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Investigation into potential cross-resistance of Enterococcus faecalis OG1RF induced by adaptation to NaCl and subsequent SDS challenge

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Abstract

The gram-positive, non-spore forming bacteria *Enterococcus faecalis* has been the interest of numerous stress studies due to its ability to survive a range of harsh environments. Its highly adapted membrane replete with specialized proteins enable *E. faecalis* to maintain homeostasis in such conditions as bile salts, low pH, and low oxygen encountered in the human gastrointestinal tract (14). Bile is a bacteriostatic agent because of its amphipathic nature and toxic amino acid moiety when in its conjugated bile acid form. Similarly, the detergent SDS (sodium dodecyl sulfate) exhibits toxic effects on the bacterial membrane of *E. faecalis* and for this reason is a comparable stressor to bile (4). Previous studies have explored the existence of cross-protection by exposure of bile salts stress to test growth in subsequent stress challenges such as heat, bile salts, SDS, and varied pH (11). We predicted that growth in NaCl may confer similar resistance to *E. faecalis* OG1RF when challenged to low concentrations of SDS in rich growth media. To examine the effects of such cross-protection by NaCl, OG1RF was grown with varying concentrations (%w/v) of NaCl then subjected to 0.01% SDS. Dilutions plated at various time points of the challenge revealed that rather than providing cross-protection, growth in salt only served to prolong the death of cells in the SDS challenge.

Introduction

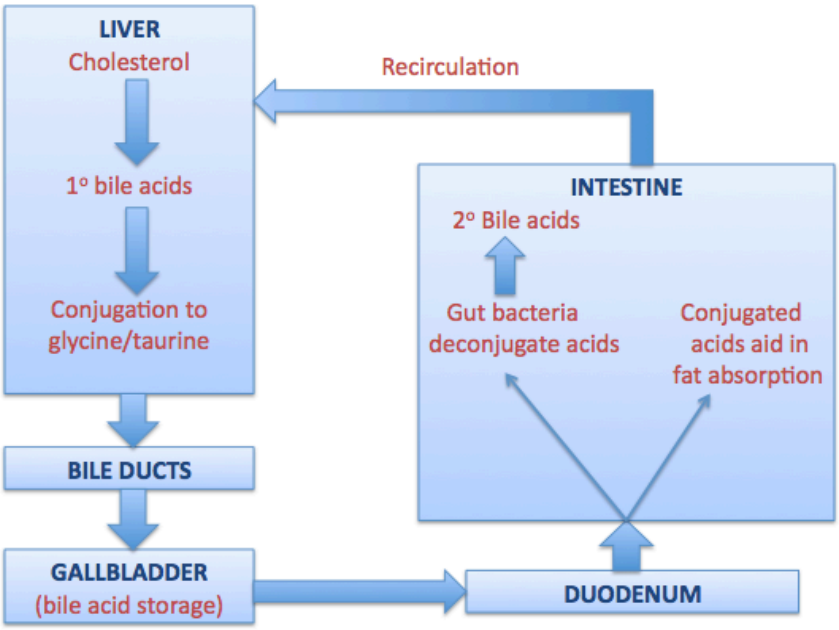
The human gut flora is composed of an amalgam of commensal organisms that have adapted to an environment designed to eliminate unwanted pathogens by competition between normal flora and invading species. An opportunistic pathogen, *E. faecalis* has become one of the leading causes of nosocomial infection in the U.S. (1). This gram-positive, facultative anaerobe demonstrates a remarkable ability to both tolerate and proliferate in high concentrations of bile acid in the human intestine through several adaptation mechanisms. Of particular interest are the resistance mechanisms to bile-induced damage through protein expression of efflux pumps as well bile salt hydrolases necessary for deconjugation of bile acids in the gut (14). The mechanism by which resistance to such surfactants as bile occurs includes up-regulation of various proteins involved in cell membrane structure, osmoregulatory signals, and efflux pumps, which are crucial to inhibiting bile's damaging effects on the cellular membrane and DNA within the cell (10).

Withstanding the environment of bile is a result of modification of bile acids within the intestine, allowing bacteria to resist its damage. Conjugated bile acid, also referred to as bile salts, is synthesized from cholesterol in the liver for later release in the intestine and in this form is able to aid in fat absorption. In the gut, however, bile is further modified by bacteria capable of removing the amino acids (glycine or taurine) to form deconjugated bile (13). In this way, *E. faecalis* adapts its membrane as well as its metabolism for survival in an otherwise bactericidal environment. Another important facet of stress response to bile salts is the osmoregulatory component which explains how hyperosmolarity (i.e. high salt environment) causes certain bacteria to increase intracellular glycine betaine and potassium to maintain proper solute concentrations and turgor pressure (7). In the same study it was found that adaptation of *E. faecalis* ATC

19433 to moderate NaCl osmotic shock conferred both significant tolerance to high concentrations of NaCl (%w/v) as well as cross-protection to 0.017% SDS challenge.

In order to determine if this holds true at less than moderate salt shock (6.5% NaCl, as established by Flahaut (2), varying concentrations of NaCl will be prepared in rich growth media as pre-adaptation conditions to test for the existence of cross-protection against 0.01% SDS detergent over a 24 hr challenge period.

