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Development of the K-state (Competence) and Mutagenesis in Stressed Bacillus subtillis Cells

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DEVELOPMENT OF THE K-STATE (COMPETENCE) AND MUTAGENESIS IN STRESSED BACILLUS SUBTILIS CELLS

By

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ABSTRACT

Development of the K-state (competence) and mutagenesis in stressed *Bacillus subtilis* cells

by

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Mutagenesis is central to the evolutionary process. We currently view evolution as a gradual process affecting all cells within a population. However, my project studied an underestimated part of the evolutionary process: mutations generated during stationary phase (caused by nutritional stress or growth arrest) within a subpopulation of cells. I used *Bacillus subtilis,* a bacterial model for cell growth and differentiation. Stationary-phase cultures of *B. subtilis* differentiate subpopulations that exhibit different survival strategies including competence, secondary metabolite production, biofilm formation, cannibalism, and endospore formation. The development of competence permits cells to uptake exogenous DNA and incorporate it into their genome. During competence, new alleles are acquired and recombined into the host's genome which leads to genetic diversity. Hence, it has been proposed that the cells that develop competence are tasked with increasing genetic diversity, which increases the potential to restore growth and escape from stress. One key process in increasing genetic diversity in the competent subpopulation is the process of recombination. Through this process, cells acquire new gene alleles and genome rearrangements with the potential to increase fitness. Interestingly, published results from my research group have indicated that increasing genetic diversity is not limited to the promotion of the recombination process and that a yet-to-be

characterized process mediating the formation of point mutations is activated in conditions of stationary phase. This concept is supported by the following observations: i) defects in genetic factors that control competence (ComK and ComA) result in decreases in mutagenesis in non-growing cells; and ii) the observed decrease is independent of recombination. We speculate that a mechanism, activated during the competence (K-state) through the transcriptional activator ComK, is responsible for the formation of point mutations acquired in stationary phase. My project seeks to bring together these separate observations into a coherent understanding of how the K-state leads to increases in mutagenesis. We tested the hypothesis that the population of cells that enter the K-state (competence) experiences increased levels of mutagenesis during stationary phase. The increase in mutagenesis levels in K-cells was affected by reactive oxygen species. We concluded that K-cells activate error-prone repair of DNA lesions caused by reactive oxygen species.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

APPENDIX

LIST OF TABLES

CHAPTER 2

APPENDIX

CHAPTER 1

LITERATURE REVIEW

Stress-induced or adaptive mutagenesis occurs when a mutation is acquired under stress or growth arrest conditions. Often, this type of mutagenesis is produced via error-prone DNA repair which increases the potential of a cell to escape stress and become better adapted to its environment (1, 2, 3). These types of mutagenic events occur in cells from all domains of life (4,5,6) and lead to phenomena such as antibiotic resistance and degenerative diseases in higher organisms (1). These mechanisms have evolved because they speed up evolution or remedy cellular conflicts that are cytotoxic. Increasing genetic diversity, however, also increases the risk of acquiring detrimental or lethal mutations (7). Published research indicates that cells that activate hypermutable states are likely to accumulate several lethal mutations before acquiring a beneficial one (7). Thus, two interesting questions in this research are: how do cells activate mechanisms that increase genetic diversity without incurring a lethal event? And, do all cells in a population activate mechanisms that increase genetic diversity?

The idea of evolution started with Charles Darwin, who developed the concept of natural selection. Natural selection posits that variation occurs randomly in natural populations. Some variants are selected against while others confer fitness and are passed on to successive generations. Darwin visualized and regarded this evolutionary process through variations in phenotypic traits in animals like the Galapagos finches (8). In finches, beak shape and size are selected based on what the finches eat. By the 1870s Darwin's theory of natural selection was well accepted by the scientific community. The basis of the natural variation observed in populations was not explained by Darwin; however, the work of a contemporary, Gregor

Mendel, suggested that such variation was the product of differences in the hereditary material (9). While it was thought that the concepts of natural selection and the units of hereditary material applied well to the study of eukaryotes, there was no clear connection to the study of bacteria. That bacteria adhered to Darwinian evolution and genetic inheritance was not determined until the 1940s.

In 1941, Luria and Delbrück, who eventually won the Nobel prize, looked at growth-dependent mutations (exponential growth phase) and how they were affected by a lethal selection pressure in bacteria (10). They specifically looked at *Escherichia coli* cells that were exponentially growing and their response to a lethal selection pressure of the T1 bacteriophage. They observed a large variation in the number of T1-resistant colonies between independent batches of cells even though the T1 selection pressure was the same across cultures. The large variance indicated that the mutations occurring in these conditions were not a direct response to the selection pressure, but instead were due to random mutations occurring prior to selection. This observation followed Darwin's theory of natural selection; however, those researchers also discussed the possibility that under non-lethal selection, growth-independent mutagenic mechanisms may increase diversity in response to stress (10).

How mutations happen in non-growing conditions in bacteria was first studied in the 1950s by Francis J. Ryan. Dr. Ryan's experiments examined mutagenesis in conditions of histidine starvation in a His^- strain of *E. coli* and observed that the number of His^+ colonies increased over time (11). This observation stood for 30 years before further research revisited the phenomenon of stationary-phase mutagenesis. Cairns et al. (1989) studied whether mutations occurring in starvation conditions were random or arose as a direct and specific response to the selection pressure. To test these concepts, they chose an experimental set up similar to the one

used by the Ryan lab, except that Cairns' selection pressure was based on lactose starvation. In this system, mutants were detected within 24 h while non-mutant cells survived on the plates, which allowed "directed" mutagenesis to occur, if there was such a mechanism (12). Since Cairns' paper, stationary-phase mutagenesis has received more attention and some important factors driving this process have been elucidated.

Most of the work in the field of stationary-phase or adaptive mutagenesis (SPM) has been done in *E. coli*, the most studied bacterium to date. In contrast, our lab uses the soil bacterium *Bacillus subtilis*, a Gram-positive, spore-forming rod. *B. subtilis* is useful because it provides a bacterial model for cell growth and differentiation, it is closely related to human pathogens and its environment is strikingly different from the one in which *E. coli* is found. *B. subtilis* has the noteworthy ability, when stressed, to differentiate into subpopulation of cells as a survival strategy. Differentiation of cells of one type into many other types has applications in understanding adaptive mutagenesis in non-prokaryotic systems since eukaryotic cells differentiate from stem cells into cells that perform specific functions.

In 2002, Sung and Yasbin designed a system to study stationary-phase mutagenesis (SPM) in *B. subtilis* using long term amino acid starvation (13). Mutations were created in *B. subtilis* that confer auxotrophy in biosynthesis of three amino acids. Amino acid starved cells are then measured for their ability to form colonies over prolonged nutritional stress. Research in *B. subtilis* demonstrated that SPM happened in this bacterial model, and that the factors influencing this process were different from those observed in *E. coli*. For example, the absence of the transcriptional factors ComA and ComK reduced SPM. ComA, part of a two-component regulatory system, and ComK, the transcriptional activator, are both required for *B. subtilis* cells to become competent, or uptake exogenous DNA from its environment. Competence is attained

by cells that undergo differentiation and it is only expressed in a subpopulation of cells in a batch culture. Furthermore, RecA, the protein required for homologous recombination of DNA, was not involved in stationary-phase mutagenesis, suggesting that stationary-phase mutagenesis proceeded in the absence of recombination, the trademark of the competence pathway. Sung and Yasbin suspected that the cause of the mutagenesis was not as a direct result of ComA or ComK but more likely caused by the gene expression and physiological changes triggered by these two transcription factors (13).

A competence gene regulation study by Berka et al. (2002), looked at ComK-dependent gene regulation. This study highlighted a regulon of over 165 genes. Interestingly, only about a dozen of these genes are necessary for the uptake and integration of DNA into the chromosome. Other genes that were upregulated were involved in repair of DNA damage and detoxification. This led Berka et al. to coin a new term for this subpopulation, the K-state, which is a "… global adaptation to stress, distinct from sporulation, which enables cells to repair DNA damage, to acquire new fitness-enhancing genes by transformation, to use novel substrates and to detoxify environmental poisons" (14). Competence, though very well studied in *B. subtilis*, is just one part of the K-state. This thesis seeks to determine whether the development of the K-state promotes mutagenic events independent of recombination. Therefore, I hypothesize that ***cells that enter the K-state (competence) show increased levels of mutagenesis during stationary phase and through a process independent of competence itself*** (Fig. 1.1).

Ultimately, the value of this research is that it examines the novel idea that there are cellular mechanisms that are activated in a subpopulation of cells to produce genetic diversity and speed evolution.

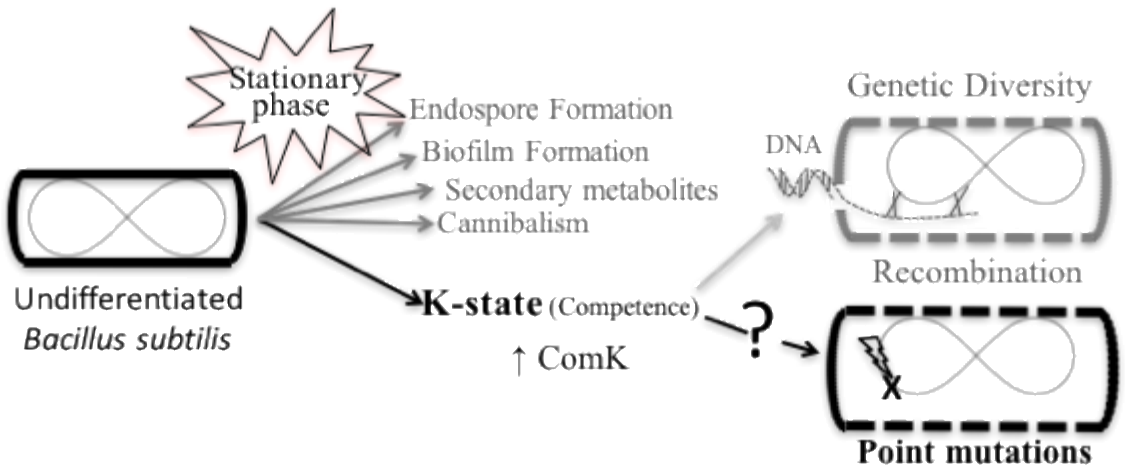

Figure 1.1. Hypothesis summary diagram. Nutritional stress (stationary phase) in *B. subtilis* activates the development of different subpopulations. Competence promotes acquisition of foreign alleles (recombination). The objective of this thesis is to determine whether the development of competence promotes mutagenic events in *B. subtilis* cells under nutritional stress. The experiments described here address the (?) in the figure.

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CHAPTER 2

THE K-STATE (COMPETENCE) SUBPOPULATION OF BACILLUS SUBTILIS PROMOTES STATIONARY-PHASE MUTAGENSIS

FORWARD

This chapter contains work conducted by me and other members of the Robleto lab: Holly Anne Martin and Carmen Vallin. Ms. Holly Anne Martin is responsible for Figure 2.1 and Table 2.2 and all research conducted to obtain these data. Ms. Carmen Vallin and Mr. John Creech are responsible for the construction of one of the strains used in this part of the study. My contribution is all other figures and writing of this section is solely my own. I do not take credit for the research presented here other than my own.

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The K-state (competence) subpopulation of *Bacillus subtilis* promotes stationary-phase mutagenesis

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Abstract

Bacillus subtilis uses cell differentiation as a survival mechanism during stress-induced conditions. Recently our view of one of these subpopulations, the competence subpopulation, was changed from a subpopulation limited to the ability to uptake exogenous DNA and incorporate it, in a recombination-dependent manner to a global stress adaptation state (K-state). K-cells rely on *comK*-dependent mechanisms for survival during stressed conditions or stationary-phase. Here, we demonstrate that the K-state subpopulation acquire stationary-phase mutations more frequently than the rest of the *B. subtilis* population. We also observe this phenomenon to be independent of recombination and DNA-uptake. Interestingly, we also observe that K-cells are more sensitive to reactive oxygen species (ROS), perhaps by changes in cell surface components that allow DNA translocation into the cell. DNA translocation-deficient cells were dramatically decreased in the accumulation of mutations compared to the parental strain during exposure to ROS. Our results support the notion that K-cells are more likely to accumulate stationary phase mutations because they activate error-prone repair of damaged DNA caused by reactive oxygen species (ROS).

Introduction

Stress-induced mechanisms accelerate mutagenesis, through activation of error-prone polymerases, transcriptional bypass, and other low-fidelity DNA repair mechanisms that are turned off during growth (1, 2, 3). From an evolutionary standpoint, this may result in increased fitness and adaption to the cell's environment. Since most mutations are deleterious, how is stress-induced mutagenesis a viable strategy for cells? Perhaps biasing mutations to a subpopulation of cells while maintaining genome integrity in the rest of the culture is one strategy that reconciles the benefits and the perils of increasing mutagenic events in stressed cells (4).

