Changing Environmental Conditions Alter the Mechanism of Lethality in *Bacillus subtilis MinD Noc* Double Mutants Grown at Elevated Temperatures

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Abstract

Climate change and its impacts has had and will continue to have unpredictable impacts on ecosystems globally. The ecosystem destabilizing effects of climate change will result in severe impacts to humans globally. Even minor changes in the function of small organisms as a result of climate change can have catastrophic effects. Microorganisms make up the vast majority of life on earth aside from plants. The unpredictability of climate change's effects can partially be attributed to our lack of understanding of how rising temperatures will affect the function of these microorganisms. *Bacillus subtilis* is one of the most-wide spread soil bacteria and the protein system by which it reproduces is poorly understood. This system, known as the Min system, has been used to demonstrate the ability of increased temperature to impact cellular function. New research has identified that the effects of temperature stress are greatly impacted by the chemical conditions of the environment *Bacillus subtilis* is grown in. In order to better understand the ways these environmental conditions influence temperature effects, a process known as transposon mutagenesis was used to find suppressor genes that allow MinD Noc double mutated *Bacillus subtilis* to grow at non-permissive elevated temperatures and chemical conditions. These suppressor genes were compared to the results of a similar experiment that utilized a different growth media. The comparison of the results of these experiments show that multiple combinations of temperature and environmental conditions can contribute to a variety of physiological stresses on bacteria. The suppressors identified in this study

also indicate that the sporulation stress response of *Bacillus subtilis* is involved in the death of cells due to certain combinations of chemical and temperature stressors.

Introduction

Approximately 30 percent of the energy from sunlight that reaches earth is reflected back into space in the form of light. The remaining 70 percent is converted into heat energy and radiated outward as infrared radiation. Some of this radiation does leave the Earth’s atmosphere however the vast majority is captured by greenhouse gasses (Denchak 2023). While this is an entirely natural process which serves a key role in allowing life on Earth, human behaviors such as the use of hydrocarbons as fuel sources has led to an exponential increase in the concentrations of CO_2 and other greenhouse gases in the atmosphere over the last 200 years (Ethridge et al., 1996). This increase in atmospheric greenhouse gasses has led to a rapid, unprecedented increase in average global temperatures as the additional greenhouse gasses trap more heat. Since 1850 global average temperature has increased by approximately 0.06 degrees Celsius per decade. In the last 40 years however this rate of change has more than tripled, with each decade bringing an increase in temperature of around 0.2 degrees Celsius (Dahlman & Lindsey 2024). This increase in temperature has resulted in the global reduction of permafrost, glacial coverage, and polar ice caps (Haeberle & Gruber 2009, Bajracharya, Mool & Shrestha 2006, Wild et al. 2003). As a direct result of this global melting process, global sea levels increased 0.19 m from 1900 to 2010 (Chuch et al., 2013). The effects of human activities can be seen through these metrics to be shaping the way the planet looks at a global level.

Climate change and its various impacts threaten to greatly affect the quality of life of humans as a whole due to the rapid changes it is already imparting on various ecosystems globally (Canadall & Jackson, 2021). Global greenhouse gas emissions increase the amount of thermal energy trapped in the Earth's atmosphere, driving climates rapidly towards the proposed 2 Celsius threshold which has been speculated as a potential point of no return that would have lethal implications for humanity (Parry, Lowe, & Hanson, 2009).

Climate change results in symptoms far more visceral than the blatant effects of a warmer atmosphere. As the name suggests, climate change results in changes in climate at the local level as well as the global level. Climate change has been linked to a variety of changes in precipitation with some areas experiencing significant decreases in precipitation and others experiencing significant increases. These new precipitation patterns happen in conjunction with changes in seasonality. Various regions now experience far different seasonal patterns than they have historically. These changes typically present as an increase in the duration of summer and a shorter, more mild winter (Portmann, Solomon, & Hegerl, 2009). These new climate patterns are expected to greatly affect human populations due to the impacts on agriculture, transportation, and water resources that they cause (Howden et al., 2007, Mills & Andrey, 2002, Leavesley, 1994).