Figure 1. Enterohepatic circulation of bile acids in humans. Synthesis begins in the liver where primary acids are synthesized from cholesterol and then conjugated to either glycine or taurine moiety. After passing through bile ducts, primary bile acids are stored in gallbladder until stimulated by food in stomach. Acid release into duodenum enters small intestine where some work as emulsifiers in fat digestion/absorption while others are deconjugated by gut bacteria to produce secondary bile acids which can re-enter circulation.



Materials and Methods

Bacterial strain used and growth medium

For this experiment, *E. faecalis* strain OG1RF was used and grown in Brain Heart Infusion (Bacto BHI) at 37 ºC without shaking. Adaptation media was prepared using solid NaCl in BHI to create appropriate %w/v conditions.

NaCl adaption

Duplicate overnight inoculums (labeled A and B) of *E. faecalis* OG1RF were prepared and diluted the following day to O.D. 0.01 in 10 ml BHI, 2% NaCl in BHI, and 4% NaCl in BHI, respectively, to create biological replicates. Growth curves were conducted prior to assay indicated time needed to reach mid-log phase for each culture. (6.5% NaCl was included in growth curve initially but due to time constraint, focus remained on effects of salt adaptation at lower concentrations).

SDS Challenge Assay

Once the culture reached mid-log phase (O.D. 0.4-0.6), they were vortexed and 10 µl of cells were placed into respective "0" time point labeled Eppendorf tubes. 2 ml of cell culture were placed into snap-cap tubes and spun down at 3500 rpm in 4ºC for 10 minutes. During this time, each "0" time point sample was diluted across in a 96-well plate filled with 90 µl 1x PBS solution. 5 µl of each dilution were plated using a multichannel pipette onto a BHI agar plate. After centrifugation, samples were decanted carefully so as not to disturb cell pellet, resuspended in 2 ml 0.01% SDS + BHI, and vigorously vortexed to re-dissolve pellet. Samples were incubated at 37ºC for 4 hours with time points taken every 15 minutes for the first hour, and then at 2 and 4 hours. After 4 hours, the remaining cultures were left to incubate until 20-24 hours later and again diluted and plated. Four trials of this assay to the 4 hour time point were conducted.

Initial assays with NaCl growth were conducted to 4 hour challenge in SDS, but with slightly inconsistent results, so the assays were conducted to 24 hour challenge in order to elucidate if cells were viable beyond a certain time or if SDS was prolonging cell death in the plate dilutions showing greater cell growth.

Results

Growth in each condition and generation times

For BHI, 2% NaCl, and 4% NaCl a growth curve was conducted to calculate the amount of time needed for each culture to reach mid-log phase. A minimum of at least two timepoints were taken between O.D. 0.3 – 0.7 so that generation times and standard deviation could be calculated (values based on Figure 3 growth curve). In BHI, cells doubled at an average of 40.2 minutes, 2% NaCl grown cells were an average of 37.8 minutes and 4% NaCl were similar with 37.4 minute doubling time.

	BHI		**2% NaCl**		**4% NaCl**	
Culture	**A1**	**B1**	**A2**	**B2**	**A3**	**B3**
b_2/b_1	1.67055394	1.6953125	1.3040201	1.33477322	1.39066339	1.27459954
$3.3*LOG(b_2/b_1)$	0.73543966	0.75652422	0.38043814	0.4138447	0.47263267	0.3477334
Generation time (min)	**40.79193**	**39.65504**	**39.42822**	**36.24548**	**31.73712**	**43.13649**
Avg Gen. time	40.2234841		37.8368519		37.4368074	
Std Dev	0.80389815		2.25053876		8.0605748	

Growth in BHI, 2% NaCl and 4% NaCl with 1 hour 0.01% SDS Challenge
After growing cell cultures (duplicates A and B for each medium) in BHI, 2% NaCl, and 4% NaCl to mid-log phase respectively, cells were challenged to 1 hour growth in 0.01% SDS in BHI and dilutions were plated at 0, 15, 30 and 60 minutes. Four initial assays using this method were conducted and showed three of the four having little to no consistency within duplicates for each medium. Some cross-protection was seen in 2% NaCl plates by higher viable cell counts than those in the control or 4% NaCl, as shown in Figure 4.

Growth in BHI, 4% NaCl, and 5% NaCl with 1 hour 0.01% SDS Challenge
The same assay was also conducted using BHI, 4% and 5% NaCl to determine if the prior salt concentrations (2% and 4%) were simply too low to have any sort of cross-protective effect. Of four assays, two conditions showed similar results of cross-protection. This was seen by higher viable cell counts in 4% NaCl over those in the control and 5% NaCl, shown in Figures 5 and 6.

Growth in NaCl with 4 hour 0.01% SDS Challenge
Having seen a difference in viable cell counts from the assays with cells grown in 4% NaCl, 5% NaCl, and BHI control, the 2% and 4% NaCl assays were revisited with the modification of a 4 hour challenge period. This was done to determine at which time point cells cross-protection, if any, was effective until. The same methods were used to grow cells in their respective salt media until mid-log, and then challenged to 0.01% SDS. The time points plated were at 0, 15, 30, and 60 minutes (for a short-term perspective of viable cell count) and then 0, 60, 120, and 240 minutes (for a long-term perspective). Of four assays conducted to the 4 hour time point, the first assay shown in Figure 7 revealed slightly higher viable cell counts in 2% NaCl over those of the control and 4% NaCl, but subsequent assays were inconsistent.