Hypermutable cell subpopulations have been reported across all three domains of life. In the human body, the immune system deploys a hypermutable strategy to increase the production of antibodies by B cells. The increase in antibody diversity allows an exquisite selection for high affinity to foreign antigens (5). In *Escherichia coli,* nutritionally stressed cells experiencing a double-stranded DNA break activate error-prone repair that increases mutagenesis near the site of such lesion (4). Because not all cells in a culture experience double-stranded breaks at the same DNA sites, this results in a fraction of cells generating mutations in different genomic locations. Further, this hypermutable state is transient because error-prone repair is no longer active once a selective growth advantage is acquired (4). Thus, transiently activating error-prone DNA repair during stress and high fidelity replication in growth conditions confers the ability to regulate how fast evolution proceeds.

During stress, *B. subtilis* develops heterogeneous subpopulations with different physiology (7). The development of different subpopulations is usually initiated through quorum sensing followed by development into the different pathways through two-component regulation

systems (7). Upon the onset of stress, cells may develop into spore formers, turn into cannibals, secrete proteases, and uptake DNA. Most of these subpopulations are mutually exclusive, however some subpopulations are transient and may sequentially activate different types of developmental states.

Competence, or the uptake of exogenous DNA from the environment, allows *B. subtilis* cells to acquire new alleles with the potential to escape adverse environmental conditions. In a study conducted by Sung and Yasbin (2002), it was hypothesized that this subpopulation was also acquiring mutations through RecA-independent means, and that ComK, a transcription factor controlling the genes for development of the K-state, was required for the accumulation of mutations (8).

After the Sung and Yasbin (2002) study, research describing the expression of genes important for the development of the K-state reported a regulon of over 165 genes (9). Interestingly, only about a dozen of the 165 had a direct link to competence itself (9). Over 100 genes were involved in regulation of other stress response systems like acid stress, detoxification, oxidative stress, and DNA repair. Based on those findings, the competence state was redefined as the K-state, which was described as "a global adaptation to stress, distinct from sporulation, which enables the cell to repair DNA damage, to acquire new fitness-enhancing genes by transformation, to use novel substrates and to detoxify environmental poisons" (9).

Oxidative stress during non-growing conditions is an important contributor to stationary-phase mutagenesis (6, 22). Cells contend with reactive oxygen species (ROS) during respiration, exposure to antibiotics, and other environmental stressors (UV, pollutants, high temperatures etc.). PerR, a transcriptional factor activated in response to increases in ROS directly stabilizes ComK levels in the cell (21). Interestingly, K-cells have different cell surface properties due to

the assembly of transformation pores in the membrane. This study explores whether the development of the K-state is conducive to stationary phase mutagenesis and examines the idea that changes in cell surface properties, effected by the assembly of transformation pores, prone cells to oxidative stress and activation of error-prone DNA repair.

Materials and Methods

Bacterial strains and growth conditions

The *B. subtilis* strains used in this study are all derivatives of strain YB955 and are described in Table 2.1. YB955 is a prophage-cured strain that contains the following auxotrophic genes: *hisC952*, *metB5*, and *leuC427* (8). A base substitution (transition mutation) was created at *leuC427* (G → A) creating a missense mutation; codon 143 changed from a glycine to an arginine within the protein. Base substitution (transversion mutations) for *metB5* (at base position 346 G → T) and *hisC952* (at position 952 C → T) created nonsense mutations.

B. subtilis strains were all maintained on tryptose blood agar base medium (TBAB; Difco Laboratories) or grown in liquid cultures in of Penassay broth (PAB) (antibiotic A3 medium; Difco Laboratories, Sparks, MD) with antibiotics as needed. *Escherichia coli* strains are maintained and grown on Luria-Bertani (LB) with antibiotics as needed.

The strain containing a defective *comEA* gene was constructed by cloning a neomycin cassette within the *comEA* gene. To do this, two sets of primers were designed (DNA sequence obtained from http://genolist.pasteur.fr/SubtiList/) to amplify two regions of *comEA* from strain YB955 along with additional sequences for recognition of restriction endonucleases compatible with plasmid pBest502 (11). The two *comEA* fragments (Table 2.1) were cloned in such manner that they flanked a neomycin cassette in pBest502. Fragments A of *comEA* was cloned using the

HindIII and *SalI* sites in of pBest502 (*E. coli* cloning plasmid with ampicillin resistance), whereas fragment B was cloned into the *BamHI* and *SacI* sites. Fragment A primers: forward agctggaagctttaaggtaacgctcttgccag and reverse agccgtgtcgacaaatttacttgcgcttcgtc, the PCR product was digested with *HindIII* and *SalI* (sites underlined above) and ligated to pBest502. Fragment B primers: Forward agtcacggatcctctgcaggacgggacagtgg and reverse aagcctcgagctctccatcagtcggcaccccaaac. The PCR product was digested with *BamHI* and *SacI* (sites underlined above) and ligated to pBest502. Ligation product was transformed into MON1 competent cells (Monserate, San Diego, CA). Colonies containing the desired recombinant plasmid were isolated by selecting on LB agar containing 100 μg/ μL of ampicillin, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) 0.01 g/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), blue-white screening. The desired plasmid was then prepared from *E. coli* and transformed into YB955; *comEA*$^{-}$ transformants were selected on neomycin and screened by PCR and restriction digest.

To construct the *gfp-comK* IPTG-inducible strain, genomic DNA was isolated from BD4010 (12) using the Wizard® Genomic DNA Purification Kit (Promega, Madison,WI). Isolated genomic DNA was then transformed into YB955 using the competence procedures for *Bacillus* (13). Briefly, YB955 was grown to T_{90}, ninety minutes after the cessation of growth (stationary phase), in GM1 broth (0.5% dextrose, 0.1% yeast extract, 0.2% casein hydrolysate, essential amino acids 50 μg/mL, 1X Spizzien salt solution (14)) and then diluted 10-fold into GM2 broth (GM1 broth plus 50 μM $CaCl_2$, 250 μM $MgCl_2$). After one hour of incubation at 37°C with aeration, genomic DNA is added. The culture was incubated for another hour, followed by the addition of 100 μL of 10% yeast extract. After an additional hour of incubation, the cells were plated on TBAB containing 100 μg/mL spectinomycin and 5 μg/mL

chloramphenicol. Transformants were verified by flow cytometry using IPTG and comparing fluorescence of induced versus uninduced versus parental (YB955).

To construct the *gfp-comK* IPTG inducible strain, *comEA* knockout, genomic DNA from YB955 *comEA::neo* (from strain described above) was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). The genomic DNA was then transformed into HAM501 using the competence protocol described above. Cells were plated on TBAB containing 100 μg/mL spectinomycin, 5 μg/mL chloramphenicol, and 5 μg/mL neomycin. Transformants were verified using the transformation assay described above and transformed using the plasmid pDG1664 (15) containing erythromycin resistance. A non-transformability phenotype was used to confirm the desired genotype.

Stationary-phase mutagenesis assay.

The procedure for this assay has been previously described in reference 8. Strain was grown overnight in 2-4 mL of PAB in a shaking incubator at 37°C with aeration (250 rpm). 2-4 mL of the overnight was transferred into 10-20 mL of PAB with 1/10 volume of Ho-Le trace elements (16) into 250 mL Erlenmeyer flask. Growth was monitored with a Genesys 10S UV-Vis spectrophotometer Genesys (Thermo Fisher Scientific, Waltham, MA) until T_{90}. Cells were then harvested by centrifugation at room temperature and resuspended in 1X Spizizen salt solution. Cells were then plated in quintuplicate (100 μL per plate) on 1X Spizizen minimal medium (SMM; 1X Spizizen salts supplemented with 0.5% glucose, 50 μg/mL of both isoleucine and glutamic acid (17) and either 50 μg/mL or 200 ng/mL of required amino acid. The lower concentration of amino acid was used to select for revertants. These plates were incubated for 9 days and revertants scored every day. To determine the initial number of cells plated, the

cell suspension was serial diluted by 6 log units and plated onto SMM containing all the required amino acids. The experiments were repeated at least three times.

To determine the survival rates of the non-revertant background cells, agar plugs were taken from each minimal plate using Pasteur pipettes every other day. These plugs were taken from areas of the plate that contained no visible colonies. The plugs were resuspended in 0.5 mL of 1X Spizizen salt solution, then serially diluted by 4 log units, and plated on SMM containing all the essential amino acids. These plates were incubated at 37°C for two days before being counted.

Oxidative-stress induced stationary-phase mutagenesis assay

To determine the effects of oxidative stress on the *B. subtilis*, cells were grown as described in the stationary-phase mutagenesis assay and when cells reached T_{90} they were treated with 1.5 mM *tert*-Butyl hydroperoxide (TBH; Luperox(R) TBH70X Sigma-Aldrich) for 2 h, double washed with 1X Spizizen salt solution, and then plated and assayed for SPM.

Transformation stationary-phase mutagenesis assay

Strains were grown overnight in GM1 with 10 μL Ho-Le trace elements in a 250 mL Erlenmeyer flask left on a bench top at room temperature for approximately 16 h. The next day the culture was incubated in a shaker at 37°C with aeration (250 rpm). Growth was monitored with a (Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, WA) until 90 min after the cessation of exponential growth (T_{90}); stationary phase. The culture is then diluted 1/10 into GM2 and incubated for 1 h as previously described. After the 1 h, the culture was split into different cultures and supplemented with 150 ng of the transforming DNA

(pHyperspank; a gift from Dr. David Rudner) is added, or no DNA control and a medium only control. Cells were incubated for an additional hour and then 10% yeast extract is than added to all three tubes. Cells were incubated one final hour and then harvested. Cells were then harvested by centrifugation at room temperature and resuspended in 1/10 of starting volume in 1X Spizizen salt solution. Cells from the DNA treatment and no DNA cultures were plated in quintuplicated (100 μL per plate) on SMM and also plated on TBAB agar with spectinomycin, which assessed transformation efficiency and spontaneous spectinomycin reversion. The SMM plates were incubated for 9 days and revertants scored every day. To determine the initial number of cells plated, the beginning cell suspension was serially diluted by 6 log units and plated onto medium containing all the required amino acids. The experiments were repeated at least three times.

To determine the survival rates or viability of the cells of the non-revertant cells, agar plugs were taken from each minimal plate using Pasteur pipettes every other day. These plugs were taken from areas of the plate that contained no visible colonies. The plugs were resuspended in 500 μL 1X Spizizen salt solution, then serially diluted 4-fold, and plated on medium containing all the essential amino acids. These plates were incubated at 37°C for two days before being counted.

To determine if prototrophic revertants were also transformants (colonies that arose on the selective minimal plates), colonies were screened for growth on TBAB spectinomycin plates. As a control, non-revertant colonies (colonies that arose from plugs that were grown onto minimal plates containing all essential amino acids) were subject to the same screen.

Transformation stationary-phase mutagenesis assay using TBH treated gDNA

Strains (YB955 and PERM1029 *mutY*) were grown overnight (approximately 16 h) and split into two batches. One batch was exposed to 1.0 mM of TBH and the other was left untreated while incubated for another 2 h. After incubation, genomic DNA from both batches were extracted and purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). This gDNA was then used in the assay described below.

A modified version of a transformation assay (previously described in the *transformation stationary-phase mutagenesis assay* section above) was conducted. A modification was done, after the first 1 h incubation in GM2, the culture was split into three different cultures/conditions: the first culture had 150 ng of the transforming DNA (pHyperspank plasmid) added as a competence control, then gDNA from cells treated with TBH and last culture had gDNA from cells that were not treated with TBH added. After a final hour of incubation, each culture was separately plated in quintuplicate on minimal media. The rest of the assay follow normal protocol as described previously.

Flow Cytometry

Strains HAM501 and AAK502 (Table 2.1) were grown to mid-exponential phase and T_{90}. The cell culture was then split at late exponential (0.7-0.8 OD_{600}) and IPTG was added to one batch and both batches (with and without IPTG) were incubated until T_{90}. 1 mL aliquot was removed at times described in previous sentence and 50 μL of 37% formaldehyde was mixed in. Then mixture was centrifuged for 10 min at 300 x g, in a microcentrifuge. Supernatant was decanted and pellet resuspended in 1 mL of phosphate-buffered saline (PBS). Sample covered in foil until ran through the BD FACSCalibur Flow Cytometer.