The abiotic effects of climate change result in numerous biotic impacts. Changes in climate result in range shifts for numerous organisms. Organisms are able to survive in areas that were previously inhabitable to them and are no longer able to survive in areas that they recently could. Organisms that are motile and fill broad ecological niches are able to survive in the face of these forced range shifts through population migration. Other organisms are not able to handle this degree of change for various reasons. Various plants lack the ability to shift their population distribution at the same rate as the climate is shifting due to the limitations of their growth rate and reproductive patterns. Other organisms such as those that are adapted to niche roles in climate islands are simply not able to move with their required environmental conditions. An example of such organisms are those species which are adapted to live in alpine regions, as their climate primarily changes vertically due to alpine glaciers shrinking from the bottom up. Such species are incapable of shifting from one climate island (mountain) to the next and thus face possible extinction as a result of climate change (MacLean & Beissinger, 2017).

A secondary effect of range shifts is the overlap of various species distributions that previously did not overlap. These species may fill similar ecological niches and the change in their ranges results in competition for limited recourses. This competition causes increased population stress in populations that are likely already stressed due to the abiotic factors that caused their initial range shift (Zielinski, Tucker, & Rennie, 2017).

Some organisms experience temporary benefit as a result of the abiotic effects of climate change. The most prevalent categories of these species are those that are able to complete additional breeding cycles as a result of increased summer durations and those that have population dips during winter. Bugs are most likely to benefit from this as they typically experience exponential population growth during the warm months followed by a gradual population decline during the winter thus placing them in both categories. Due to the exponential nature of reproduction, a slight increase in their breeding season lengths results in a huge increase in their total population prior to the start of winter. Exponentiating this effect is the reduced winter die off as a result of shortened winter months and a larger initial population at the start of summer. This results in a cyclic feedback loop which produces a population that is only limited by resource availability. In these instances, the limiting resources are often completely depleted. In this way such feedback loops can result in complete ecosystem collapse. This exact effect has already been observed in some pine forests as a result of this feedback loop increasing pine beetle populations to unprecedented size (Sambaraju & Goodsman, 2021). Such examples make it clear that the benefits experienced in certain populations are almost always short lived as such shifts in individual populations are capable of destabilizing ecosystems to the point of collapse.

The unpredictable biotic impacts of climate change are likely to be even more harmful to humans than the abiotic impacts. Crop fields are just as vulnerable to increasing pest populations as pine forests. One of the most directly harmful effects of climate change is the emergence and increased prevalence of infectious disease. Shifting climates and resulting range shifts put humans in contact with new disease vectors, opening the possibility of novel diseases to jump to humans. Climate change may also result in increased populations of disease vectors such as mosquitos. Increased precipitation and longer summer seasons contribute to larger mosquito populations, thus increasing the risk for transmission of malaria and other blood borne diseases (Zell 2004).

While there are extensive observed and predicted impacts as a result of climate change, the complexity of ecosystems makes it impossible to truly foresee the extent by which human driven climate change will impact ecosystems and human quality of life globally. Minor changes in the function of even the smallest of organisms can lead to feedback loops that destroy ecosystems and environments. Knowledge limitations regarding the physiology and function of any given organism can prove catastrophic given the important roles each component of complex biological systems play. This is especially true of micro-organisms. Despite being unseen by the naked eye, bacteria make up 81.06 percent of all non-plant biomass on earth (Bar-On, Phillips, & Milo, 2018).

These microorganisms fill various, essential roles that contribute greatly to the stability of ecosystems (Briones & Raskin, 2003). It is difficult to quantify the cumulative environmental impact of microbes due to the enormous diversity of microbe species. It is clear however, that microbes are vital due to their key roles in nutrient cycling, soil health, and plant-microbe interactions. Soil microbes in particular are responsible for driving various biologic transformations that convert soil organic matter into readily available pools of micro and macro nutrients (Sahu, Vasu, Sahu, Lal, Singh, 2017). Various soil bacteria form complicated metabolic relationships with plants that contribute greatly to the stress resistance of the plants and their ability to uptake nutrients from the soil (Mahapatra, Yadav, Ramakrishna, 2022). Due to a combination of these factors, soil microbes are a key marker of overall soil health (Kennedy & Stubbs, 2006). Due to the extensive supporting role that microorganisms play in every ecosystem, climate impacts on their populations have the potential to destabilize and destroy ecosystems as well as cripple agricultural production from the bottom up (Jansson & Hofmockel, 2020). In the face of climate change, the roles that microbes play have become increasingly vital to maintain, and yet, the exact way that increasing global temperatures will affect these species is understudied.