Viable cells after 4 hour SDS Challenge plated after 24 hours
Each of these 4 hour challenges were also subject to continued incubation for 20-24 hour to determine if salt adaptation only prolonged death. The results of each of the four respective 4 hour assays was allowed to grow for 24 hours and plated in dilutions. The results showed no consistency, as some experienced completed cell death in all media while others showed complete growth. Two of these assays are seen 24 hours later in Figure 8a (no growth in 4% NaCl) and 8b (growth in all media).

Discussion

Although the results of OG1RF growth in varied %w/v NaCl + BHI media did not show consistency, the viable cell counts following SDS challenge revealed that cells did not completely experience death upon exposure. While salt and SDS detergent are chemically

two different substances (one affects osmolarity while the other disrupts membrane due to its amphipathic nature), the growth of cells in salt first seemed to induce some change that prevented the harsh effects of SDS from completely destroying the bacterial membrane. Hyperosmolarity in *E. faecalis* induces an increase in potassium and glycine betaine (7) which prevent a sudden significant water loss which would lead to fallen turgor pressure and eventual cell death. This mechanism is not unique to *E. faecalis,* however. The gram positive *Bacillus subtilis* utilizes a similar means to handle high salt concentrations. By synthesizing proline and using the already present glycine betaine, it is able to maintain water pressure and prevent desiccation (**5**). It should be noted that *B. subtilis* is normally found in soil and is capable of forming endospores (which confer protection through an entirely different means), however, its response to salt stress suggests that this is a common mechanism among other gram positive organisms.

Studies that have measured which proteins are upregulated and downregulated in response to bile reveal that in the gram positive organism *L. johnsonii,* proteins involved in cell division are diminished whereas those involved in metabolism had increased **(8**). In high stress conditions of bile salts where the cell membrane is directly exposed to harsh agents (detergents capable of phospholipid disruption), bacterial cells conserve their energy towards metabolic functions. In a similar study measuring transcriptional responses of *E. faecalis* to SDS and bile (14), it was also found that genes necessary for energy metabolism were also upregulated. The above findings taken into consideration with the results of our study suggest that the bacterial response to detergents is sufficient for cells to survive for some time under a bile-like stress condition, but not permanently. The changes associated with the cell membrane as a response to NaCl (not amphipathic, therefore not as deleterious to phospholipid bilayer) were just enough to slow down the damage induced by SDS.

While these results suggest that variables such as O.D. of cells harvested, consistency in pipetting dilutions (i.e. same number of up-down pipetting each time) and media preparation (precision in measuring exact %w/v NaCl) may have influenced the ability of the salt growth to induce cross-protection, it may also suggest that cross-protection does not exist at such low levels of salt. Although some protection was seen in at most, 50% of the assays conducted (as in the 4% and 5% salt adaptation to 1 hour SDS challenge), was not conclusive evidence to state that 4% NaCl induced cross-protection to *E. faecalis* once subjected to SDS. This is not to say that salt growth is not altering membrane proteins, because it is clear that adapted cells sustained some viability in the SDS challenge beyond 1 hour which suggests that the bacteria is upregulating specific functions that prevent desiccation by osmotic shock and enable growth in the presence of membranolytic detergent.

As well, it should be noted that strain specificity may have significance in the adaption mechanism of *E. faecalis* OG1RF to stress conditions (14). Differences in protein expression of *E. faecalis* ATCC 19433 versus OG1RF may explain how in former studies certain strains displayed tolerance while the later did not in this experiment. It would be beneficial to test the effect of 6.5% NaCl adaptation to determine if perhaps 2-5% NaCl was too low to initiate significant protein modification and thus show tolerance in SDS

over the control culture in BHI. The importance of such investigation would indicate whether or not *E. faecalis* can gain the advantage of adaptation if exposed to high stress conditions before introduction into the human body, where colonization may lead to infection (2).

Figure Legends

Figure 2. As noted in methods, initial growth curve was conducted prior to assays in three salt media and a control. Although *E. faecalis* growth in BHI + 6.5% NaCl was not used in final assay challenges, preliminary growth curve is shown below. Biological duplicates for each media were created by two overnight cultures of OG1RF diluted to O.D. 0.01 in BHI (A1, B1), BHI + 2% NaCl (A2, B2), BHI + 4% NaCl (A3, B3), and BHI + 6.5% NaCl (A4, B4). Cell growth measured every hour.

Figure 3. Growth curve conducted using only two salt media and a control is shown below using same procedure as above. Curves represent the following cultures: BHI (A1, B1), BHI + 2% NaCl (A2, B2), BHI + 4% NaCl (A3, B3) monitored every hour for the first three hours, then every thirty minutes until each respective medium neared stationary phase.

Figure 4. OG1RF growth in BHI (A1, B1), 2% NaCl (A2, B2), 4% NaCl (A3, B3) subjected to 1 hour SDS challenge. Assay results plated for each duplicate at four time points- 0, 15, 30 and 60 minutes. Cross-protection seen by higher viable cell count in 2% NaCl growth.

Figure 5. *First assay with 4% and 5% NaCl*. OG1RF growth in BHI (A1, B1), 4% NaCl (A2, B2), 5% NaCl (A3, B3) subjected to 1 hour SDS challenge. Time points plated at 0, 15, 30 and 60 minutes. Cross-protection seen by higher viable cell count in 4% NaCl growth.

