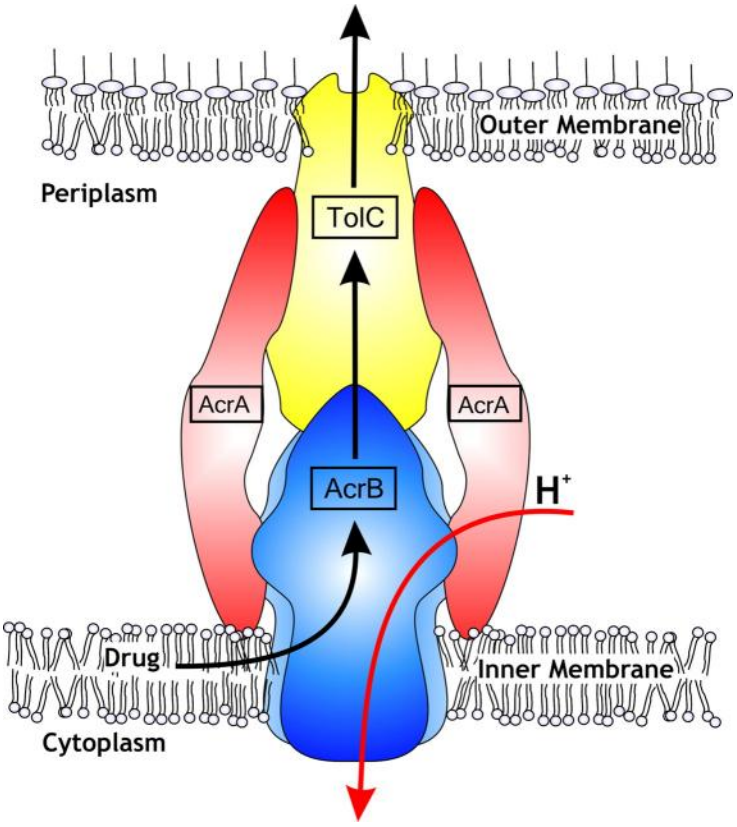
Lab 3. Bacterial Growth and Drug Resistance. Background Reading: S&F Chapters 4, 5

Bacteria evolved to grow at various ranges of environmental conditions, such as temperature, salinity, and pH. Other conditions include the concentrations of organic nutrients and organic antimicrobial agents (antibiotics) produced by competing bacteria. Bacteria make special protein complexes to adapt to these conditions.

E. coli bacteria synthesize protein complexes that export antibiotic drugs, such as TolC-AcrA-AcrB. The drug export is driven by import of H^+. This kind of transport is called **antiport** (exchange of one solute for another).



TolC-AcrA-AcrB drug efflux pump.
Klaas Pos et al. 2009. PNAS 106:6893

What happens to bacteria that lack part of the TolC complex, and therefore lack this means of exporting antibiotics?

Do they also lack the H^+ influx capability?

Or might H^+ influx occur without regulation?

What would be the effect on bacterial growth in acid or base?

We will address the questions about pH dependence and TolC by these experiments:

(1) Perform growth curves of *E. coli* W3110 compared to strain W3110 *tolC*::kanR, in medium at pH values from pH 4.5 - pH 9.0.

(2) Streak strains W3110 and W3110 *tolC*::kanR on plates containing antimicrobial agents: MacConkey agar (contains bile salts, which the TolC pump exports) and Kanamycin plates (plates containing an antibiotic that only KanR mutants grow on).

The Spectramax microplate spectrophotometer is used to measure optical density of bacterial cultures. The class will obtain data together, using a shared microplate. Then the data are exported into Excel and combined in one table. Students work together to complete the table, **but each student generates graphs on their own.**

Procedure.

1. Overnight cultures of bacteria strains have been grown in unbuffered LBK broth. Obtain one MacConkey plate, one TSA plate (tryptic soy agar), and one TSA Kan plate. With a marker, divide each plate down the middle. Streak strains W3110 and W3110 *tolC* on the two halves of each plate.

2. Each student dilutes ONE of the two strains at ONE pH value. Obtain 1 dilution tube containing 5 ml LBK buffered at a particular pH. Different pH values require different buffers. (Why? Think about possible effects of buffer on your experiment.)
   If you get done early, do another strain-buffer combination, until the class plate is full.

