Different RsbR Paralogs in Bacillus subtilis Affect Cell Viability When Exposed to Environmental Stress

Abstract

Every living organism must find a way to survive stressful situations and bacteria are no different. Bacteria deal with many kinds of stress, including energy stress like starvation or ATP depletion and environmental stress like acids, alcohols, salts, or lack of oxygen. *Bacillus subtilis* is a model organism that displays a unique stress response involving a large protein complex called a stressosome composed of multiple copies of several different proteins. The RsbR proteins in this stressosome are the stress sensors of the cell and elicit a response through a signal cascade once stress is sensed. There are four different types of RsbR proteins named RsbRA, RsbRB, RsbRC, and RsbRD that are present in the wild type (WT) cell. Considered individually, each of these sensors responds slightly differently to incoming stress, but it is not known whether these differences in response pattern affect cell viability. By isolating each RsbR protein into separate strains and competing them against one another under 4% Ethanol and pH 6.25 HCl stress, I have developed a hierarchy that shows which RsbR proteins outcompete the others. In 4% Ethanol, RC>RD~RB>RA>WT. At pH 6.25 (acid stress), RC>WT>RB>RD>RA. All of the stress hierarchies from competition assays show RC outcompeting the other strains, even though the WT was hypothesized to win because it exists in nature. This is most likely because of the slower, but more sustained stress response pattern of RC compared to the other strains.

Introduction

All living things must deal with stress to survive, including bacteria. Common environmental factors that induce bacterial stress include alcohols, salts, acids, and antibiotics. In fact, the ability of bacteria to respond quickly and efficiently to stress greatly affects their chance of survival. Because of this, all bacteria must have a mechanism for dealing with environmental stress. *Bacillus subtilis* is a well-known, gram-positive bacterium that uses a complex called a stressosome to deal with stress. The stressosome is a large protein complex composed of at least 60 proteins which include RsbR (R), RsbS (S), and RsbT (T) proteins. A given wild-type cell has 10-20 stressosomes in its cytoplasm (Marles-Wright, et al. 2008), and each stressosome is thought to contain a different mixture of these proteins. The R proteins make up 40 of these 60 proteins and comprise four different paralogs: RsbRA (RA), RsbRB (RB), RsbRC (RC), and RsbRD (RD), which are the stress sensors of the cell. The T proteins are bound to the stressosome by R and S proteins when the cell is unstressed, causing them to remain inactive. Upon stressor exposure, the R proteins activate the kinase activity of T, causing T to phosphorylate R and S when it is released from the