Figure 6. *Second assay of 4% and 5% NaCl*. OG1RF growth in BHI (A1, B1), 4% NaCl (A2, B2), 5% NaCl (A3, B3) subjected to 1 hour SDS challenge. Time points plated at 0, 15, 30 and 60 minutes. Similar cross-protection to assay in Figure 5 seen by higher viable cell count in 4% NaCl growth.

Figure 7. OG1RF growth in BHI (A1, B1), 2% NaCl (A2, B2), 4% NaCl (A3, B3) subjected to 4 hour SDS challenge. Time points shown at 0, 60, 120, and 240 minutes. Slightly higher viable cell count seen in 2% NaCl.

Figure 8a. *First 4 hour SDS Challenge assay plated at 24 hour time point*. Left plate: A1 (BHI), A2 (2% NaCl), A3 (4% NaCl) from same overnight culture. Right plate: B1(BHI), B2(2% NaCl), B3 (4% NaCl). No growth in 4% NaCl media subjected to challenge.
b. *Fourth 4 hour SDS Challenge assay plated at 24 hour time point*. Sample labeling same as above. Growth seen in all media subjected to challenge.

Figure 2.

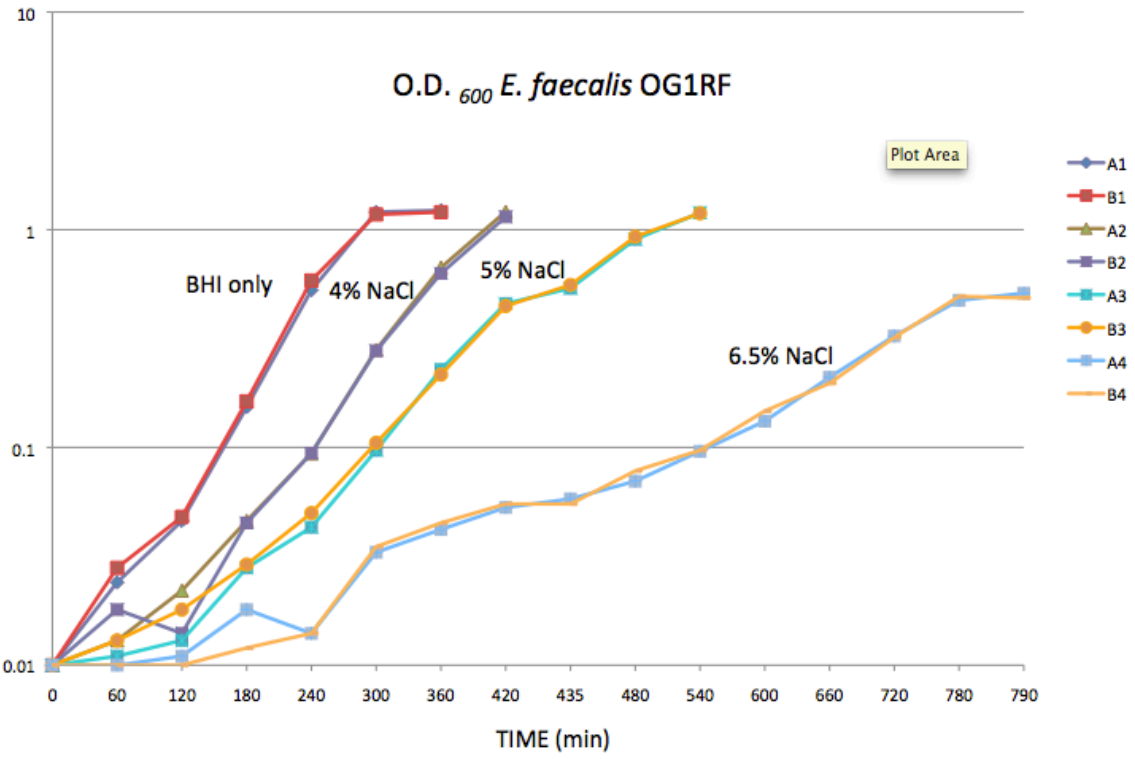


Figure 3.