Analysis of mutation rates

The growth-dependent reversion rates for the His, Met, and Leu alleles were measured by fluctuation tests. The Lea-Coulson formula, r/m-ln(m)=1.24 (18) was used to calculate rates by determining, m, the number of mutations per culture. The median, r, is the number of mutant colonies from the 18^{th} culture after all cultures are ranked. The bacterial strains were grown (8) in PAB for 12 h at 37°C with aeration. The saturated cultures were then diluted 10^{-4}-fold into fresh PAB and 1 mL aliquots were transferred into 38 18-mm test tubes. These test tubes were then incubated for another 12 h at 37°C with aeration. Once saturated, 35 test tubes were then pelleted and resuspended in 100 µL of 1X Spizizen salt solution. Cells were then spread onto selection medium as described previously in the stationary-phase assay section. Revertant colonies were scored after 48 h of incubation at 37°C. To determine the total number of CFU plated (N_t), three of 38 the cultures were serially diluted and spread onto minimal media containing all the required amino acids. The mutation rates were calculated with the formula $m/2N_t$ (8, 18, 19).

Statistical analysis

Statistical significance was determined performing either a Student's t-test or a Chi^2 test. Statistical significance was tested at $p < 0.05$ or lower.

Results

Amino acid starvation was used to determine stationary-phase mutagenesis in *B. subtilis* cells. A strain of *B. subtilis* was created in a previous study (8) that contains point mutations in three amino acids genes histidine, methionine, and leucine. Cells were subjected to prolonged

starvation of amino acids. Cells were starved for 9 days and mutant colonies were counted during the duration of the starvation assay. The non-revertant cell population was also monitored (for full experimental description see Materials and Methods) to measure survivability of cells during the assay period. Mutants were also assessed for mutation type and sequenced for location as described previously (8). As demonstrated previously, mutants arising on days 1-4 are from exponential growth while days 5 and later are the result of mutations occurring in stationary phase.

K-cells are more likely to be stationary-phase mutants

To determine if K-cells were more likely to also be stationary-phase mutants over the rest of the *B. subtilis* population, a modified stationary-phase mutagenesis assay in combination with a transformation assay was conducted. Competence was used as a tool to determine whether a cell had entered the K-state. A stationary-phase mutagenesis assay combined with a transformation assay created an environment that promoted competence and transformability of the cells and while being able to assess the accumulation of stationary-phase mutations. YB955 (parental strain) was grown in competence medium along with a transformation marker the integrative plasmid pHyperspank (Table 2.1). pHyperspank contains spectinomycin resistance which was used to select for transformant cells (K-cells) immediately following completion of the 9-day starvation period of either histidine, methionine, or leucine (Fig. A1). His^+, Leu^+, Met^+, cells from the amino acid starvation plates were patched on complex medium containing spectinomycin to determine resistance. Table 2.2 shows the combined percent of stationary-phase mutants from all amino acid starvation assays which were also spectinomycin resistant. Results demonstrated that if a cell was a K-cell (Sp^r), the cell was more likely to be a His^+, Met^+

and Leu$^+$ mutant as compared to the rest of the *B. subtilis* population. When DNA was added, early revertants (mutants from exponential growth) were 10.9% likely and late revertants (stationary-phase mutants) were 18.1% more likely to also be stationary-phase mutants. Actually, they were up to 10 times more likely to be K-cells. Though spectinomycin resistance can occur spontaneously (no DNA added row Table 2.2), that effect appears to be minimal at only 4% and 1% of the cells respectively. These data revealed that K-cells are more mutagenic than the rest of the *B. subtilis* population and prompted further investigation and characterization of this subpopulation.

Stationary-phase mutagenesis in K-cells occurs independently of DNA-uptake

In this part of the study, we determined whether the increased ability in stationary-phase mutagenesis, previously observed in K-cells, was independent from DNA uptake. The subpopulation of K-cells is part of bistable system in which 10-20% of the total population develops the K-state (7,9,12). K-cells gain the ability to take up DNA from the environment by creating up to 20 transport systems that bind and translocate DNA into the cell (21). To address whether the ability to take up DNA affected SPM, we generated a strain with a knock-out in *comEA,* this gene codes for a protein component of the transformation pore, and measured SPM.

A stationary-phase assay (8) was conducted on YB955 and a ComEA$^-$ strain, JC101. ComEA is expressed late during the K-state and involved in stabilization and transport of dsDNA for uptake into the cell during competence (21). JC101 cannot transformed (data not shown) and based on this observation, we speculate that the JC101 strain is defective in its ability to assemble transformation pores and therefore unable to take up DNA. The strains used in this experiment were also tested for their ability to activate *comK*-dependent gene expression.

This was to ensure that any effects seen were solely attributed to differences in ComEA and not due to differences in K-state development. We used a flow cytometry experiment in YB955 (HAM501) and JC101 (AAK502) (Table 2.1). These strains contain a transcriptional *gfp* fusion to *comK*. There were no notable differences in *comK* expression levels between strains (Fig 2.1).

Results from the stationary-phase mutagenesis assay (Fig. 2.2), as expected, showed no significant differences in the frequency of reversions for JC101 versus the parental strain YB955 in the three amino acid genes tested. While not statically significant, JC101 accumulated fewer mutations than the parental strain consistently over several trials. This was unexpected since there were no differences in cell viability (Fig. 2.2, right side) and there were no differences seen in the mutation rates during exponential growth between the two strains (Table 2.3). We interpreted these results to mean that the stationary-phase mutagenesis promoted by ComK was independent of DNA uptake. However, the slight differences in SPM levels observed in strains differing in ComEA warranted further examination.

K-cells (competence) and oxidative stress in *Bacillus subtilis*

Oxidative stress during non-growing conditions is an important contributor to stationary-phase mutagenesis (6, 22). Cells contend with reactive oxygen species (ROS) during respiration, exposure to antibiotics, and other environmental stressors (UV, pollutants, high temperatures etc.). PerR, a transcriptional factor activated in response to increases in ROS directly stabilizes ComK levels in the cell (21). Interestingly, K-cells have different cell surface properties due to the assembly of transformation pores in the membrane. Considering the connection between PerR and ComK, and the different cell surface properties of K-cells, we speculated whether K-cells experience increased exposure to ROS and DNA damage. In conditions of increased

exposure to ROS in non-growing cells, we tested the idea that K-cells may experience increases in error-prone repair of DNA. To test this concept, a stationary-phase mutagenesis assay was conducted in the presence of the oxidant *tert*-Butyl hydroperoxide (TBH) in strains that differ in their ability to translocate DNA (transformation pore) into the cell.

Results showed a drastic difference in SPM between YB955 and JC101 (Fig 2.3 A-C). In methionine reversion, YB955 accumulated 100-fold more revertants than JC101 (Fig. 2.3 A). We interpreted these results to mean that K-cells that translocate DNA into the cell experience oxidative stress and error-prone repair in response to such damage (6,22, 23, 24).

One possibility that could explain the differences in mutagenesis between YB955 and JC101 was the inability of JC101 to take up DNA containing oxidative damage from lysed cells treated with TBH, which could lead to reversions. We then conducted a follow-up experiment to test if the uptake of damaged DNA increased mutagenesis in YB955 and a strain affected in repair of oxidative in DNA. An overnight culture of YB955 was either untreated or treated with TBH and prepared to extract gDNA. Of note, treatment with TBH resulted in more than 90% kill of cells, which suggest that DNA contained oxidative damage. The gDNA from untreated and treated cells was used to transform into a population of *B. subtilis*. The results from this experiment showed no differences in the accumulation of mutations between cells transformed with gDNA from undamaged cells and cells transformed with gDNA from damaged cells (Fig 2.5 A). As an additional control, we also used a strain defective in the ability to repair DNA (PERM1029 *mutY*) containing lesions caused by oxidative damage (Table 2.1) (Fig. 2.4B). We saw no differences in SPM. These results suggested that the cells' ability to translocate DNA have no effect on SPM. Moreover, the ComEA-dependent effect on SPM previously described is more in line with the idea that assembly of a DNA translocation pore in membrane leads to an

increase exposure to ROS, a subsequent increase in error-prone repair of DNA lesions and an increase in mutagenesis.

Discussion

Competence has been extensively studied in not only *B. subtilis* but in many other naturally competent organisms (9,12,25). However, none of the studies thus far have addressed the role of competence in stationary-phase mutagenesis. The results presented here provide evidence that K-cells promote genetic diversity independently of the uptake of DNA as a response to stress. First, our data demonstrated that SPM mutants were more likely to have undergone transformation, a trademark of the K-state, than the background population. Our data showed that stationary-phase mutants were up to 10 times more likely to be K-cells (Table 2.2). Our studies used conditions that optimized transforming efficiency, however, we could not determine the total number of K-cells in the population only which K-cells integrated the transforming DNA. Thus, our 10-fold increase estimate on the likelihood that stationary-phase mutants underwent the K-state is an underestimate.

Next, our study built upon previously published work, in which stationary-phase mutagenesis was ComK- dependent, but recombination-independent (8). To further test the idea that stationary-phase mutagenesis in K-cells was transformation-independent and ComK-dependent, a transformation pore-defective mutant was measured in stationary-phase mutagenesis. The data demonstrated that the transformation-defective K-cell subpopulation showed no impairment in the ability to accumulate mutations in stressed cells (Fig. 2.2). However, there was a trend showing a consistently lower level of mutagenesis in JC101 than the

parental strain (YB955) in all three alleles tested. This warranted further investigation on the effect of defects of DNA translocation on SPM.

Previous reports that connected oxidative damage to K-cells suggested that K-cells were more susceptible to ROS (Fig. 2.3), perhaps due to changes in cell properties mediated by the assembly of transformation pores in the membrane. Interestingly, our results showed that mutagenesis in the presence of the oxidant TBH was strikingly decreased in the transformation pore-defective strain. Further, these results are in full agreement with studies in which deficiencies in the ability to repair oxidative damage in DNA significantly reduced SPM (9, 25). We also eliminated the possibility that ComEA was translocating damaged DNA that confers the ability to accumulate mutations (Fig. 2.4).

We summarized the results presented here in the context of a cellular strategy that promotes escape from environmental stress. Stressed *B. subtilis* cells differentiate into different subpopulations and those that developed into the K-state promote mutagenic events and therefore increase the adaptive potential of the cell. K-cells incorporate new DNA into their genome by the process of recombination. At the same time, however, the physiological changes required for cells to take up DNA from the environment are conducive to oxidative stress. The oxidative stress results in DNA lesions that are fixed via error-prone repair and subsequent formation of mutations that confer fitness. By limiting this type of mutagenesis to K-cells, the culture ensures that only a fraction of cells "experiment" with their DNA to generate new genes that confer fitness while protecting genome integrity in the rest of the culture. Thus, in the case of stressed *B. subtilis*, the formation of a fraction of differentiated cells that promote genetic diversity (K-cells) and a fraction of cells that becomes dormant (endospores) is a powerful adaptive trait that reconciles rapid evolution and protection of the germline.

On a broader note, this study provides insights into mutagenesis in all domains of life. How mutagenic events can actually be initiated by cells in response to stress leads to an understand an organism's ability to respond to dynamic environmental changes. Mutator phenotypes and hypermutation have been reported in bacteria experiencing antibiotic treatment stress or targeting by immune systems (26, 27). Thus, understanding how cells control increases in mutagenic events will allow us to design strategies that ameliorate the formation of cancers, and antibiotic resistance.

Figures

Table 2.1. *B. subtilis* strains, plasmids and primers used in this study.

Strains	**Relevant genotype**	**Reference or source**
YB955	*hisC952 metB5 leuC427* xin-1 Spβ^{SENS}	8
JC101	*hisC952 metB5 leuC427* xin-1 Spβ^{SENS} *comEA::neo*	This study
HAM501	YB955 *amyE*::P_{hs}—*comK* (sp) *comK*-gfp (CBL, cm)	This study
AAK502	YB955 *amyE*::P_{hs}—*comK* (sp) *comK*-gfp (CBL, cm) *comEA::neo*	This study
PERM1029	YB955 *mutY::em*	24
Plasmids	**Description**	
pHyperspank	Integrative vector, sp	David Rudner
pDG1664	Integrative vector, em	Bacillus Stock Center
Primers	**Sequence**	
Fragment A	F 5'- agctggaagctttaaggtaacgctcttgccag	This study
	R 5'- agccgtgtcgacaaatttacttgcgcttcgtc	
Fragment B	F 5'- agtcacggatcctctgcaggacgggacagtgg	This study
	R 5'- aagcctcgagctctccatcagtcggcaccccaaac	

Cm, chloramphenicol; Em, erythromycin; Neo, neomycin; Sp, spectinomycin

Table 2.2. Percentage of spectinomycin-resistant cells. Survival and revertant colonies were patched onto TBAB with spectinomycin to determine if the colonies were transformants. The proportion of Sp^r in early and late revertants was compared to the proportion of Sp^r in non-revertant cells using a Chi^2. The Chi^2 test value was 353 ($p < 0.005$) and indicated that the proportion of revertants that were transformants was significantly different from the proportion of transformants in the non-revertant population.