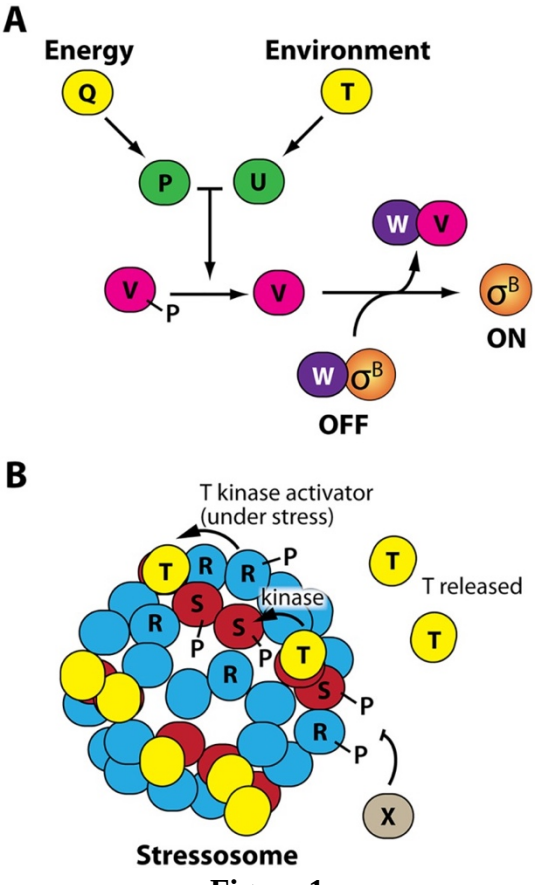

Figure 1

stressosome. The released T can then activate the phosphatase RsbU to dephosphorylate the anti-anti-sigma factor RsbV. RsbV can then bind the anti-sigma factor RsbW, freeing the sigma factor (σ^B) to direct transcription of the general stress response (Hecker, et al. 2007). The stress response is reset when the phosphatase RsbX dephosphorylates R and S and T is recaptured. We know that each of the four RsbR paralogs exhibit their own unique response pattern to an identical input stressor (Cabeen et al. 2017), but we do not know how those differences in patterns make a difference in the survival of the cell. **Figure 2** shows the unique response patterns of each of the R proteins in the general stress response of *B. subtilis* (figure taken from Cabeen et al. 2017). WT and RA share the most similar responses of all the paralogs. Both strains turn on and off quickly and never turn back on. RC takes longer to turn on than RA and WT, but once the stress response is on, it stays on. RB is similar to RC, but the intensity of the response is much lower. RD turns on and off much like RA and WT, but also stays active like RC over time, demonstrating a hybrid response pattern. By looking at each RsbR paralog individually, we can begin to understand whether each paralog has a different effect on survival and whether those effects also differ among stressors. My project is designed to answer this question by detecting differences in the fitness of strains containing only a single RsbR sensor.