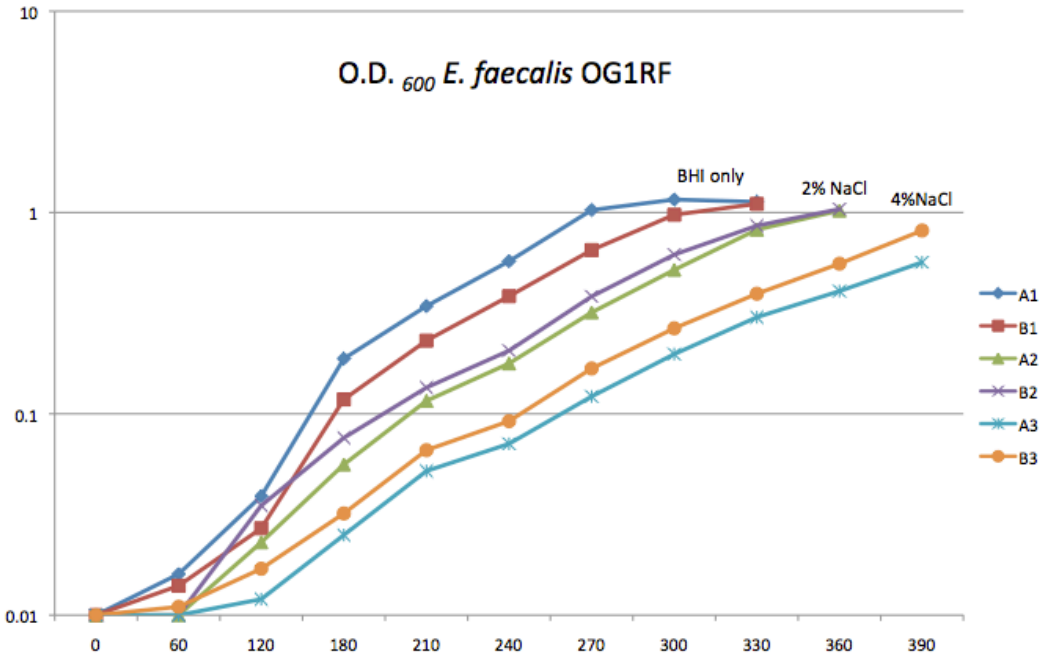
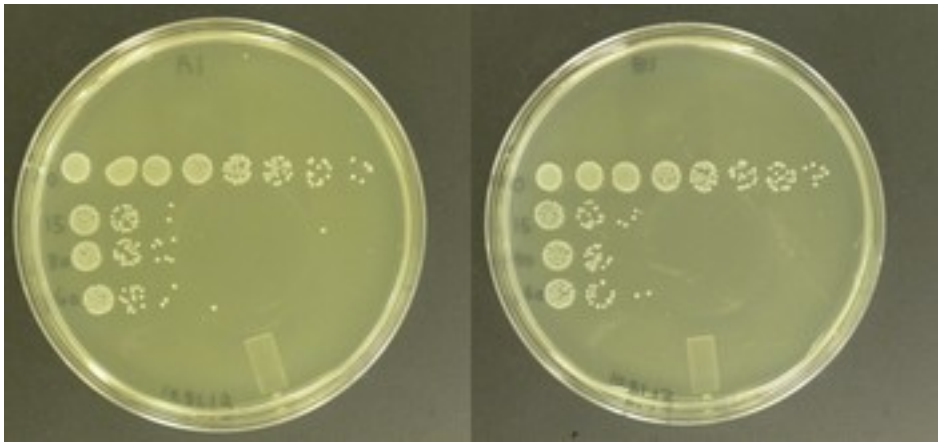
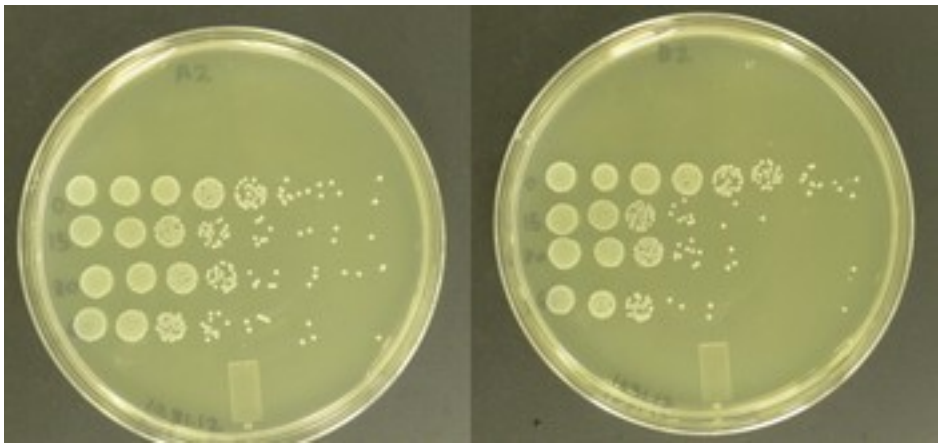


Figure 4: BHI, 2% NaCl, 4% NaCl to 1 hr SDS challenge assay

a.



b.



c.