Strains	Early Revertant (From days 1-4)	Late Revertant (From days 5-9)	Non-revertants
DNA added	10.9% n = 550	18.1% n = 545	1.7% n = 1755
No DNA added	4.0% n = 100	1.0% n = 100	1.6% n = 440

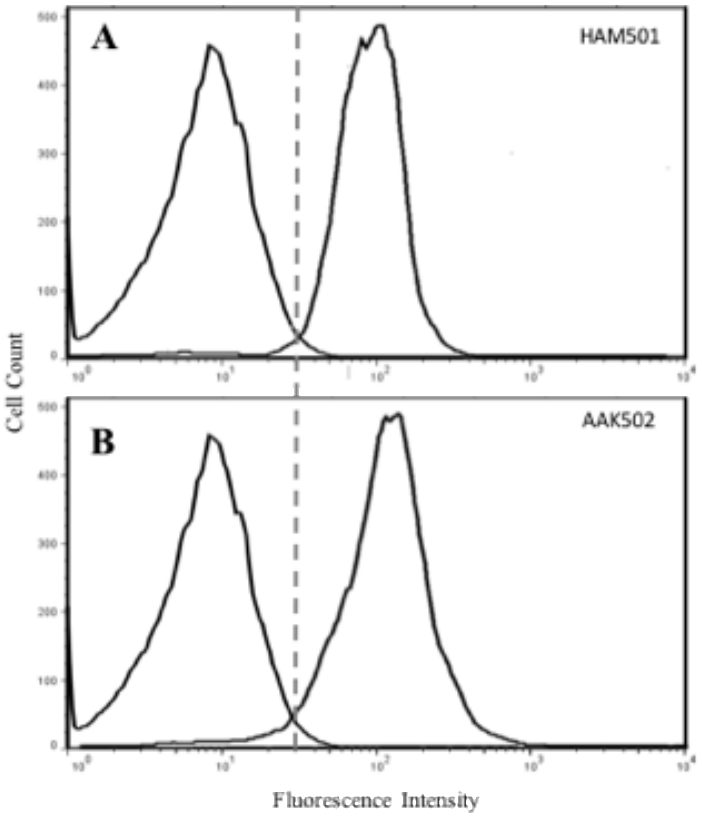


Figure 2.1. Relative expression of *comK* during stationary-phase in isogenic strains of YB955 (HAM501) and JC101 (AAK502) with an inducible *comK-gfp* fusion. (A) YB955 (HAM501) and (B) JC101 (AAK502). Left peak is relative fluoresce in uninduced culture and right peak is relative fluoresce of induced culture. Strain and experiment information in Materials and Methods.

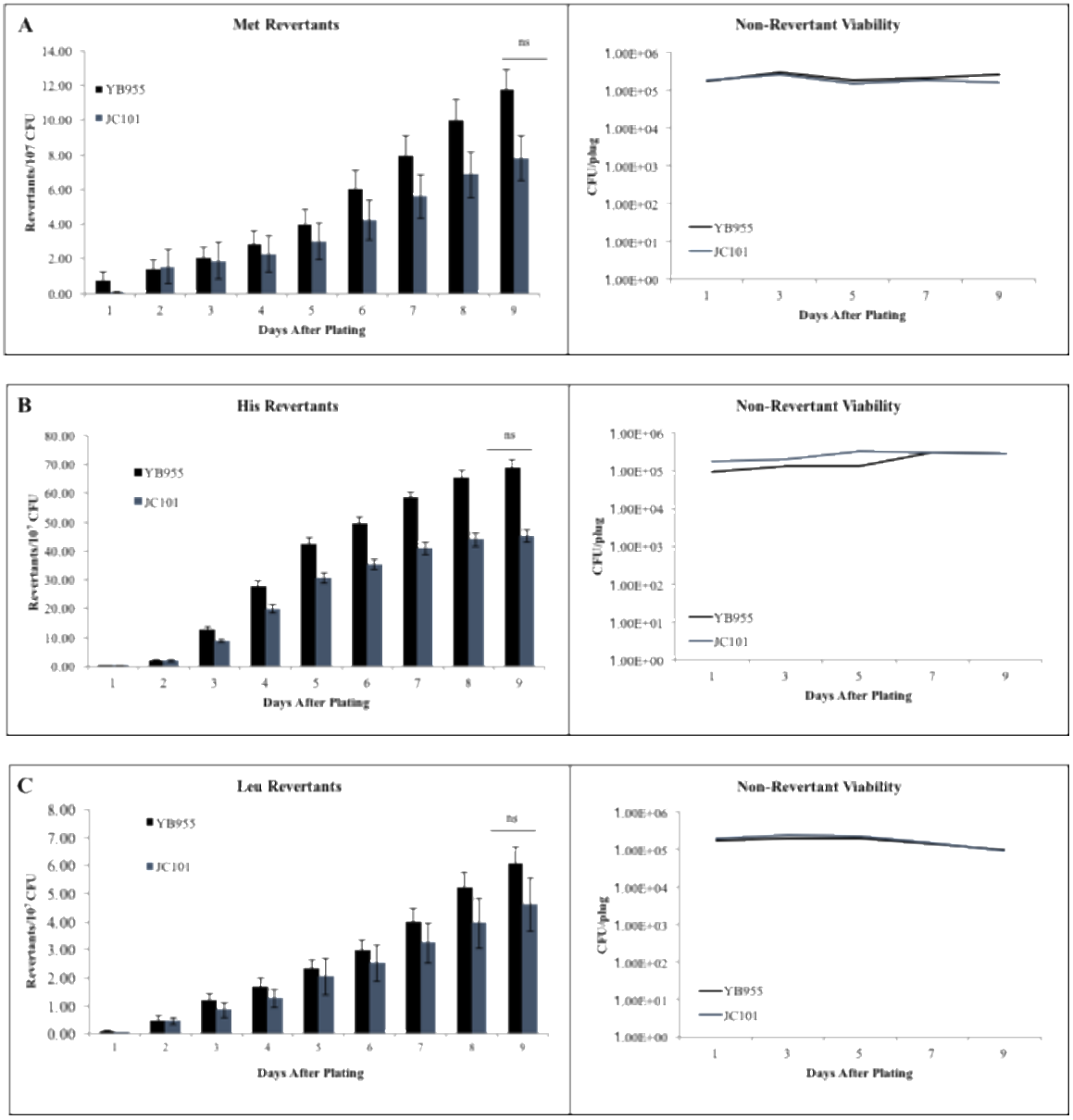
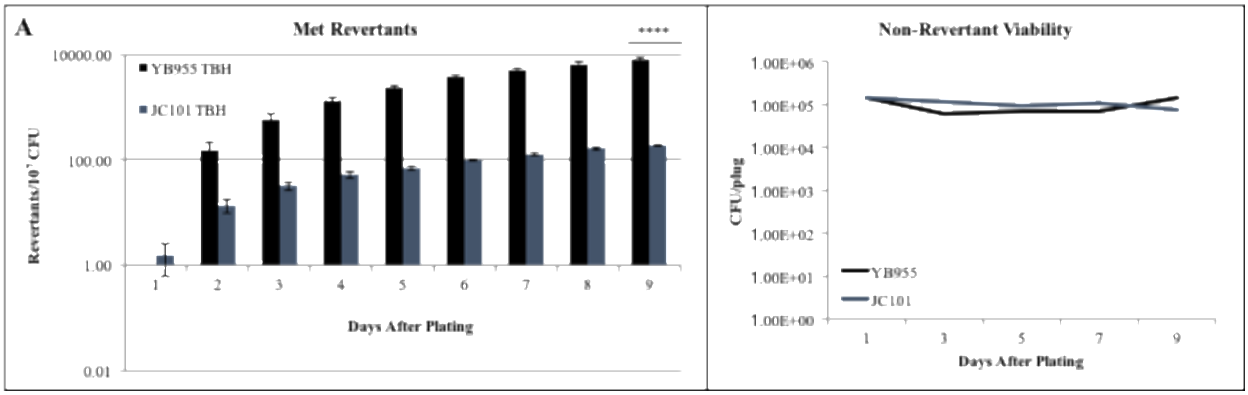


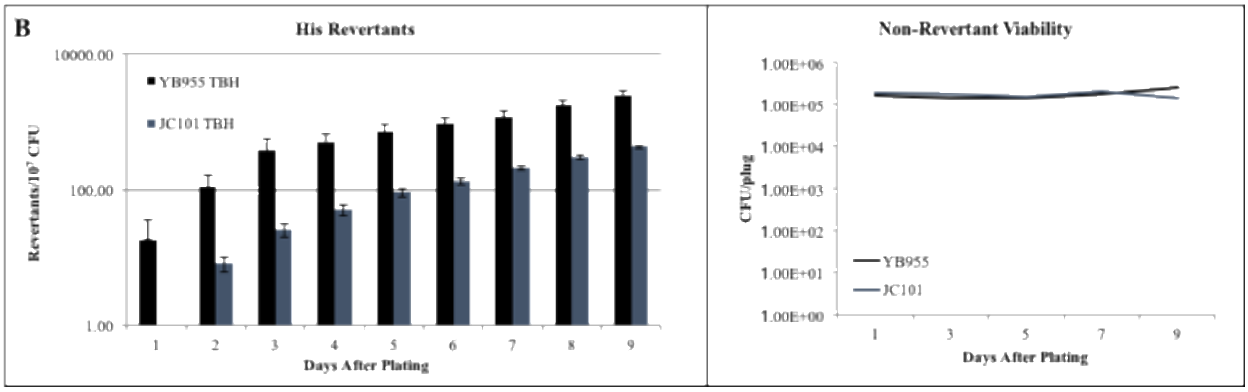
Figure 2.2. DNA-uptake stationary-phase mutagenesis assay. The accumulation of stationary-phase mutations under conditions of amino acid starvation in YB955 (parental strain) and JC101 (*comEA*). (A) The accumulation of (A) Met^+, (B) His^+ and (C) Leu^+ revertants and the non-revertant background. Left panels represent the average of at least three separate tests ± standard error of the mean (SEM). Student's t-test conducted.

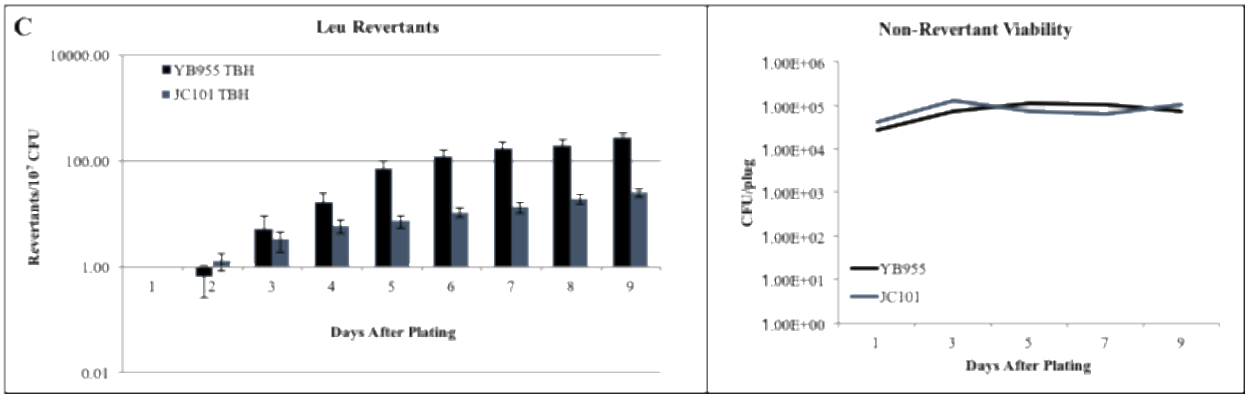
Table 2.3. Rate of mutations for strains used in this study. Rates were calculated based on reversion to prototrophy in amino acid biosynthesis.

Strain	Mutation Rate		
	His$^+$	**Met$^+$**	**Leu$^+$**
YB955	7.24E-09 ± 0.10	4.82E-10 ± 0.71	2.35E-08 ± 1.42
JC101	2.94E-09 ± 0.11	6.80E-09 ± 1.33	1.21E-09 ± 0.24
HAM501	nd	1.02577E-08 ± 0.24	nd
AAK502	nd	1.08E-09 ± 0.71	nd

Error is representative of the 95% confidence value.
nd, not determined
Estimated mutation rates calculation described in materials and methods.







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Strains	Average Percent Kill Met Trials	Average Percent Kill His Trials	Average Percent Kill Leu Trials
YB955	99.99 ± 0.00	99.98 ± 0.04	99.98 ± 0.04
JC101	99.97 ± 0.06	99.96 ± 0.03	99.96 ± 0.03

Figure 2.3. Oxidative damage stationary-phase mutagenesis assay. The accumulation of stationary-phase mutations after treatment with *tert*-Butyl hydroperoxide (TBH) under conditions of amino acid starvation in YB955 (parental strain) and JC101 (*comEA*). (A) The accumulation of (A) $Met^{+\cdot}$ revertant data was log transformed and analyzed using a Student's t-test. The Student's t-test showed significance at $p < 0.0001$ for day 9 only, (B) His^{+}, and (C) Leu^{+} revertants and the non-revertant background. (D) Percent kill of cells after 1.5 mM TBH treatment ± standard deviation (SD). All right panel data represents the average of at least three separate tests ± standard error of the mean (SEM).