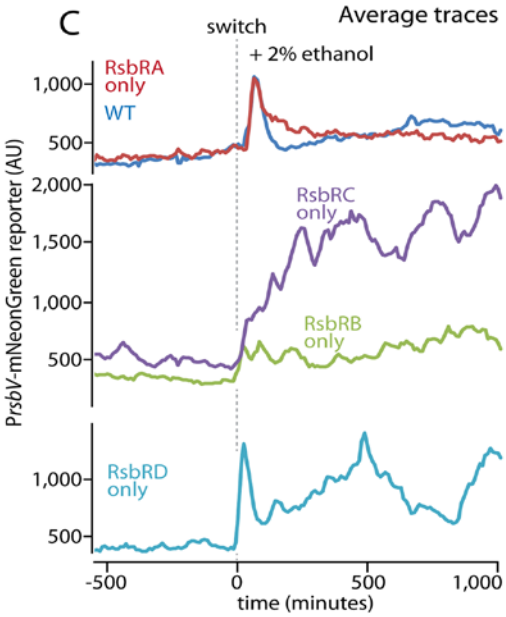

Figure 2

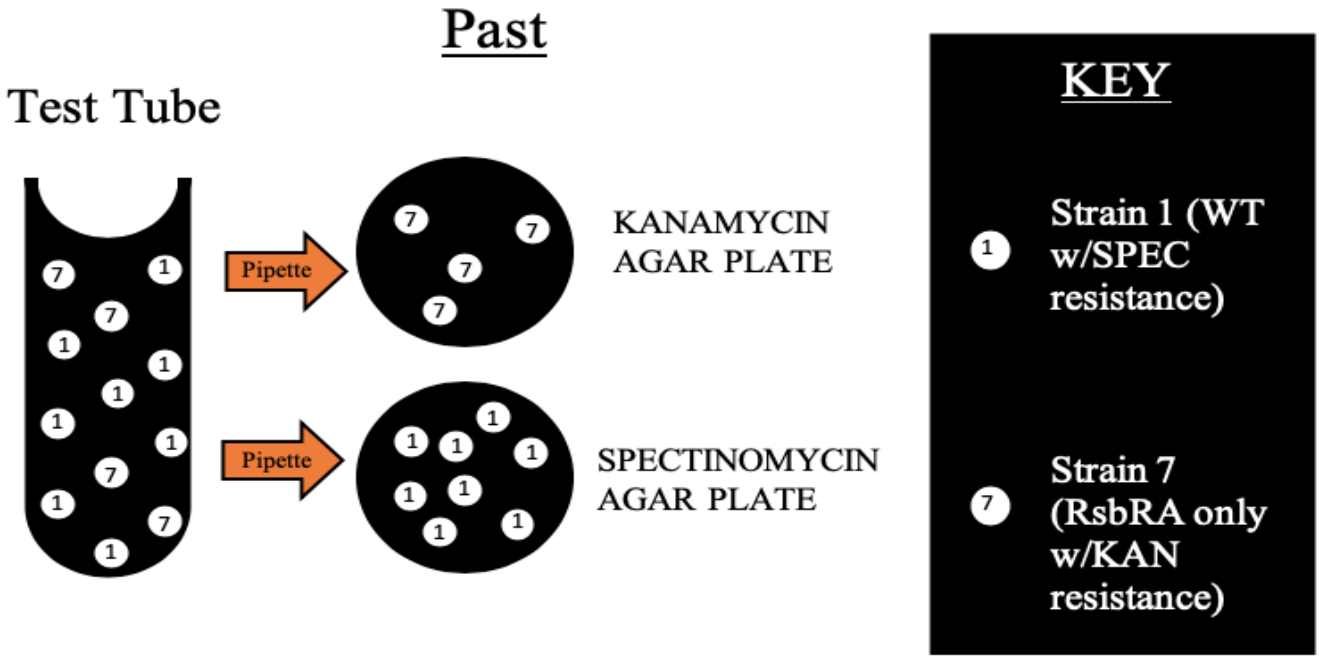

Figure 3

In our past experiments, we have competed every pairwise combination of strains (that contain only one of the four R proteins) against each other using different antibiotic resistance

markers in order to be able to distinguish the two strains on separate plates. Only the strain with the corresponding antibiotic resistance would survive on each plate. For example, in **Figure 3** above, strain 1 (Wild Type (WT) – all four RsbR paralogs) contains an antibiotic resistance marker for spectinomycin and strain 7 (RsbRA only) contains an antibiotic resistance marker for kanamycin. So, when we pipetted equal amounts of culture from the test tube to each plate, only strain 1 survived on the spectinomycin plate and only strain 7 survived on the kanamycin plate. This allowed us to quantitatively view the results of our competition assay by counting the number of colonies on each plate while maintaining the competing strains in exponential phase throughout the experiment. The "winning" strain at each timepoint was defined as producing the most colonies. After allowing the strains to grow on the plates overnight, we counted all of the