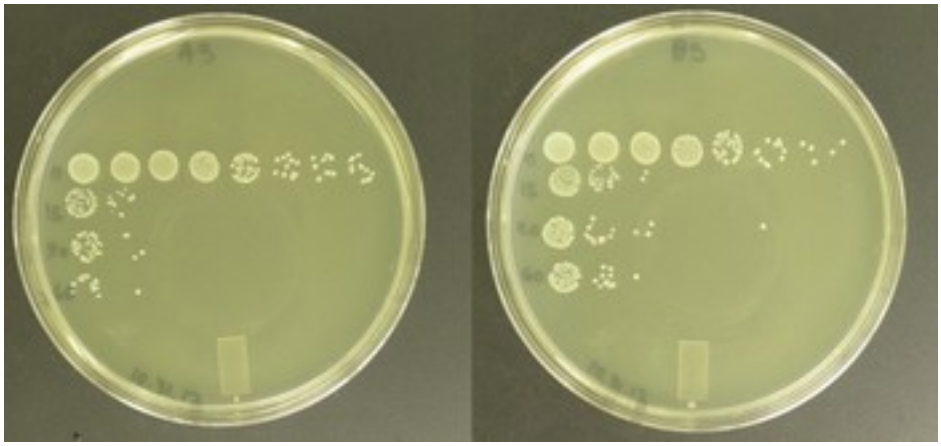
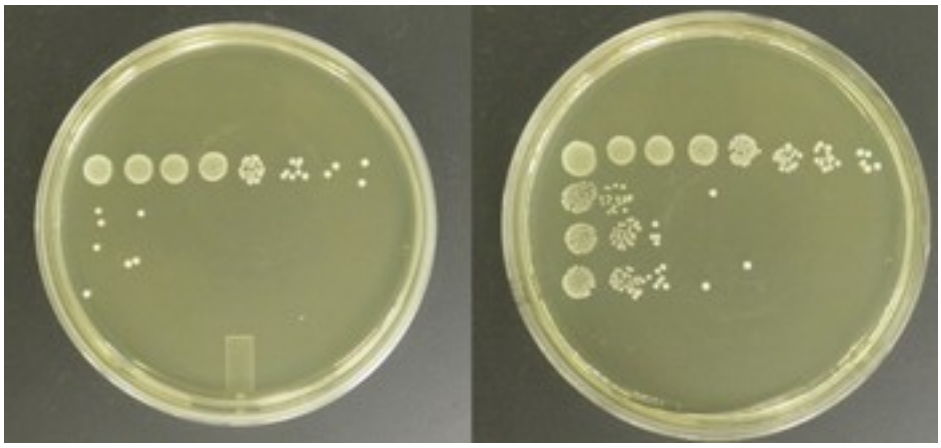
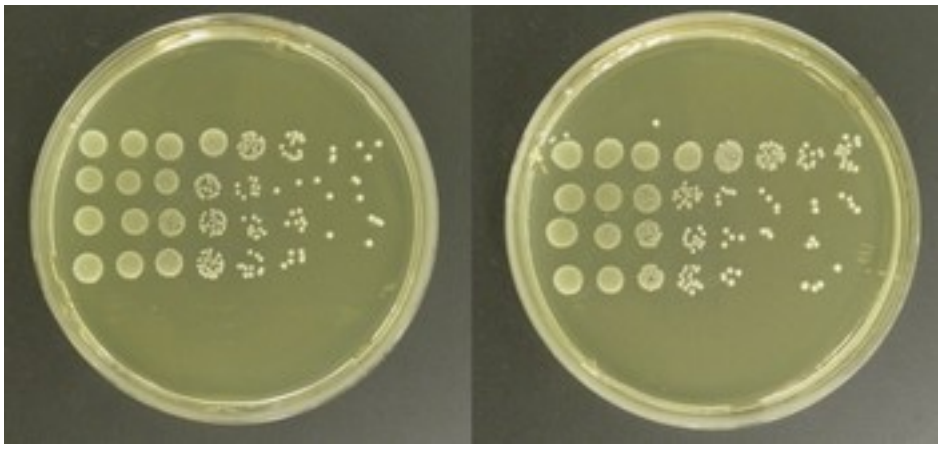


Figure 5: BHI, 4% NaCl, 5% NaCl to 1 hour challenge assay

a. A1, B1 left to right (BHI)



b. A2, B2 (4% NaCl)



c. A3, B3 (5% NaCl)

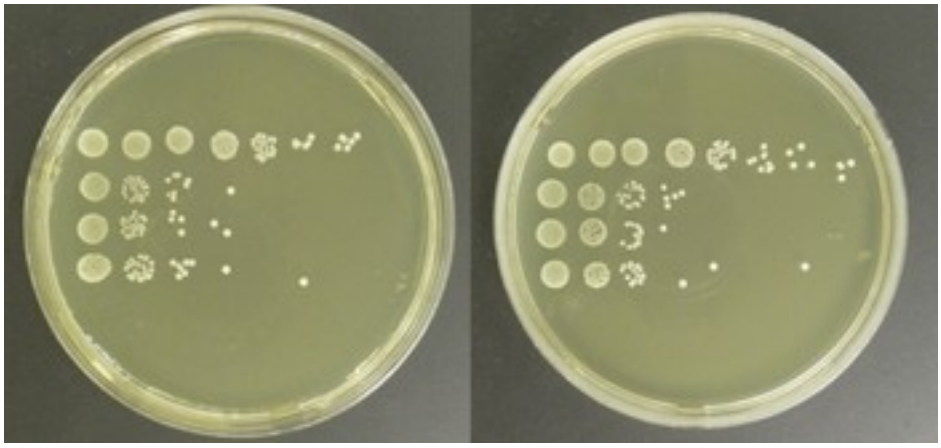
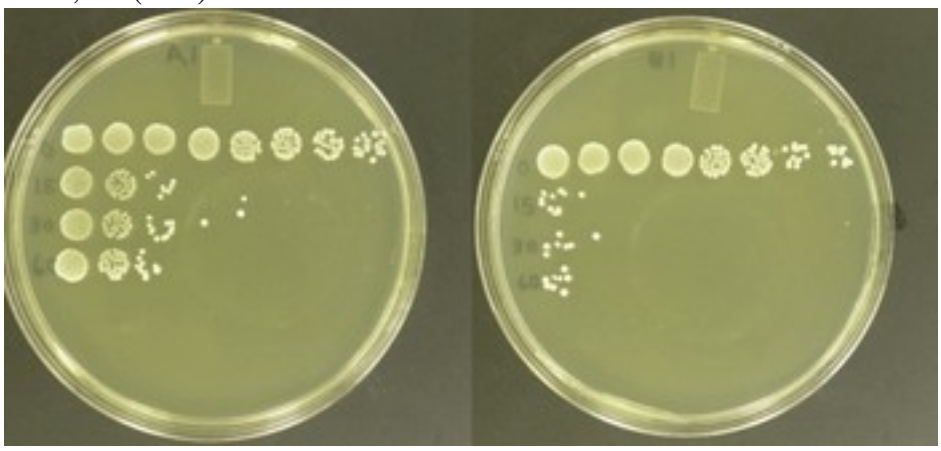
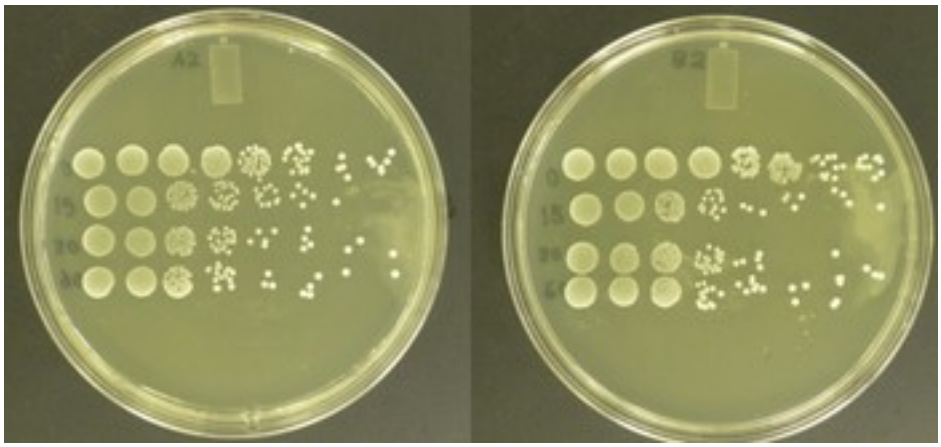