Of the known soil microbes, *Bacillus subtilis* is the best studied (Errington and Aart 2020). The Bacteria is found extensively throughout the world in the soil, air, and water, and grows extremely fast, making it an attractive research tool. As a result of these features, *Bacillus subtilis* is commonly used as an analogue for the numerous similar bacteria which function and behave comparably to it (Earl, Losick, Kolter, 2008). *Bacillus subtilis* is known to have a highly prevalent environmental role, through both increasing the resilience of plants to abiotic and biotic stressors through the relationships it forms with them, and in revitalizing degraded soil which allows plants to colonize it (Mahapatra, Yadav, Ramakrishna, 2022, Ramakrishna, Rathore, Kumari, Yadav, 2020). In the context of climate change, understanding how the important environmental player *Bacillus subtilis* functions at a molecular level, especially in regards to its response to the conditions it grows in, is crucial in order to accurately predict how the increase in global temperatures will affect not only *Bacillus subtilis,* but soil bacteria as a whole.

Cell division is the most crucial molecular process for the survival of any microbe. The cell division process in *Bacillus subtilis* is guided by two primary systems: the Min system, and the nucleoid occlusion system (Noc). These two protein systems work together to ensure that cell division occurs at the center of the rod-shaped bacteria and does not compromise the integrity of either of the genomes (Yu 2021). Three proteins make up the Min system: MinC, MinD, and MinJ. A recent research project investigating the function of these systems has found that while strains with mutations in the genes coding for either MinD or Noc are able to function semi-regularly, strains with both of these genes mutated are incapable of surviving at elevated temperatures. This study incorrectly assumed that temperature was the only stressor contributing to the deaths of these compromised cells (Yu et al. 2021). This study performed its experiments using a form of growth media known as LB media. A key component of this media is yeast extract. While LB media is highly utilized in the field of microbiology, the exact chemical makeup of various yeast extracts are subject to some minor variation due to their biotic source. New, unpublished research has found that that this high temperature lethality phenotype is not found when MinD Noc double mutated strains are grown on LB media made with some varieties of yeast extract (Kearns, Adebiyi, unpublished). This is largely unprecedented and demonstrates that minor variations in the chemical composition of the environments that *Bacillus subtilis* grows in can have huge impacts on the outcomes of stress due to increased temperatures. While complete lethality would not be expected in healthy strains of bacteria grown in comparable environmental conditions, the lethal outcome for the weakened mutant strain grown in these conditions suggests that the same combination of abiotic stressors would result in decreased function and reproductive capacity for healthy strains. The existence of this relationship between chemical environmental conditions and temperature conditions may be crucial in the prediction of climate change’s effects on countless bacteria strains in addition to *Bacillus subtilis*. Due to the observed potential for minor shifts in population sizes of small organisms to destroy ecosystems from the bottom up, it is imperative that this interaction between environment and temperature be explored further.

Transposon mutagenesis is a process used to randomly disable individual genes in a bacterium. A transposon, a DNA sequence which codes for proteins that insert the transposon itself into the chromosome, is added to a growing cell that contains known mutations. The gene that the transposon randomly inserts itself into becomes non-functional due to the addition of the new DNA sequence within the reading frame of the gene. The majority of these mutations caused by the transposon have no impact or a negative impact on the already mutated cell. In some instances, however, by disabling an additional gene the cell begins to function as if the pre-existing mutations were not present. Using genetic sequencing, the insertion site of the transposon is identified and a suppressor gene is discovered (Voelker & Dybvig, 1998). In this paper transposon mutagenesis was used to find various suppressor genes which allow the MinD Noc double mutated strain of B. subtilis to survive at high temps on a non-viable growth media that mimics the lethality of the LB media used for the 2021 Yu et al. paper. Because the suppressor gene being disabled restores functions that were disabled by the mutation of the MinD and Noc genes, the protein that the genetic mutation affects can provide clues towards the mechanism of lethality. As the exact chemical makeup of the new media, called CH media, is known, a link can potentially be made between the components of the media and the predicted functions of the suppressor genes identified. A connection between a component found in the media and the known function of a suppressor gene would help to identify what specific compounds are causing the increase in stress as a response to temperature. Additionally, because suppressor genes are intrinsically linked to the mechanism of lethality, the suppressors identified in the 2021 Yu et al. paper for the mutants grown on LB media would be expected to be the same as the suppressors for the mutants grown on CH media if the stress mechanism was the same. The most common suppressor genes are those that, when mutated, allow for an increase in the concentration of stress response proteins in the cell. A gene known as clpX is most commonly identified acting as a suppressor gene in this way.