3. Inoculate your LBK tube with 50 µl of overnight culture. For the class as a whole, we need to make sure that there is one tube from each strain for each of the pH values.

4. Measure 200 µl samples in triplicate for your particular combination of bacteria (W3110 or W3110 *tolC*) and pH. As each sample is measured, place it in a well of the Spectramax plate.

Spectramax Plate: Loading Positions

(pH values across) **Rows A, B, C: W3110** **Rows F, G, H: W3110 *tolC***

	1	2	3	4	5	6	7	8	9	10	11	12
A	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**		
B	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**		
C	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**		
D	**Blank**	**Blank**	**Blank**	**Blank**	**Blank**	**Blank**	**Blank**	**Blank**	**Blank**	**Blank**		
E												
F	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**		
G	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**		
H	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**		

5. When all the plates are loaded, the Spectramax plate reader will read the OD600 (optical density at 600nm wavelength) for all samples at 37ºC every 15 minutes for 6 hours.
   During the lab period, we will discuss Excel calculations.
6. On Friday, check your plates. Which bacteria grew on which plates? What do you conclude? Obtain class data and think ahead about analysis.

7. **The following week Thursday: Analyze your data.** Determine where (if any) you see log phase growth. Plot your growth curves in Excel to obtain slope and k (gen/hour). Note any evidence of lag phase or stationary phase. Explain evidence in your report.
8. Use your data to determine:
   (A) The dependence of log-phase growth rate on pH, for both strains.
   (B) The effect (if any) of the *tolC* mutation on the pH dependence of growth.
9. In your discussion, speculate on how the TolC complex might interact with pH homeostasis.

Calculations

We will discuss Excel calculations, using the sample data sheets linked to your syllabus.

Use your own PC, or one of the department PCs. The data reduction involves:

(1) Rearrange all data from plate reads into columns as a function of time.

(2) Plot all growth curves from W3110 and *tolC*::kanR strains in one graph together. Do you observe any interesting trends? Select subgroups of growth curves to plot together, to clarify interesting connections. Decide whether to plot average curves for replicates; or individual replicate curves. Defend your decision (under Discussion). Discuss other aspects of the curves, as noted in original handout.

(3) Select portions of curve that appear to be logarithmic. Convert data to log2 values and obtain best fit line. Calculate growth rates.

(4) Plot growth rates as a function of pH. Plot the pH dependence for growth rate of strains W3110 and *tolC*::kanR.

For the second Thursday:

Use the lab period to <u>finish all calculations</u>. Do not leave until you have all your graphs through Step 4.

We will also address the question: Does the *TolC* complex help *E. coli* resist a number of different antibiotic substances, besides the bile salts of MacConkey agar?

Test the ability of the *tolC*::kanR defective strain to grow in the presence of various antibiotics.

1. Obtain two TSA plates. Mark the bottom of each plate to divide into 8 sectors. Spread one plate with 100 µl strain W3110, and the other plate with 100 µl strain *tolC*::kanR.

2. Use sterile forceps to place a blank control BBL disk at the center of each plate. What do you expect to observe with bacterial growth over the plate?

3. Use dispenser to dispense one disk of each type antibiotic onto the surface of each bacteria-spread plate. The antibiotics are: chloramphenicol (30 µg), erythromycin (15 µg), kanamycin (30 µg), neomycin (30 µg), novobiocin (30 µg), penicillin G (10 standard units), streptomycin (10 µg), tetracycline (30 µg). Incubate plates overnight at 37°C.

On Friday, remove your plates and store in the cold room. Measure the diameters of clearing, in millimeters. Interpret all your results for your report. Propose explanations for any differences observed between the two strains.

References

Vassilis Koronakis, Jeyanthy Eswaran, and Colin Hughes. 2004. Structure and function of TolC: The bacterial exit duct for proteins and drugs. Annu. Rev. Biochem. **73:**467–489.

Kari N. W. Deininger, Akina Horikawa, Ryan D. Kitko, Ryoko Tatsumi, J. Lee Rosner, Masaaki Wachi, and Joan L. Slonczewski . 2011. A Requirement of TolC and MDR efflux pumps for acid adaptation and GadAB induction in *Escherichia coli*. PLoS ONE **6:**e18960.