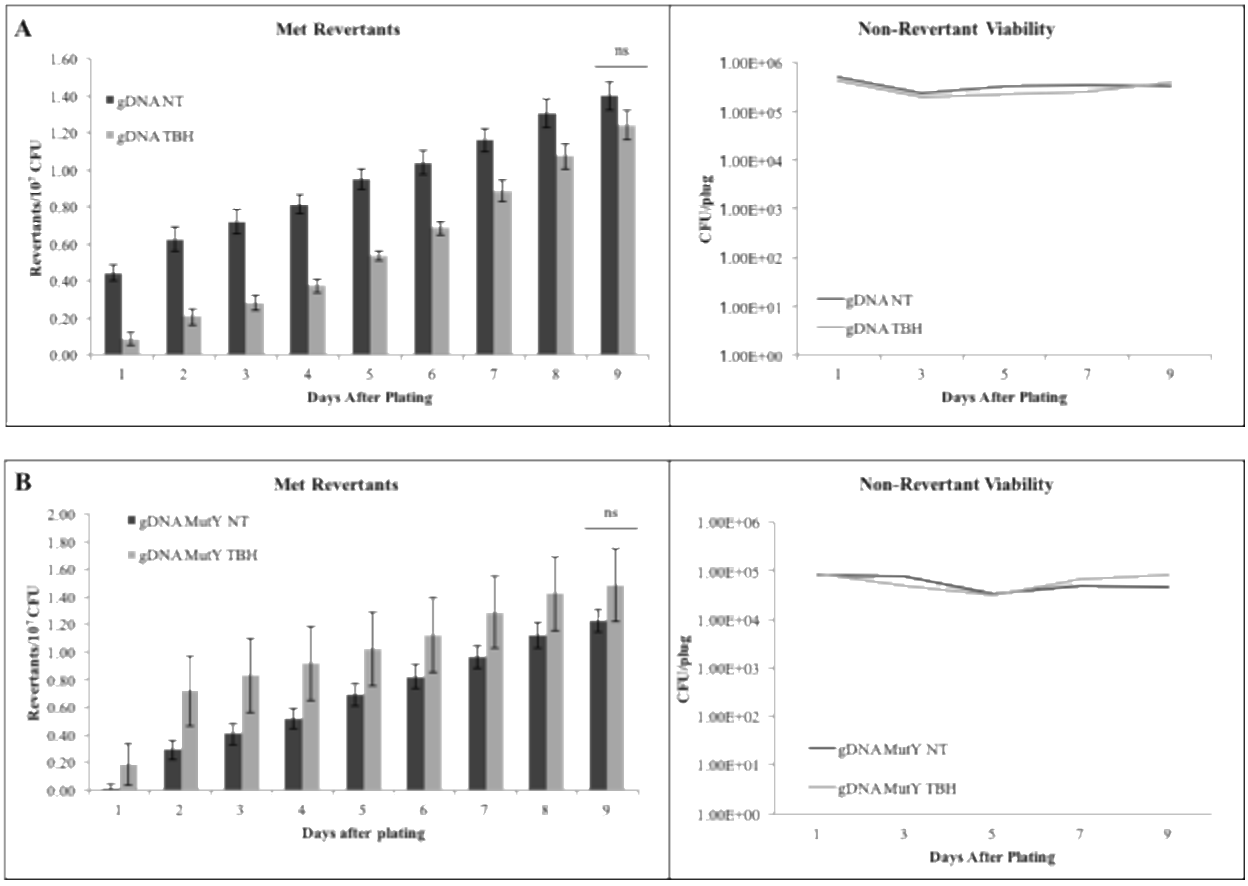

Figure 2.4. Oxidative damage gDNA stationary-phase mutagenesis. The accumulation of

stationary-phase mutations under conditions of methionine starvation in YB955 (parental strain) after transformation using damaged and undamaged gDNA. (A) The accumulation of Met^+ revertants after transformation into YB955 using damaged and undamaged (A) YB955 gDNA or (B) PERM1029 (*mutY*) gDNA and the non-revertant background. Data represents the average of at least three separate tests ± standard error of the mean (SEM). Student's t-test conducted.

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CHAPTER 3

CONCLUSION AND FUTURE DIRECTIONS

Stationary phase *Bacillus subtilis* cells develop subpopulations that exhibit different survival strategies including competence, secondary metabolite production, biofilm formation, cannibalism and endospore (1). Regulation of cell differentiation from a vegetative, phenotypically and genetically homogenous population of cells to a heterogeneous population of cells types, listed above, is a well-coordinated and complex process controlled by environmental signals and quorum sensing (2). Those signals are picked up by sensory kinases that activate the major differentiation regulators (Spo0A, DegU, and ComA) and determine a cell's fate. What "decides" if cells are competent versus another subpopulation type are fluctuations in "noise" of gene expression that lead to fluctuations in ComK levels within the cell (2). Cells that reach threshold levels of ComK commit to competence and enter the K-state. Though this state is mutually exclusive from the other subpopulations, it is transient (2).

Our understanding of the competence subpopulation has changed from being limited to recombine exogenous DNA in to the genome to an overall response to stress and adaptation that includes increases in recombination-independent mutagenesis. Competence is only a part of a larger network of adaptation stress responses (3). We learned that competence itself does not play a major role in stationary-phase mutagenesis in amino acid-starved cells (Summarized in Fig. 3.1 *Past Studies*).

Another interesting aspect of this state, not mentioned previously, is that K-cells activate proteins like Maf and ComGA which halt cell division (4). This may play a role in the accumulation of mutations in the absence of chromosome replication and active cell growth.

Previous studies indicated that oxidative damage is significant in the formation of stationary-phase mutations (5,6,7); however, whether the development of K-cells is part of the mutagenic pathway was not apparent. PerR responds to oxidative stress and controls the regulon responsible for oxidative stress adaptation (8). Surprisingly, the protein encoded by this gene indirectly stabilizes ComK levels in the cell and therefore entry into the K-state. This process occurs through activation of an operon in which the product of *comS*, a gene required for stabilization of ComK, is activated. ComS competes for binding of the MecA/ClpC/ClpP protease complex, which also degrades ComK and keeps ComK protein levels high in the cell. As oxidative stress increases so does PerR's response which leads to development of the K-state. Since this work showed that the K-state influences SPM, an interesting set of follow-up experiments is to study the effects of PerR on SPM.

If K-cells are more prone to oxidative damage, as a result of their transformation pores allowing more ROS into the cell, then error-prone repair can step in allowing for increases in mutagenesis (hypermutation) in hopes of acquiring a beneficial mutation and eventually allowing to be better adapted to their environment and grow. (Summarized in Fig. 3.1 *Present Study*)

Future directions of this project (Summarized in Fig. 3.1 *Future Studies*) will include using strains of *B. subtilis* that allow for stabilization of ComK levels and modulation of the proportion of K-cells in a population. This goal can be achieved through modulation of ComS in the cell. By increasing ComS we decrease ComK degradation. This will allow for better detection of a K-cell's abilities and increasing K-state subpopulation numbers to work with. We can then determine, by directly increasing the number of K-cells in a population if there are also increases in the accumulation of stationary-mutations as a direct result.

Next, to elucidate the ComK-dependent mechanism involved in SPM, mutants of several oxidative and ComK-dependent genes can be mutated to see the role each plays. PerR, an oxidative stress response protein is high priority for this study. Since it can directly stabilize ComK levels in a cell it would be interesting to see how the cells respond with respect to mutations with PerR is removed or induced in the cells. Then KatA (part of the PerR regulon), *B. subtilis* primary catalase can also be removed to look at the affect, of more physiological levels, in which *B. subtilis* is exposed to during both exponential and stationary-phase life cycles. Last but not least RadC, a hypothesized DNA damage repair protein (8, 9), which is directly induced by ComK seems to be an interesting candidate to determine its involvement in stationary-phase mutagenesis if any.

Finally, determining if not having a functional ComEA contributes to the protection of K-cells exposed to ROS. ComEA is an integral membrane protein (10), therefore when we knocked it out it probably destabilized the transformation complex which lead to our non-transformable phenotype. To test if our mutation itself was the cause of decrease in mutagenesis seen in the data or due to some other side effect of the mutation we can create a transformation mutant by knocking out an essential transformation gene but not one so integral to the pore itself. NucA is the membrane-associated nuclease required for digestion of dsDNA into ssDNA prior to entrance into cell (11). NucA, though essential for a cell to transform DNA into it, not such a large protein as compared to ComEA. This experiment is a way to test if we still allow pore to hold its form and does not have the ability to successfully transfer DNA into the cell if there is any change in mutagenesis.

Understating the mechanisms involved in stationary-phase mutagenesis can lead to our understanding of not only evolutionary processes, effects of oxidative damage, cell

differentiation and its role in adaptability. But potentially can lead to treatments for antibiotic resistance, diseases caused by oxidative damage and even our understanding of cancer occurrences and treatment potentiality. This work along with other in our field aim to expand our knowledge of DNA repair and mutagenesis in hopes use in research and the betterment of the human race far beyond our current understanding and application.

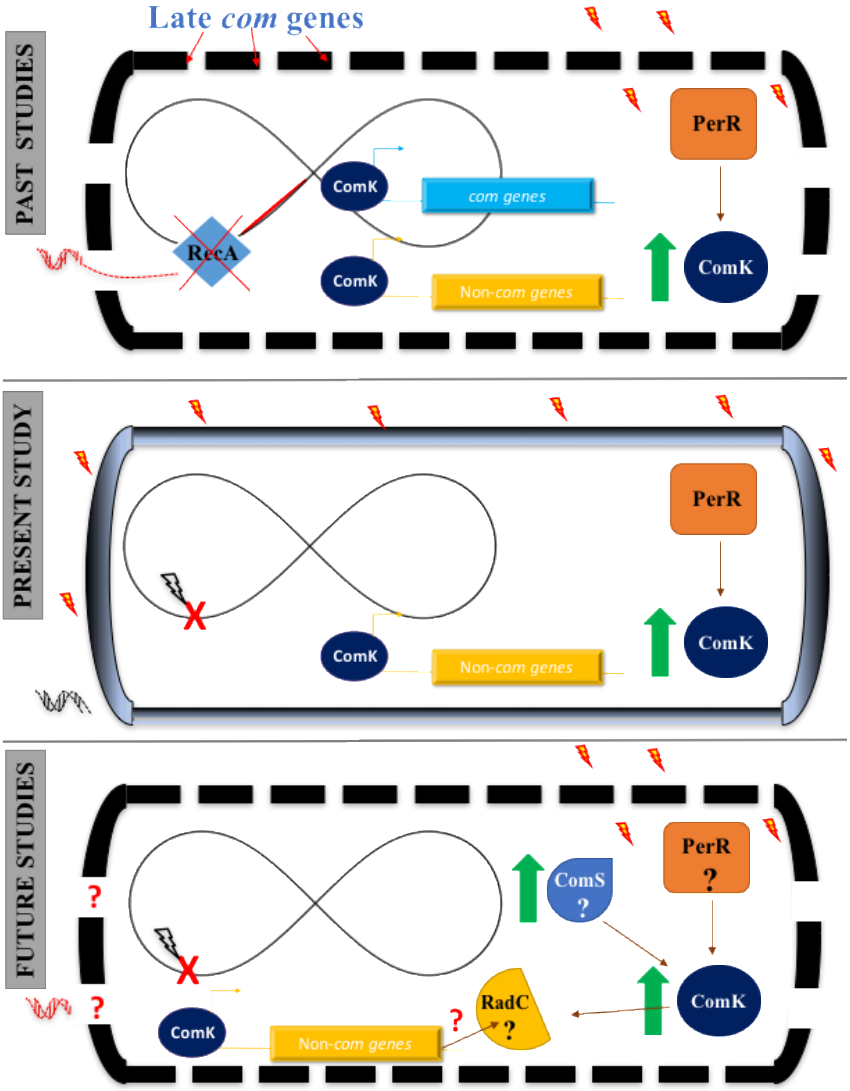

Figure 3.1. Summary figure of information gained from previous, current and future studies on the K-state and SPM**.** Past studies helped our understanding of the importance of ComK beyond competence and prompted us to hypothesize that ComK was important for SPM (7). The current study conducted in this paper built upon the previous findings and determined that K-cells were more prone to mutagenic events than non-K cells. This increase in mutagenic events due to oxidative stress. Finally, future experiments that build on the current work can examine the effects of PerR, ComS, and RadC on SPM.