Methods

In order to gather information on the molecular function of the Min system in *Bacillus subtilis*, a process known as transposon mutagenesis was performed. This process identified genes that, when mutated, allowed for the survival of a MinD Noc double mutant grown on CH media at high temperatures.

Transposon Mutagenesis

The strain DK6659, which contains a transposon plasmid and has the genes coding for both MinD and Noc mutated from the wild type strain DB1042, was cultured at room temperature in LB media for 48 hours. 3 ml of this high-density culture was spread on a CH media plate and incubated at the non-permissive temperature of 42°C overnight. The transposon inserted into the genome of the colony forming unit for each resulting colony and disabled a gene which permitted growth at this temperature.

Inverse PCR

Inverse PCR was utilized in order to identify the insertion site of the transposon for each novel strain generated using the transposon mutagenesis. DNA was extracted from each strain. 6 μL of this gDNA was ligated using 0.5 μl of the restriction endonuclease Sau3AI at 37°C for 1 hour with a heat kill step at 65°C for 20 minutes. The resulting digested DNA was ligated with 0.5 μl of T4 DNA ligase at room temperature for 1 hour. Inverse PCR was performed with 2 μl of ligated DNA with oligonucleotides 6212 and 6420 in an Eppendorf thermocycler running a standard PCR program. Each amplified DNA fragment was sequenced by Eurofins Scientific with the universal primer 6224. The sequencing results were compared with the genome of DK1042 and matching regions identified the gene which the transposon had inserted within.

Results

Transposon insertion site LB media (Yu et. al. 2021)	Annotation of gene product	Transposon insertion site CH media	Annotation of gene product
rho	Transcription terminator (Bidnenko et al., 2017)	gerAA	Germinant receptor (Mongkolthanaruk et Al.2011)
ggaB	Teichoic acid biosynthesis (D'Elia et al., 2006)	yvcB	Sporulation cascade initiator (Shi et al. 2020).
yaaT/ricT	Regulator of RNase Y/Spo0A (Dubnau et al., 2023)	tig	Trigger factor homologue ((Matavacas, Hallgren, Wachenfeldt, 2023)
hemX	Regulator of heme biosynthesis (Schröder et al., 1994)	yraO	Gene product unknown
clpX	AAA+ unfoldase (Yu et al., 2021)	clpX	AAA+ unfoldase (Yu et al., 2021)
Upstream clpP	Polar on clpP, AAA+ protease (Yu et al., 2021)		
YjbH	Proteolytic chaperone for Spx ((Larsson, Rogstam, & Von Wachenfeldt, 2007)		
YjbI	Polar on yjbH, hemoglobin-like (Imai et al., 2022)		

Table 1: Results comparison of Transposon insertion sites between MinD Noc mutants grown on LB and CH media

Of the insertion sites found in this study, only clpX was conserved between the MinD Noc mutants grown on LB and CH media. The remaining suppressor genes were entirely unique.

Predicted functions of suppressor genes for MinD Noc mutants grown on CH media

clpX is the best studied suppressor gene identified through the transposon mutagenesis. This gene is involved in the degradation of misfolded and old proteins. Its mutation results in the accumulation of stress response proteins in the cell that make *Bacillus subtilis* highly tolerant towards numerous genetic and environmental stressors. The mutation of clpX through transposon mutagenesis results in an accumulation of the stress response regulator protein spX within the cell as old spX proteins are not degraded in the absence of clpX***.*** Spx is known to bind to the alpha subunit of the RNA polymerase and lead to the transcription of numerous stress response proteins that greatly increase the survival capability of *Bacillus subtilis* (Schäfer & Turgay, 2019).