Figure 6: (a) BHI **(b)** 4% NaCl **(c)** 5% NaCl to 1 hour challenge assay

a. A1, B1 (BHI)



b. A2, B2 (4% NaCl)



c. A3, B3 (5% NaCl)

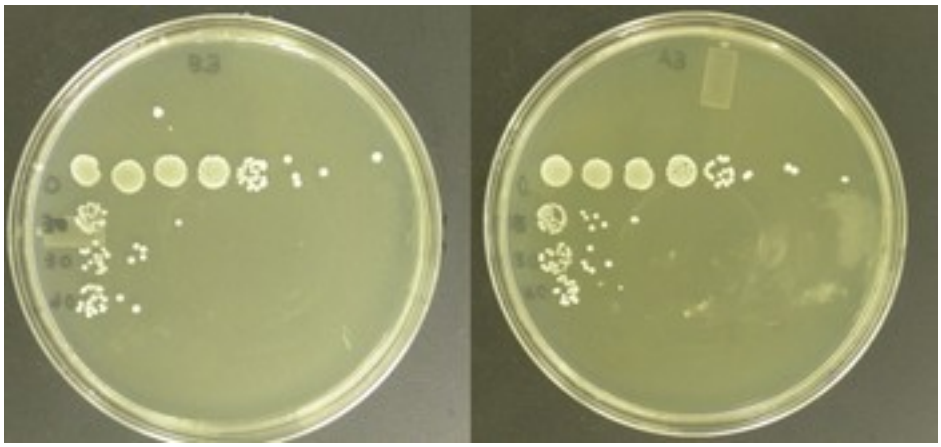
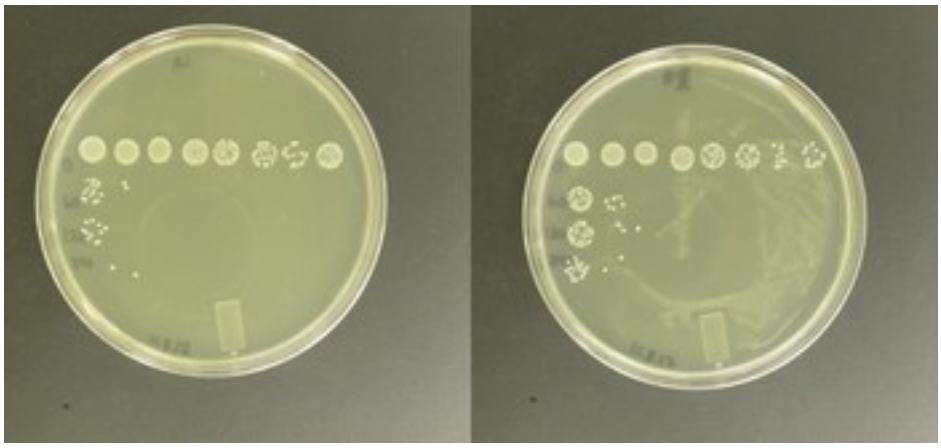
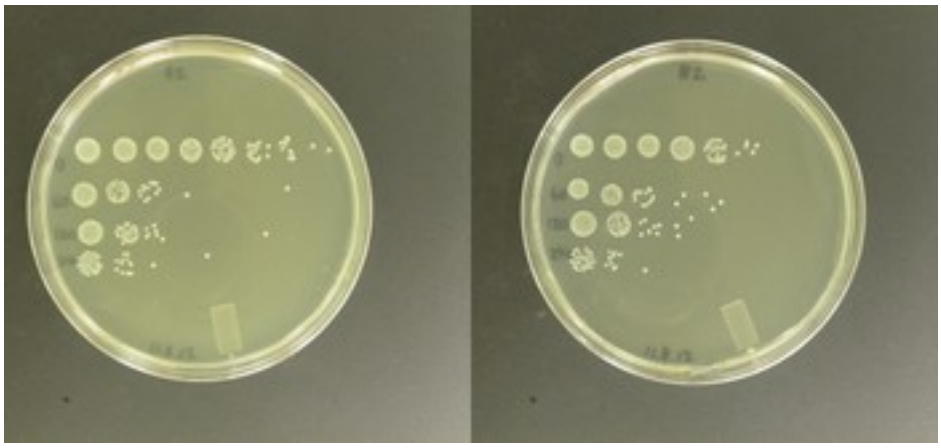