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APPENDIX

SUPPLEMENTAL DATA

FORWARD

This appendix contains methods, figures and tables that were produced as part of the research project for this thesis. However, these data, because of the formatting requirement for Chapter 2 to the Journal of Bacteriology, was not included in that section. This section contains work conducted by me and Holly Anne Martin. Ms. Holly Anne Martin is responsible for Figure A1 and all research conducted to obtain these data. My contribution is all other figures and writing of this section is solely my own. I do not take credit for the research presented here other than my own.

This appendix is formatted, in conjunction with Chapter 2 for submission to the Journal of Bacteriology as supplemental.

Methods

Suppressor analysis. The procedure discussed in Sung and Yasbin (2002) was used. Met^+ and His^+ colonies are screened for growth on medium lacking histidine or methionine, respectively. This is to determine whether the Met^+ mutation is a true reversion or a suppressor mutation. Revertant colonies selected for prototrophy to one amino acid that subsequently screen positive for another amino acid protorophy are likely caused by tRNA suppressor mutations. True revertant colonies were stocked and sent for sequencing.

Oxidative-stress induced stationary-phase mutagenesis assay. To determine the effects of oxidative stress on the *B. subtilis*, strains cells were grown as described in the stationary-phase mutagenesis assay and when the cultures reached T_{90} they were treated with 1.5 mM *tert*-Butyl hydroperoxide (TBH; Luperox(R) TBH70X Sigma-Aldrich) for 2 h, double washed 1X Spizizen salt solution (SMS), and plated as described above. If strains were used with an IPTG-inducible promoter cultures cells were grown to 0.7-0.8 OD_{600}, split into two batches. One batch was supplemented 0.1 mM IPTG for induction of *comK*.

UV Damage. Strains were grown to mid-exponential phase and T_{90}. IPTG was added to inducible strains at late exponential as described previously. Cells were harvested by centrifugation, resuspended in 1X SMS, serially diluted to 10^{-7}, and Then plated as 3-10 μL dilution on TBAB with required antibiotics. Once dried plates were exposed 0, 25, and 50 $J/m^2/s$, of UVc respectively.

Figures

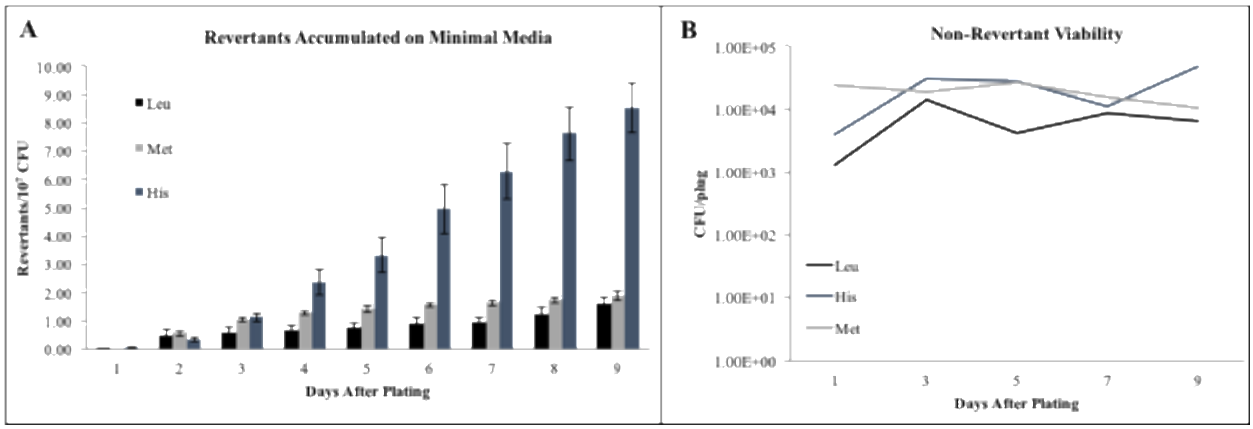


Figure A1. The accumulation of mutations in YB955 using transformation stationary-phase mutagenesis assay and transformation marker (pHyperspank) (See Material and Methods in Chapter 2). This data accompanies Table 2.1. (A) The trend of the accumulation of revertants is constant with previous SPM experiments. (B) The non-revertant background remains constant throughout the 9 days. Data represents the average three separate tests ± standard error of the mean (SEM).

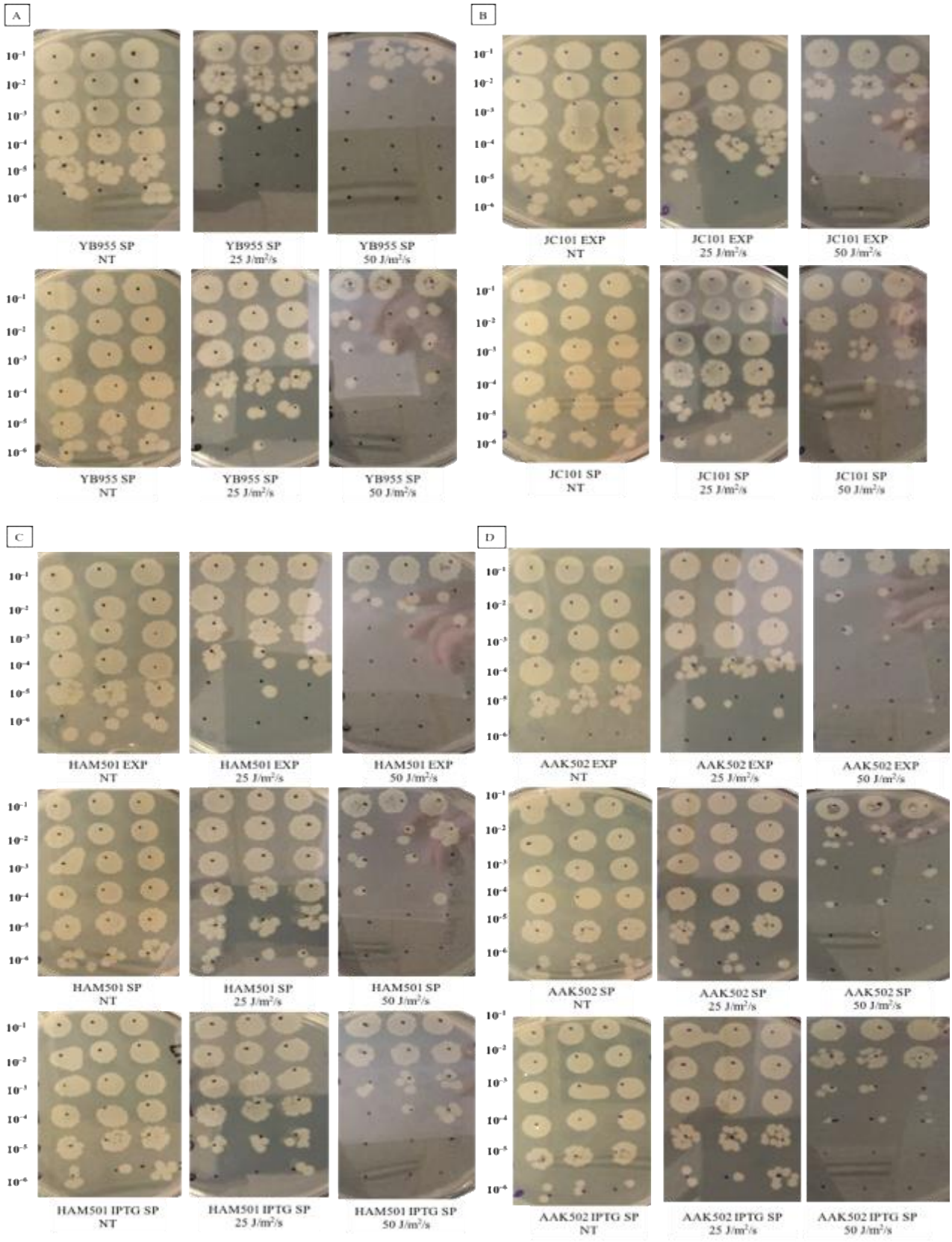

A
B
C
D
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
YB955 SP NT
YB955 SP 25 J/m²/s
YB955 SP 50 J/m²/s
JC101 EXP NT
JC101 EXP 25 J/m²/s
JC101 EXP 50 J/m²/s
JC101 SP NT
JC101 SP 25 J/m²/s
JC101 SP 50 J/m²/s
HAM501 EXP NT
HAM501 EXP 25 J/m²/s
HAM501 EXP 50 J/m²/s
HAM501 SP NT
HAM501 SP 25 J/m²/s
HAM501 SP 50 J/m²/s
HAM501 IPTG SP NT
HAM501 IPTG SP 25 J/m²/s
HAM501 IPTG SP 50 J/m²/s
AAK502 EXP NT
AAK502 EXP 25 J/m²/s
AAK502 EXP 50 J/m²/s
AAK502 SP NT
AAK502 SP 25 J/m²/s
AAK502 SP 50 J/m²/s
AAK502 IPTG SP NT
AAK502 IPTG SP 25 J/m²/s
AAK502 IPTG SP 50 J/m²/s

Figure A2. UV-damage exposure to *B. subtilis* strains. Strains showed no differences in cell survival when exposed to UV damage**.** (A) YB955 during exponential and stationary-phase exposed to 0, 25, and 50 J/m^2/s, of UVc. (B) JC101 during exponential and stationary-phase exposed to 0, 25, and 50 J/m^2/s, of UVc. (C) HAM501 during exponential and stationary-phase (uninduced and induced) exposed to 0, 25, and 50 J/m^2/s, of UVc. (D) HAM501 during exponential and stationary-phase (uninduced and induced) exposed to 0, 25, and 50 J/m^2/s, of UVc.

Table A1. Suppressor analysis for DNA-uptake stationary-phase assay (Fig. 2.2).

Results show more suppressor mutations are acquired during exponential growth than stationary-phase. This is consistent with previously published results (Sung and Yasbin 2002).

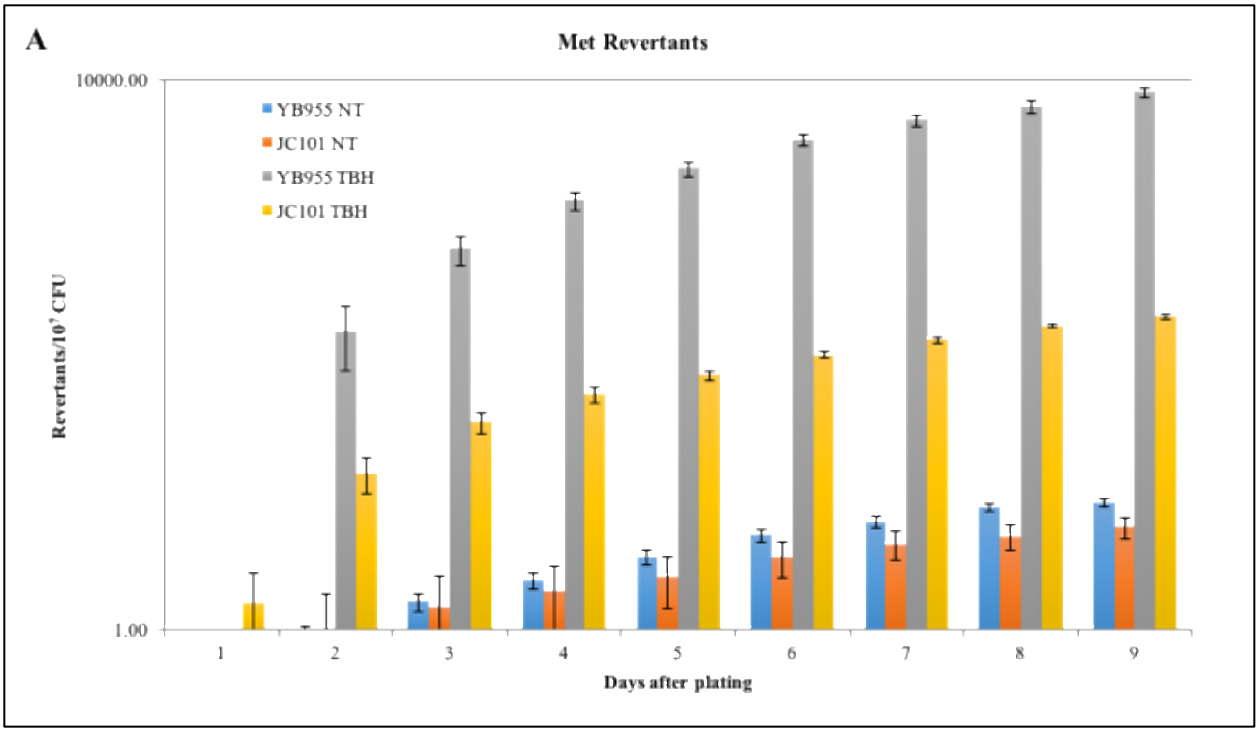
Amino Acid	Strain	Early Revertant (From Day 1-4)	Late Revertant (From Day 5-9)
Methionine	YB955	45%	19%
Methionine	JC101	32%	30%
Histidine	YB955	6%	4%
Histidine	JC101	39%	8%