gerAA is known to be involved in the ending of sporulation. GerAA is thought to form a functional unit in conjunction with gerAB that senses nutrient changes in the environment that Bacillus spores are located in. In response to appropriate nutritional changes, this subunit signals for the termination of the spore phase (Mongkolthanaruk et Al.2011).

yvcB is a largely unstudied gene which is found on an operon which is known to be involved in the sporulation cascade in Bacillus subtilis (Shi et al. 2020).

tig encodes a trigger factor that is involved in both protein folding during translation as well as management of misfolded proteins (Matavacas, Hallgren, Wachenfeldt, 2023). The mechanism of this suppressor is likely comparable to the mechanism of clpX as mutation of tig results in an increase in SpX due to inhibited degradation.

yraO has been identified through the sequencing of the entire Bacillus subtitles however no research has been done which links it to any known process or function.

Discussion

The results of the transposon mutagenesis on MinD Noc double mutated *Bacillus subtilis* grown on CH media provides two key insights into the interaction of *Bacillus subtilis* and environmental stressors. First, the presence of two suppressor genes, gerAA and yvcB, that are known to be involved in sporulation implies that the stress induced sporulation response of *Bacillus subtilis* is involved in the lethality of CH media and high temperatures in the MinD Noc mutated cells. Second, and more surprisingly, the lack of overlap between the suppressor genes found to restore function in these cells when grown in the LB media used in the 2021 Yu et al. paper and the suppressor genes found to restore function in these cells when grown in CH media implies that different mechanisms cause the deaths of cells grown in each respective media. The presence of tig and clpX as suppressor genes does not allow for any additional extrapolation on the causes and mechanisms of lethality in these cells as their mutation allows cells to survive given a wide variety of environmental and genetic stressors. YraO similarly provides little insight into the cause of lethality in these cells due to knowledge limitations regarding its function in *Bacillus subtilis.* It may be concluded that its appearance as a suppressor gene is indicative of a novel mechanism of lethality that has not been paralleled through other transposon mutagenesis experiments.

Sporulation is a stress response induced as a result of environmental conditions by *Bacillus subtilis.* This behavior involves the formation of a round highly reenforced spore that has no known metabolic activity and very low internal water content. Spores are capable of surviving for years in conditions that would quickly result in the death of a normal cell. These conditions include both high temperatures and insufficient nutrients in the environment (Higgins & Dworkin, 2020). YvcB is known to be involved in the genetic cascade which allows for normal spore formation in *Bacillus subtilis* (Shi et al. 2020). Mutations in MinCD are known to result in reduced ability to form proper spores as the division septum is misplaced during the asymmetrical cell division process necessary for the completion of spores (Barák, Prepiak, & Schmeisser, 1998). This failure to properly place the division septum could potentially be lethal in conjunction with high temperatures and the additional mutation of Noc. The combination of temperature and environmental stressors experienced by bacterium grown on CH media at 42 Celsius may induce a sporulation cascade that the bacteria are not capable of properly completing due to the mutations in MinD and Noc. While seemingly possible, this proposed mechanism is insufficient to explain the appearance of gerAA as a suppressor gene. GerAA is known to be involved in sporulation, however it is thought to be involved exclusively in the germination of spores back into metabolically active cells (Mongkolthanaruk et Al. 2011). If the initiation of sporulation resulted in the death of the cells due to a misplaced division septum, gerAA's function in the cell would not occur in the first place. Both suppressor genes being linked to sporulation does strongly implicate the sporulation process as a component of lethality in the cells grown on CH plates at high temperatures however yvcB's role occurring at the initiation of sporulation and gerAA's role occurring at the termination of sporulation does more to obscure the mechanism of lethality than it does to reveal it.