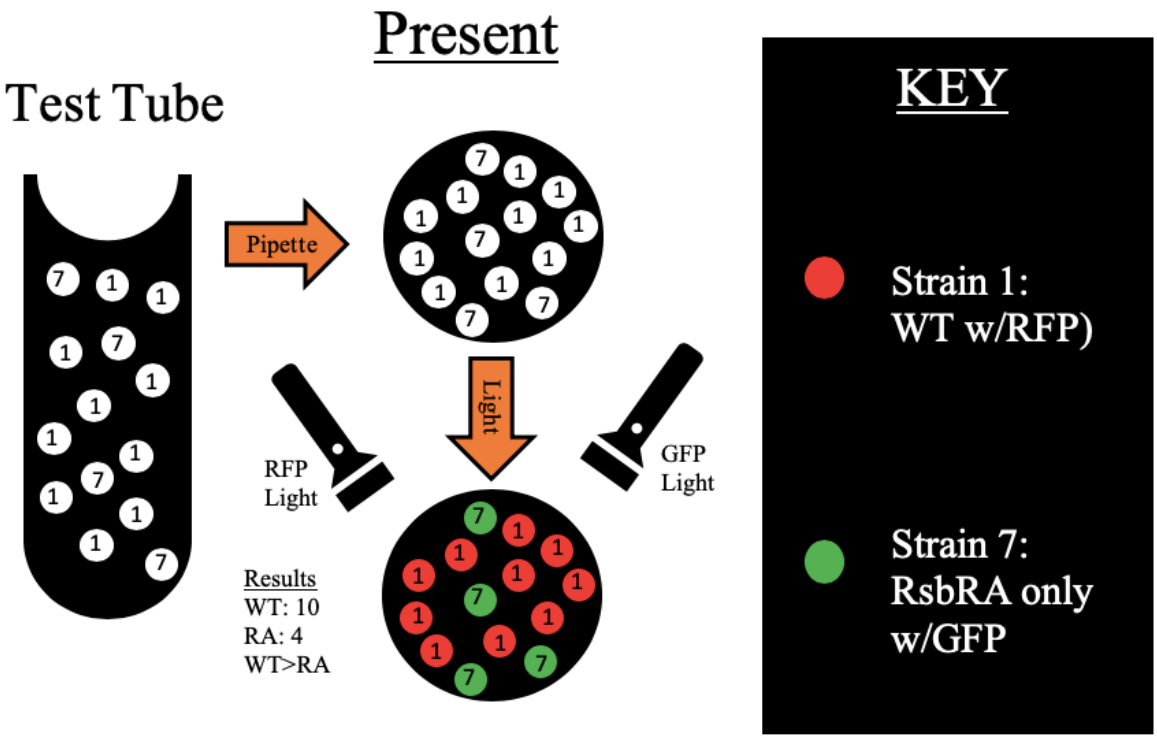

Figure 4

plates at each timepoint to see which strain "beat" the other by the end of the experiment.

As shown in **Figure 4**, we are currently using a fluorescence technique instead of antibiotic resistance to differentiate the strains from each other. In collaboration with my mentor, we developed a new assay that allows us to assess each competition assay on only one agar plate. This is important because pipetting once onto one plate removes the possibility of error from pipetting slightly different amounts onto each of two plates. It will also reduce the time spent counting colonies so more time can be allocated towards performing experiments and analyzing data. This new assay will allow us to continue our previous work much more efficiently because we do not have to pour as many plates, which means less time and money will be spent preparing materials.

I hypothesized that the WT strain would beat out all of the other R strains because there must be some advantage to having all four paralogs otherwise the WT would not be found in the natural environment.

Methods

Antibiotic Resistance

We engineered 10 strains: 5 with spectinomycin resistance (SpecR) and 5 with kanamycin resistance (KanR). The 5 strains included a WT, RA-only, RB-only, RC-only, and RD-only strain, with the WT containing RA, RB, RC, and RD. We competed any pairwise combination of a SpecR strain and a KanR strain in the presence of either 2% Ethanol or pH 6.25 HCl. Both strains were grown on agar plates and then inoculated in a LB broth overnight. We diluted the strains in the morning and let them grow until they were in exponential phase ($0.1 < OD_{600} < 0.5$ from the spectrophotometer). Once in exponential phase, we took the ODs of both strains and diluted them to 0.1 in a 250mL Erlenmeyer flask containing 25mL of sterile LB broth. Then, we placed the flask in a shaking incubator for 60 seconds before removing it and taking a 1mL aliquot for the first timepoint (T_0=0hrs) and replacing the flask in the shaking incubator until the next timepoint. We took 500uL of the aliquot to use in the spectrophotometer to get a starting OD. Then, we diluted the aliquot by a factor of 10, 100, and 1000 into separate microcentrifuge tubes. We plated two 100uL samples of each of these dilutions onto both a Kanamycin agar plate and a Spectinomycin agar plate, for a total of six plates, so only one of the strains would survive on each plate. We used glass beads to spread the sample around on the plate and then incubated the plates in a 37°C incubator for 24 hrs. We repeated these steps for 0.5 hr, 1 hr, 2 hr, 4 hr, and 24 hr timepoints. At each timepoint (besides T_0 and T_{24}) after taking the to-be-plated aliquot, we diluted another aliquot into a new 250mL Erlenmeyer with 25mL sterile LB to 0.1 OD in order to keep the culture in exponential phase for the duration of the experiment. This new flask was placed in the shaking incubator and used until the next timepoint and the old flask was discarded. After the colonies grew, we counted each plate to determine the "winner" of the competition based on fitness levels. Before starting the competitions between different strains, we first competed all of the same strains against each other with different opposite antibiotic resistances (in the absence of a stressor) in order to confirm that there was no intrinsic advantage of one strain over the other. Observing equal amounts of colonies during these control experiments was necessary for ruling out any intrinsic bias between the strains.