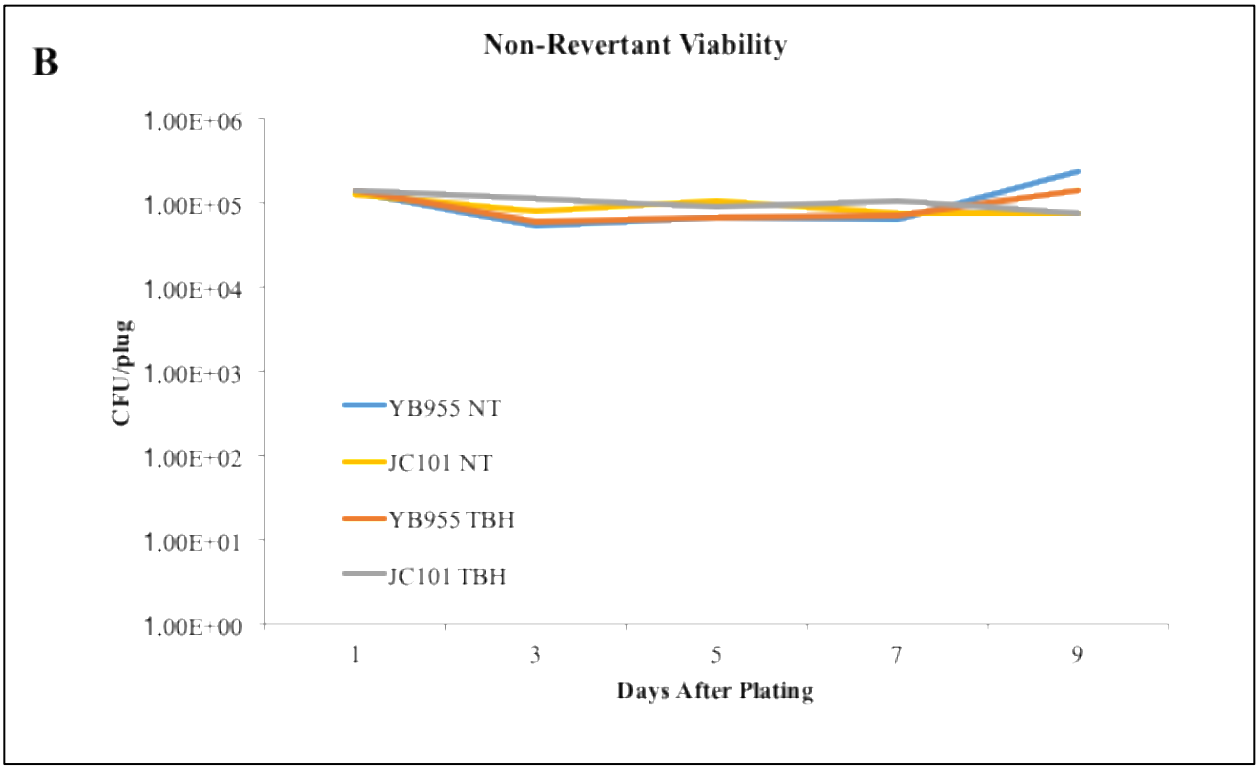
Table A2. Suppressor analysis for oxidative damage stationary-phase mutagenesis assay (Fig. 2.3). Results show more suppressor mutations are acquired during exponential growth than stationary-phase. This is consistent with previously published results (Sung and Yasbin 2002).

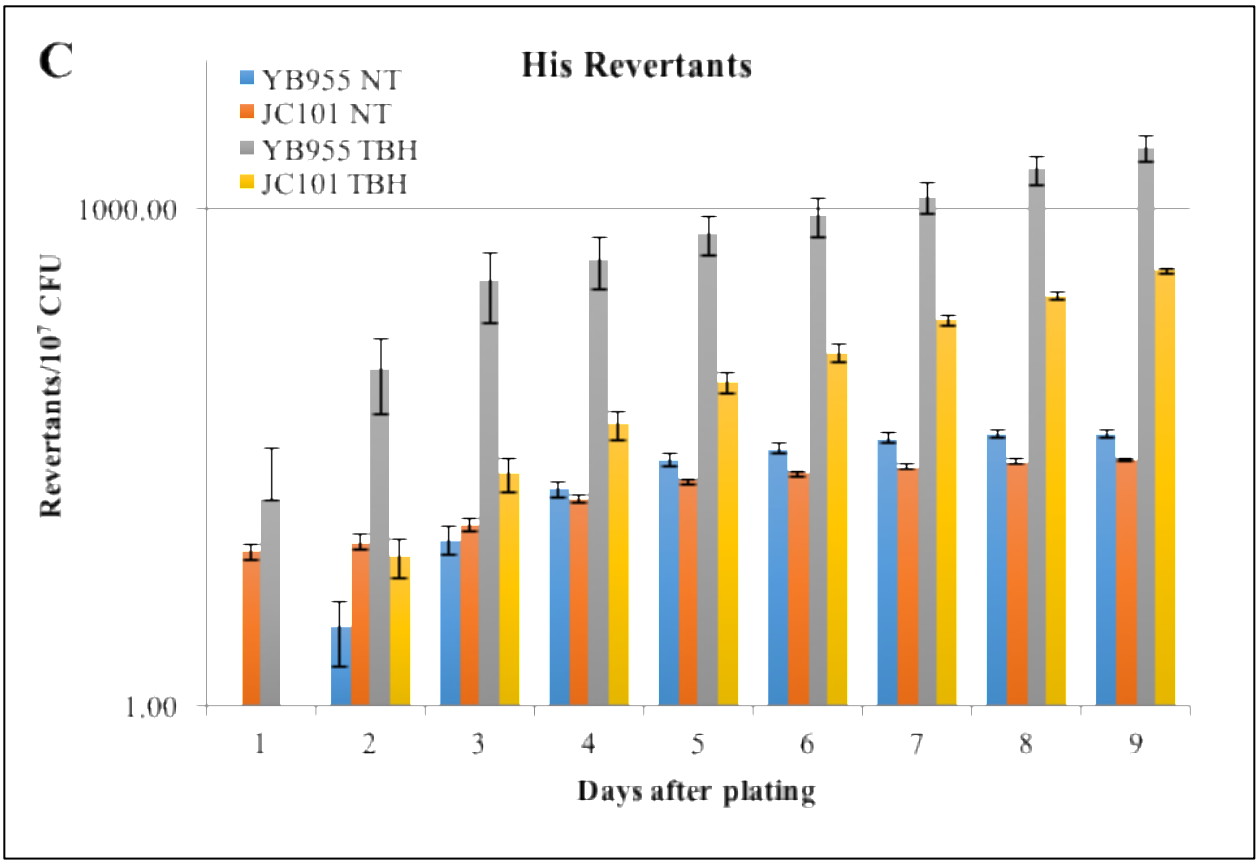
Amino Acid	Strain/Condition	Early Revertant (From Day 1-4)	Late Revertant (From Day 5-9)
Methionine	YB955 NT	64%	27%
Methionine	JC101 NT	80%	45%
Histidine	YB955 NT	11%	4%
Histidine	JC101 NT	31%	5%
Methionine	YB955 TBH	51%	25%
Methionine	JC101 TBH	68%	44%
Histidine	YB955 TBH	12%	8%
Histidine	JC101 TBH	25%	13%

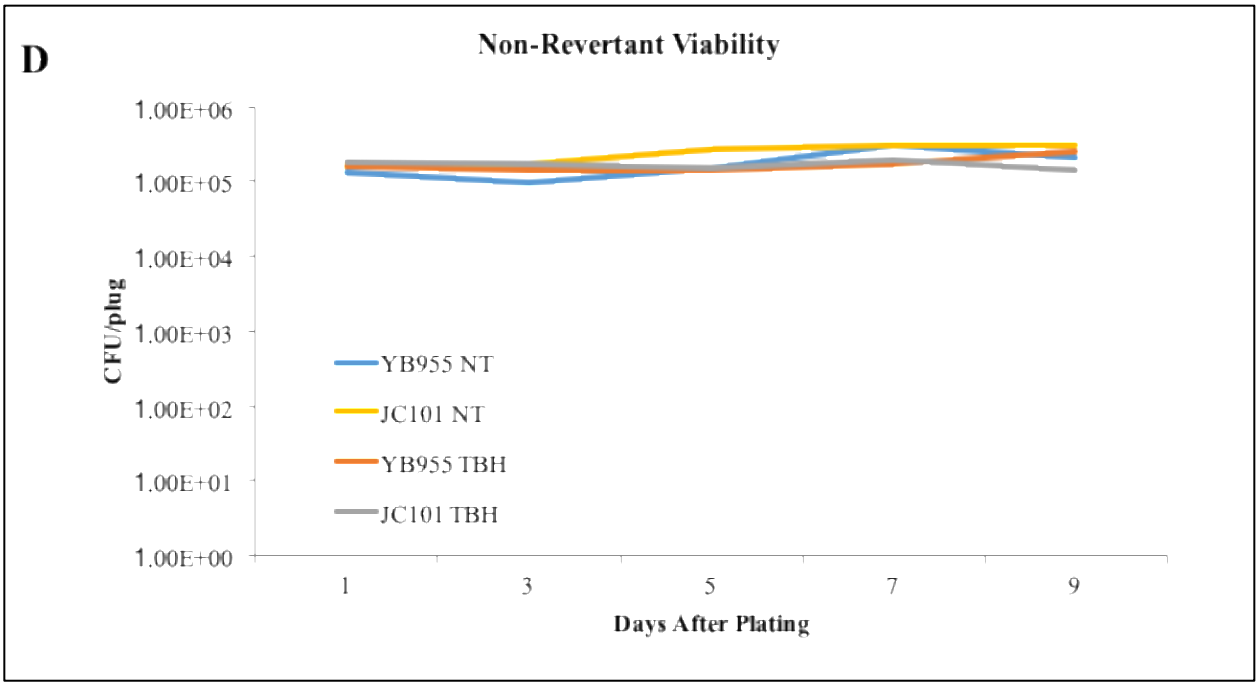
Table A3. Suppressor analysis for oxidative damage gDNA stationary-phase mutagenesis assay (Fig. 2.4). Results show more suppressor mutations are acquired during stationary-phase growth than stationary-phase (top two rows) which is not consistent with previously published work (Sung and Yasbin 2002). However, Bottom two rows show more suppressor mutations from exponential growth then stationary-phase and this is consistent with previously published results (Sung and Yasbin 2002).

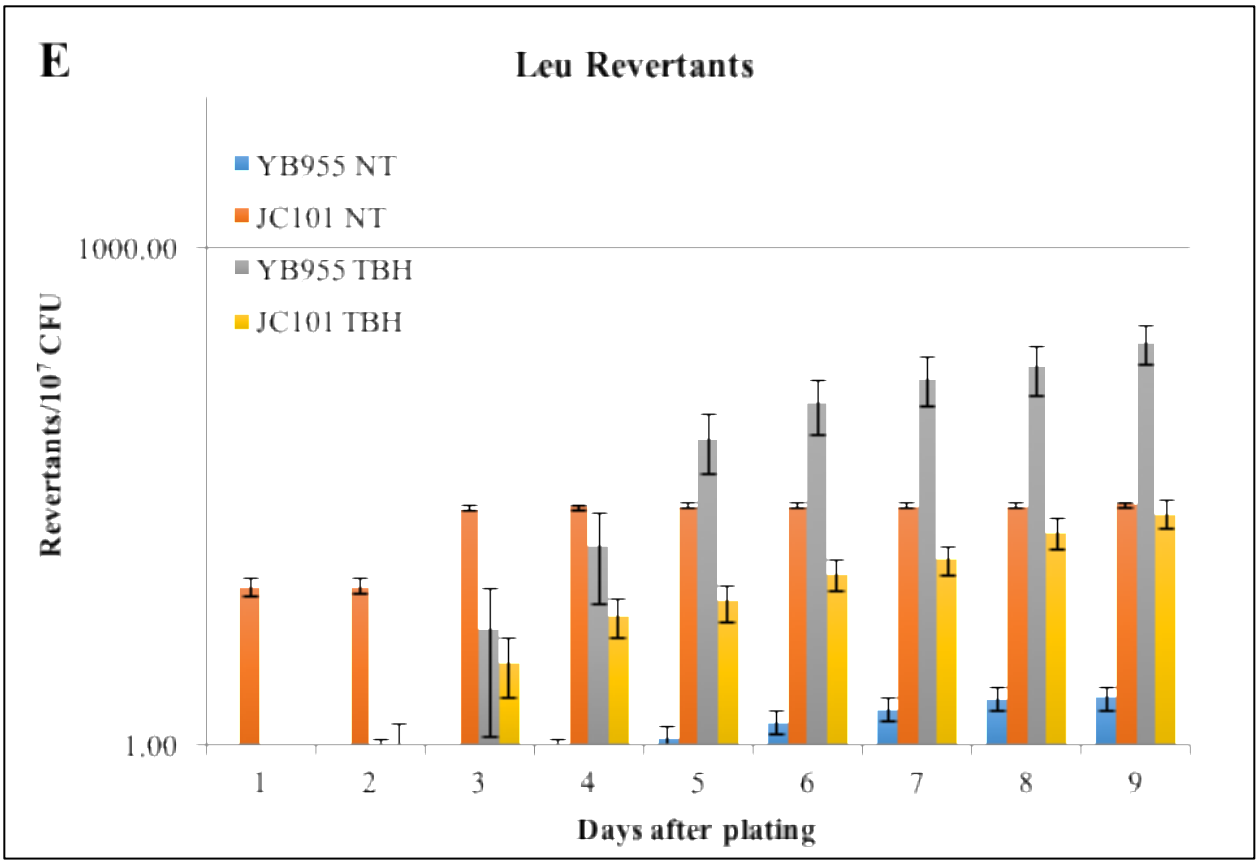
Strain/Condition	Early Revertant (From Day 1-4)	Late Revertant (From Day 5-9)
YB955 gDNA NT → YB955	20%	44%
YB955 gDNA TBH → YB955	25%	35%
PERM1029 gDNA NT → YB955	55%	42%
PERM1029 gDNA TBH → YB955	51%	37%