The lack of overlap between the suppressor genes identified for the mutated genes grown on LB media and CH media is indicative of two separate mechanisms by which exposure to unknown environmental compounds in conjunction with heat stress results in lethality. This implies that various combinations of environmental factors result in a wide variety of stress mechanisms which further demonstrates the vulnerability of soil microbes, and thus ecosystems as a whole, to changing climates. With multiple poorly understood mechanisms causing cell death in these mutated cells it is clear that predicting the impacts of climate change on microbe populations poses a tremendous challenge for scientists. While it is possible that chemical contributors to increased heat stress are linked to the natural makeup of the environment, it is equally if not more likely that human behaviors such as the use of a wide variety of chemical compounds in the production of crops and the addition of thousands of compounds into the environment from the industrial production and transportation sectors will have an even greater impact on the population dynamics of wild bacteria. Both LB media and CH media are made up almost exclusively of nutrients necessary for the growth of cells and yet in conjunction with increased temperatures their specific chemical composition contributes to a least two unique mechanisms of chemical stress for bacteria. By further modifying the chemical compositions of the ecosystems that humans rely on, there arises an increased vulnerability towards unpredicted bacterial responses to climate change. Due to the cornerstone roles that bacteria fill in ecological systems, even small changes in the way that they function can result in catastrophic impacts on ecosystems.

Conclusion

The lack of overlap between the transposon insertion sites in LB media and CH media in addition to the changing response of MinD Noc double mutated *Bacillus* subtilis as a result of variance in the makeup of LB media exemplifies how knowledge limitations increase the human risk of climate change due to the difficulty of predicting how various factors will combine to affect bacteria in the wild. The current lack of understanding of how *Bacillus subtilis,* as well as other bacteria species function at a molecular level make it impossible to accurately identify the specific environmental influences that can dictate their response to climate change.

Extensive research in this area is necessary in order to identify chemical markers which indicate regions and environments that will be most affected by climate change as a result of changing bacteria population dynamics. Through similar research the specific chemical factors that contribute to increased thermal stress in bacteria can be identified. This research will be necessary in order to identify environments that already contain these compounds in order to plan for the possibility of destabilizing climate impacts on their microbiomes. Additionally, this research will allow for the identification and mitigation of possible human actions that release these compounds into the environment.

While the probable existence of multiple environmental factors each contributing to their own unique effects makes identifying all possible stressors impossible, simple experiments can be used to identify some of the factors. Given the known lethal response of the MinD Noc double mutated strains of *Bacillus subtilis* to high temps on CH media, it would be possible to alter the composition of the media in order to find what individual compounds in it causes lethality at high temperatures. Through manipulation of the makeup of the media, compositions could be found that allow for growth at high temperatures. These media compositions could then be compared to the original composition in order to identify which factors result in the death of the cells. Ideally reducing or increasing the concentration of one compound in the media would restore survival and thus be identified as the factor contributing to increased environmental stress. It is just as likely that a ratio of chemicals results in the lethality at high temperatures.

Once a non-variable media was identified which allows for survival at high temperatures in these mutated cells, further research could be performed in order to identify other environmental constituents that can impact the effects of increasing temperatures on bacteria. Addition of compounds known to be present in the environment can be used to test for and predict possible compounding impacts of combined thermal and chemical stress.

Once the combinations of chemical and thermal stressors are better understood it will be important to plan for the possible changes to ecosystems that will result from climate change's effects on bacteria. In order to mitigate impacts on humans, it may be necessary to artificially augment the chemical conditions found in especially vulnerable ecosystem in order to limit the changes that bacteria populations will undergo in response to climate change. It will almost certainly be necessary to undergo this augmentation process in vulnerable lands used for crop production. If the services that soil bacteria provide were decreased as a result of increasing temperatures and compounding environmental condition, the impact on humanity's food production capacity would almost certainly outweigh the cost of environmental inputs.

In addition to the pressing need for more research in this area, a key takeaway of this study is the unpredictable nature of climate change impacts. The complexity of ecosystems and the organisms and abiotic factors that make them allows for infinite possible effects as a result of climate change. Unknown and understudied interactions can lead to complete ecosystem collapse as a direct result of human actions. If human behavior does not change, extremely harmful climate impacts are certain. While researching and identifying compounding factors that can exasperate the impacts of climate change helps to better understand and mitigate harmful impacts, it is necessary to limit the root cause of climate change. It is pressing to transition away from the reliance on hydrocarbons as a fuel source. Energy sources such as nuclear and wind need to be pushed for rapidly in order to stop the exponential increase in greenhouse gas concentrations that is currently occurring. It is impossible to plan for every impact of climate change so the actions that can be taken now must be taken. A minor decrease in quality of life now is to be greatly preferred to total global ecosystem collapse.

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