Dilution Key

OD	Dilution
0 or -0	-1
.001	-2
.003	-2
.007	-2
.014	-2
.026	-3
.043	-3
.061	-3 to -4
.067	-3 to -4
.088	-4
.146	-4
.407	-4
.45	-4

Figure 5

Fluorescence

Each of the 10 strains was engineered with a chloramphenicol antibiotic resistance (ChlorR) and 5 of them contained GFP while the other 5 contained RFP. We competed any pairwise combination of the GFP and RFP strains in 25mL of sterile LBK pH=6.25 in a 250mL Erlenmeyer flask. Both strains were initially grown overnight and diluted in the morning until the spectrophotometer showed they were in exponential phase. Then both strains were diluted into 25mL LBK pH=6.5 at 0.1 OD. The T_0 1mL aliquot was taken after 60 seconds in the shaking incubator. After the aliquot was removed, we added 12uL of 37% HCl to the flask to

shock the strains into acid stress before replacing it in the shaking incubator. Samples for each timepoint were all diluted to the same dilution based on the dilution key shown in **Figure 5** and plated onto three agar plates containing chloramphenicol. Timepoints were taken at 0, 3, 6, and 9 hrs. After the 3-hr and 6-hr timepoints, we diluted into another pre-made flask containing 25mL LBK pH=6.25 to 0.1 OD in order to keep the cells in a pH=6.25 environment and in exponential phase for the entire experiment. The plates were incubated for 24 hrs at 37°C. In order to count the colonies to determine a "winner", we took pictures (placing either a green light filter or red light filter over the lens of the camera) of each of the plates illuminated by a green-light-only flashlight and a red-light-only flashlight in a dark room. This allowed us to count the number of red and green fluorescent colonies on each picture using the ImageJ program in order to determine a winner.

Results

2% Ethanol Stress

Our results indicate a hierarchy of fitness in 2% ethanol, as follows: RC>RD~RB>RA>WT, meaning that the RC strain demonstrates the most fitness and the WT strain shows the least fitness. As described in the methods, control experiments were performed before starting competition experiments to make sure that there was no innate advantage of having one antibiotic resistance marker in one strain versus the other. The final-timepoint colony