Figure 7: BHI, 2% NaCl, and 4% NaCl to 4 hour challenge assay

a. A1, B1 (BHI)



b. A2, B2 (2% NaCl)



c. A3, B3 (4% NaCl)

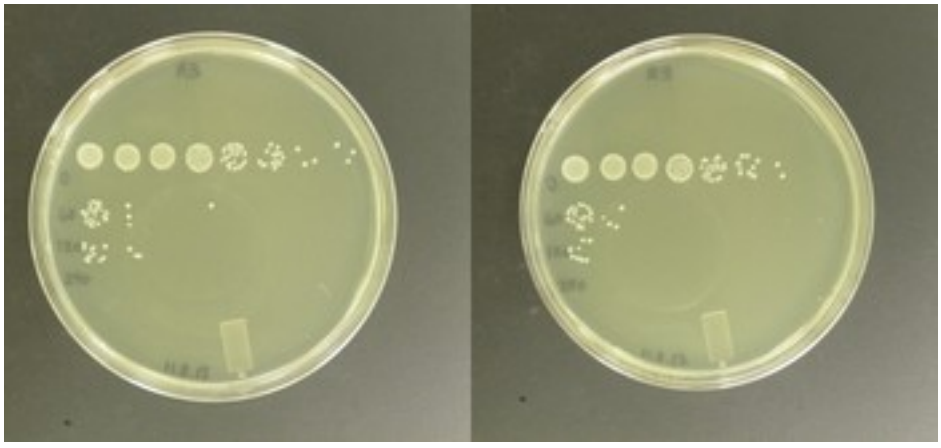
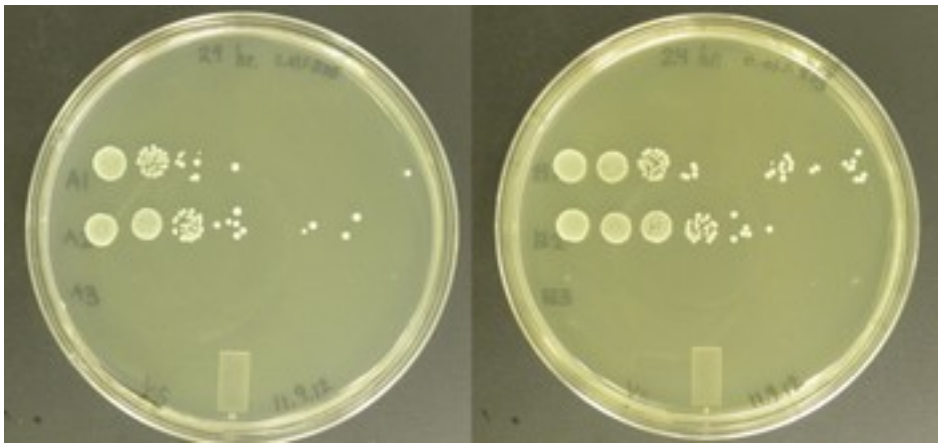
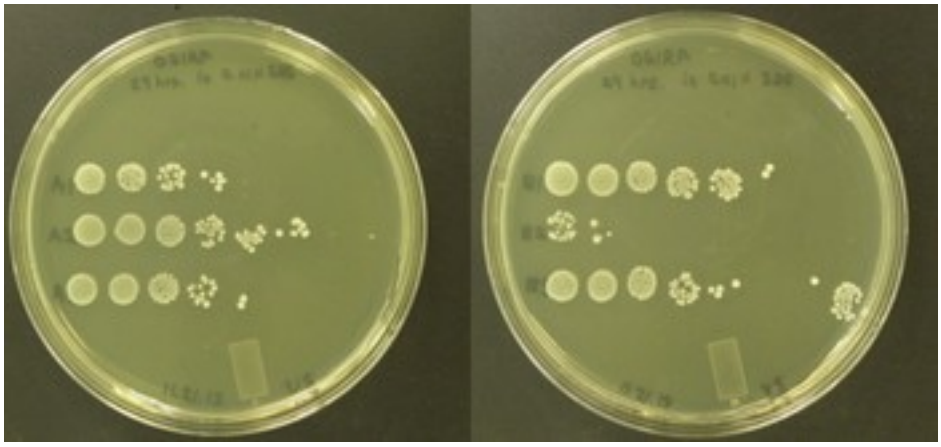


Figure 8: 24 hour SDS Challenge

a. First 4 hour assay seen 24 hours later



b. Fourth 4 hour assay seen 24 hours later



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