A
Met Revertants
10000.00
1.00
YB955 NT
JC101 NT
YB955 TBH
JC101 TBH
Revertants/10⁷ CFU
1
2
3
4
5
6
7
8
9
Days after plating


B
Non-Revertant Viability
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU/plug
YB955 NT
JC101 NT
YB955 TBH
JC101 TBH
1
3
5
7
9
Days After Plating


C
His Revertants
YB955 NT
JC101 NT
YB955 TBH
JC101 TBH
1000.00
1.00
Revertants/10^7 CFU
1
2
3
4
5
6
7
8
9
Days after plating


D
Non-Revertant Viability
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU/plug
YB955 NT
JC101 NT
YB955 TBH
JC101 TBH
1
3
5
7
9
Days After Plating


E
Leu Revertants
YB955 NT
JC101 NT
YB955 TBH
JC101 TBH
1000.00
1.00
Revertants/10^7 CFU
1
2
3
4
5
6
7
8
9
Days after plating

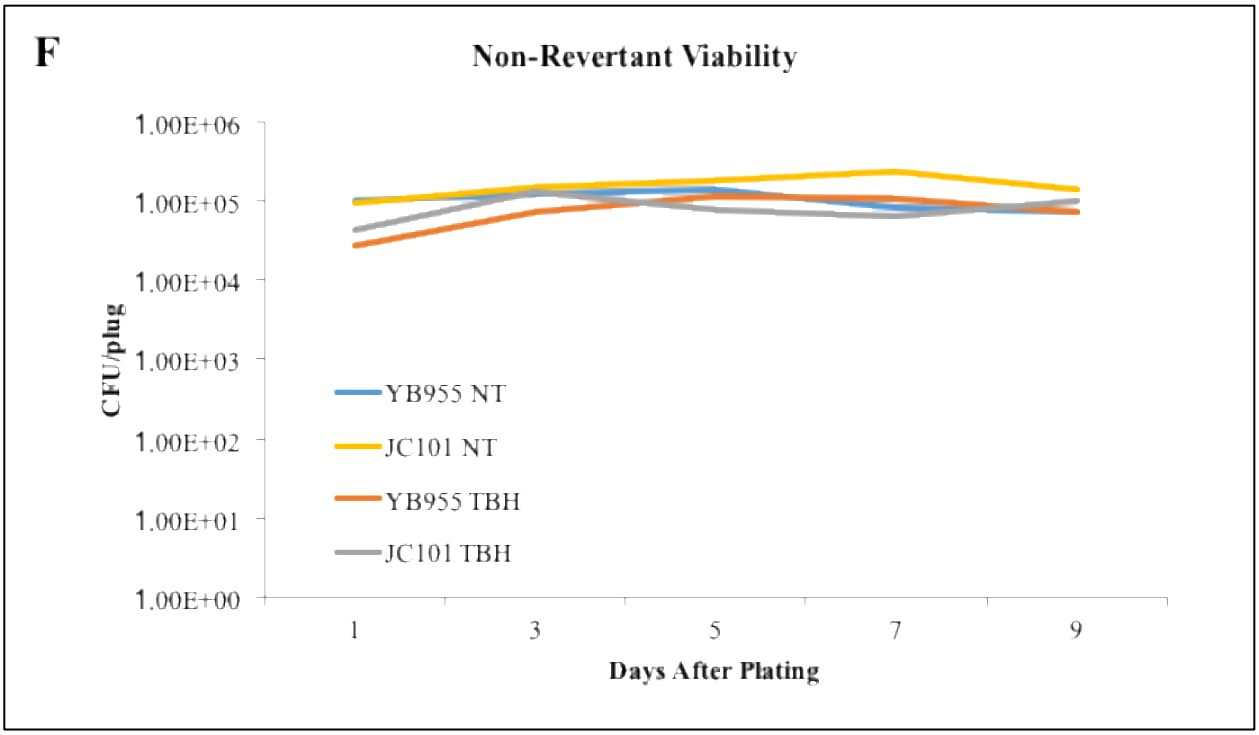
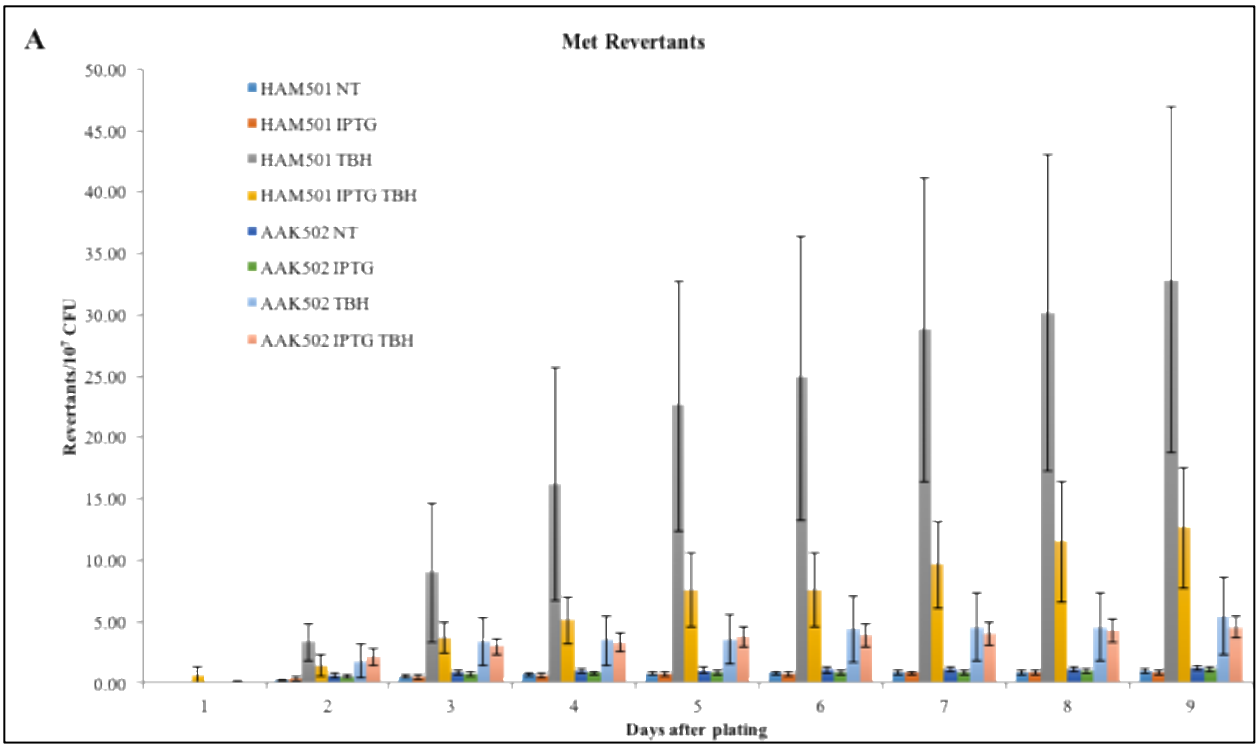
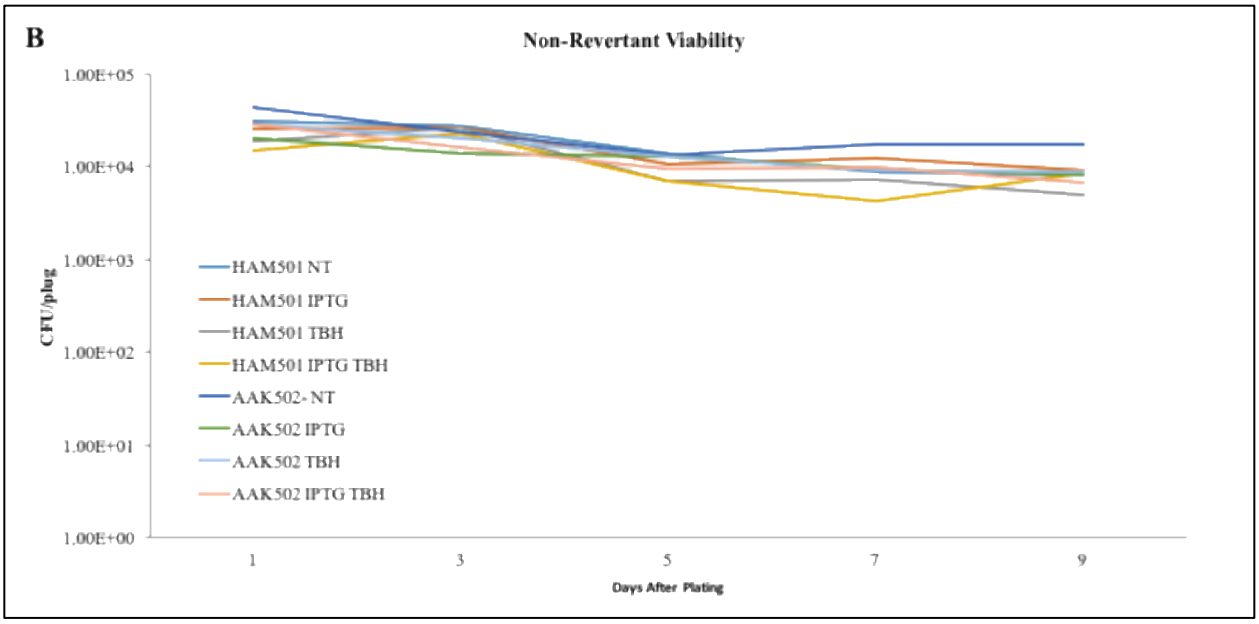

F
Non-Revertant Viability
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU/plug
YB955 NT
JC101 NT
YB955 TBH
JC101 TBH
1
3
5
7
9
Days After Plating

Figure A3. Oxidative damage stationary-phase mutagenesis assay with additional treatments. The accumulation of stationary-phase mutations after treatment with and without *tert*-Butyl hydroperoxide (TBH) under conditions of amino acid starvation in YB955 (parental strain) and JC101 (*comEA*). (A, B) The accumulation of Met^+ revertants and the non-revertant background. (C, D) The accumulation of His^+ revertants and the non-revertant background. (E, F) The accumulation of Leu^+ revertants and the non-revertant background. Data represents the average of at least three separate tests ± standard error of the mean (SEM).





C

	HAM501	**HAM501 IPTG**	**AAK502**	**AAK502 IPTG**
Average percent kill	99.99 ± 0.00	99.99 ± 0.01	99.98 ± 0.06	99.98 ± 0.11

Figure A4. Oxidative damage stationary-phase mutagenesis assay on inducible-*comK* strains. The accumulation of stationary-phase mutations after treatment with *tert*-Butyl hydroperoxide (TBH) under conditions of starvation in HAM501 and AAK502. (A) The accumulation of Met^+ revertants and the non-revertant background. (B) Non-revertant cell viability. (C) Percentage kill of cells after 1.5 mM TBH treatment ± standard deviation (SD). Data represents the average of three separate tests ± standard error of the mean (SEM).

Table A4. Suppressor analysis from TBH SPM Assay on HAM501 and AAK502 (Fig. A4). Results show notable trend and results are not consistent with previously published data (Sung and Yasbin 2002)

Strain/Condition	**Early Revertant (From Day 1-4)**	**Late Revertant (From Day 5-9)**
HAM501	53%	50%
HAM501 IPTG	53%	70%
HAM501 TBH	13%	36%
HAM501 IPTG TBH	67%	17%
AAK502	47%	100%
AAK502 IPTG	50%	57%
AAK502 TBH	42%	70%
AAK502 IPTG TBH	40%	29%

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Publications:
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