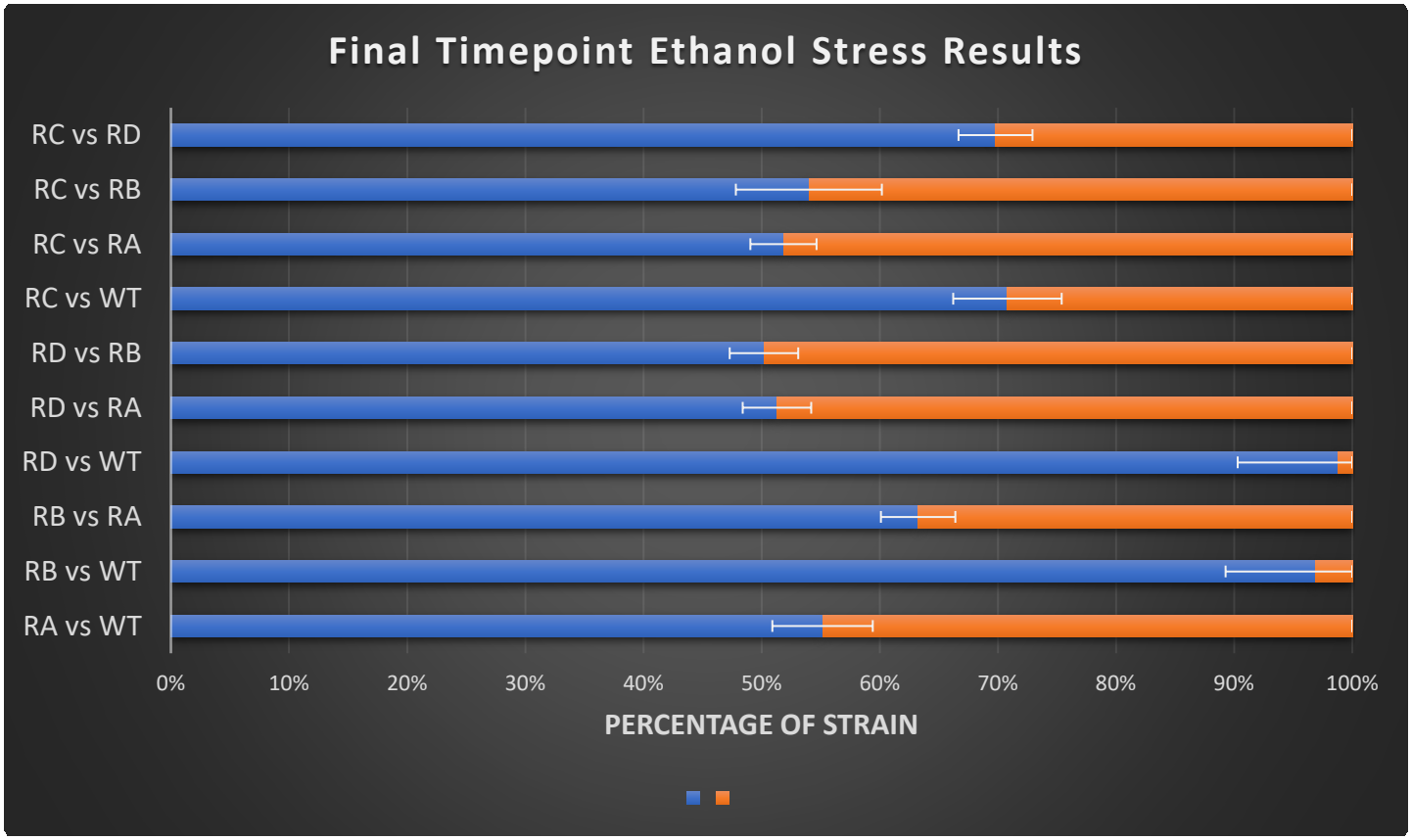

Figure 6

counts for each of the competition assays were converted to percentages and are listed in **Figure 6** below.

pH 6.25 HCl Stress

The hierarchy in pH 6.25 HCl is as follows: RC>WT>RB>RD>RA, showing RC to be on top again, followed closely by the WT strain. As mentioned in the methods, the acid stress assays started with antibiotic resistance markers to differentiate the strains but switched to fluorescence markers RFP and GFP for differentiation. The colonies were counted for each strain and converted into percentages to show which strain won and by how much. These results are shown for the final timepoints in **Figure 7**.

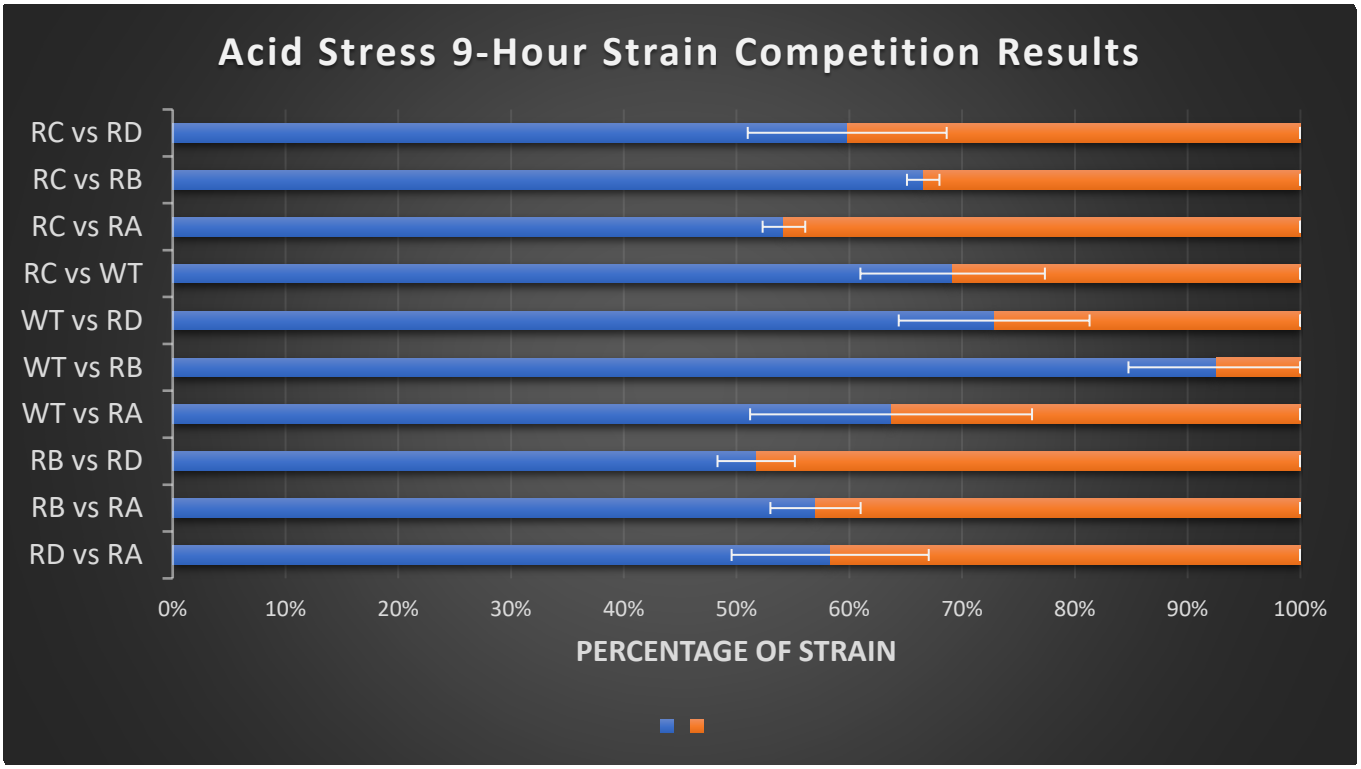

Figure 7

Supporting Documents

Spectinomycin and Kanamycin Antibiotic Controls:
https://benchling.com/s/etr-hodVeUz4YA9pAleIKuGU

Kanamycin and Chloramphenicol Antibiotic Controls:

Kan WT (6) vs Chlor WT (156):
https://benchling.com/s/etr-YFmKskZFJ30mRXiQGSHD

Kan RA (7) vs Chlor RA (157):
https://benchling.com/s/etr-lwppZ0lpTa9X6eRlg9Cu\

Kan RB (8) vs Chlor RB (158):
https://benchling.com/s/etr-jOkzuqQPkSmJ2JZOx8az

Kan RC (9) vs Chlor RC (159):
https://benchling.com/s/etr-W7noAjjrl0zvR58W3OHA

Kan RD (10) vs Chlor RD (160):
https://benchling.com/s/etr-YNC7SfCTJl8kmkH36rID

Discussion

My hypothesis first proved incorrect after finishing the ethanol trials where the WT strain placed last. This is quite interesting because the WT has all four paralogs and theoretically, should be at the greatest advantage to handle any kind of stress. While there is no single reason for WT being outcompeted by the other strains, an attractive hypothesis for the reason that WT exists naturally is that having all four strains provides a broader resistance to many different environmental stressors by sacrificing a more effective output. For example, RC showed the greatest output in both acid and ethanol stress, but there may be other environmental factors that RC would succumb to while the WT could still survive. Another hypothesis is that the strains will all perform differently as the stress is increased. My colleagues have conducted further research on this topic with 4% ethanol and demonstrated the following hierarchy in which RC wins again: RC>RB>RA>WT>RD. Shown again is **Figure 2**, which shows the stress response patterns of each of the paralogs in a microfluidic device. In all of the ethanol assays, the WT and RA strains were very close to each other in colony counts, which was expected because both WT and RA turn on and off quickly and stay off. RC has the most different and interesting response pattern of all of the paralogs. RC turns on gradually but stays on and continues to heighten the stress response as time continues. This is a very plausible explanation for why RC is at the top of all of the hierarchies, meaning that while all of the other strains exhibit initial stress responses of the same magnitude, only RC continues to display and increase the magnitude of that response, so by the last timepoint in the experiment RC was more active than when it started while its competitor had already passed its peak activity time.

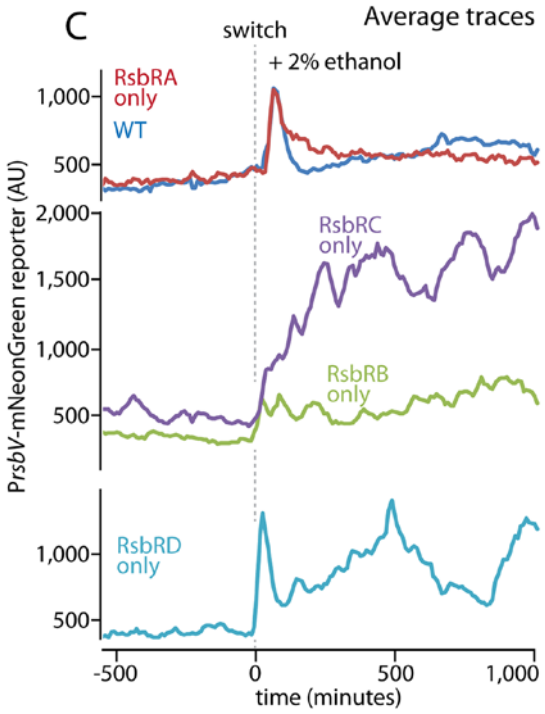

Figure 2

While these response patterns allow certain predictions to be made, the hierarchies we developed do not always agree. In ethanol stress, both RD vs WT and RB vs WT show WT losing by a large amount. This could be because these last timepoint taken in these experiments was at 24hrs which allowed RB and RD to be able to outgrow the wild type for a longer period of time. By the timepoint taken at 4hrs, RB and RD had already outcompeted WT thus confirming that they would still be winners during a 9hr timepoint. In acid stress, WT and RA are very far apart even though they have almost identical responses to stress. Also, the WT strain seems to perform much better in acid stress than in ethanol given that it is second only to RC. It is interesting to see the WT vs RB experiment so lopsided in favor of WT when RB follows right behind WT in the hierarchy. This could be because in the WT vs RB experiment, WT's quick stress response initially dominates RB and RB's low intensity fails to recover. This is further seen in WT vs RD, where RD's hybrid response competes against WT much more effectively. Given this, RD would presumably

dominate RB, but since there competition assays are so similar in acid and ethanol, additional experiments would prove useful to discover the cause of their similarity. In the 4% ethanol hierarchy developed by my colleagues, RD is much lower than expected because RD is seen as a hybrid response of RC and RB, and 2% ethanol and HCl stress show that RB and RD are right next to each other and even next to RC in 2% Ethanol.

This stress response is important to understand given *Bacillus subtilis*'s relation to *Listeria monocytogens*, a common bacterium that causes food poisoning. Understanding more about the function of these RsbR proteins and how they respond to different environments will help us understand how *Listeria monocytogenes* and other similar organisms are able to survive and adapt to different environmental stressors.

References

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