ABSTRACT

PEACOCK, TRENT JARRELL. Biological Role of Mn-catalase in Select Lactobacilli. (Under the direction of Hosni M. Hassan.)

Mechanisms for protection against oxidative stress within species of *Lactobacillus* vary widely, encompassing manganese accumulation, peroxidases, and both heme and non-heme (Manganese containing) catalases. While most species of *Lactobacillus* accumulate manganese, to mM levels, and contain peroxidases, heme and manganese catalases are limited to a select few lactobacilli. Furthermore, manganese catalases are documented in only two *Lactobacillus* species, one of which is *Lactobacillus plantarum* ATCC 14431. Therefore, the presence of catalases in lactobacilli represents a unique opportunity to investigate both the biological role and potential advantages associated with having Mn-catalase in the native host as well as in *Lactobacillus* species utilized in the food industry and as probiotics, that are normally lacking catalases.

To address the biological role of Mn-catalase, a *mnkat*$^-$ strain of *L. plantarum* ATCC 14431 was constructed through insertional inactivation. Findings show that Mn-catalase is essential for normal growth of *L. plantarum* ATCC 14431 under aerobic conditions and that the protein is critical for removing H_2O_2 generated during aerobic growth. Additionally, the inactivation of *mnkat* results in an increased sensitivity to exogenous H_2O_2, though growth in Mn-rich media does improve both general growth and growth in the presence of H_2O_2.

It is also of interest to be able to successfully obtain multiple strains of *Lactobacillus* that have potential use as starter cultures or probiotics that have an improved antioxidant capacity. To address this, the 1449-bp manganese catalase gene from *L. plantarum* CECT 221 (ATCC 14431), including its native promoter, has been cloned into the shuttle vector pTRK563. The resulting pMnKat was transformed into *L. reuteri* NCK 932 and *L. gasseri* NCK 334. Manganese catalase (Mn-catalase) activity was assayed and detected in both species. Furthermore, expressing MnKat leads to increased growth rate (μ_{max}•hr^{-1}), increased resistance to H_2O_2 concentrations as high as 10 mM, and increases long term survival under aerobic conditions.

Biological Role of Mn-catalase in Select Lactobacilli

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DEDICATION

To Jesus Christ, my Lord and Redeemer, you are the same yesterday, today and forever. To my Dad and Mom, who always pushed me to pursue the challenges and always see things through to the end. With great admiration and appreciation to my wife and son, who have endured this calling in my life, which seemingly kept me away and occupied a great majority of the time, thank you for your sacrifice. To my dear friends in the Hassan, Grunden, Olson, and Hyman labs who were always willing to listen, provide input/expertise, and in general keep me balanced; thank you. To Dr. Hassan, thank you for allowing me to be a part of your lab, allowing me to make mistakes and learn from them and pulling me back in when I needed it. My committee members, Dr. Brown, Dr. Grunden, Dr. Klaenhammer, and Dr. Miller, each of you at some point has taught me valuable lessons that pertain to both science and life by which I was able to become more mature and well-rounded. To T.J., your experience in life and science has been refreshing and I look forward to longer conversations in the future. To Cindy, thank you for your instincts, they were always right. Lastly, to Ryan, José, and Matt thank you for the time and effort you put in to get me where I needed to be, but most of all your friendship is what I cherish most.

BIOGRAPHY

Life is to be cherished, but can so easily be wasted away by frivolous pursuits that are lost in the wastelands of neglected history. So as it is written, “And it is appointed for men to die once, but after this the judgement, so Christ was offered once to bear the sins of many. To those who eagerly wait for Him He will appear a second time, apart from sin, for salvation.”(Hebrews 9:27-28(NKJV)). My biography is thus, God, Family, and Country.

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CHAPTER I

Literature Review

1.1 Lactic Acid Bacteria – An Overview

Lactic acid bacteria (LAB) are a diverse group of low G+C, Gram-positive obligate fermenters that occupy several environmental niches from foodstuffs to plants to animal/human GI and urogenitals. Phylogenetically, this group of organisms overlaps with both the aerobic and facultatively anaerobic organisms that compose the Gram-positive low G+C group (i.e. *Bacillus, Listeria, Staphylococcus*) (17), contrary to the original thought that LAB formed their own phylogenetic supercluster that was clustered between the aerobic and strictly anaerobic microorganisms (phylogenetic tree referenced per 17).

By definition LAB are considered a group of catalase negative fastidious non-sporulating Gram-positive microorganisms that are obligate fermenters lacking a respiratory chain as well as the ability to synthesize heme (17, 91). A key component to the traditional definition of LAB is that they lack the ability to synthesize heme, and catalase activity is absent. However, some members of the group have the ability to synthesize heme- and non-heme-catalases, a key differential biochemical attribute in the traditional definition, and express cytochromes, key to a functional electron transport chain (37, 50, 61, 68, 96-98, 115, 117, 131, 159, 160, 195-197, 199). Instead, LAB is a heterogeneous mixture of twenty different genera, grouped into three different categories; obligate homofermenters, obligate heterofermenters, and facultative heterofermenters (17, 100) based upon individual organisms primary sugar fermentation pathways (17). Due to the heterogeneity of the group in both physiology and ecology, they must endure a variety of stressors (i.e. acid, osmotic, cold, salt/bile-salt, oxygen, heat, starvation). As a result, this group of organisms has developed specific systems to combat the different stressors (122, 181). With such diversity among LAB, they are used in a variety of applications to include, imparting food properties (i.e. flavoring, aroma, acidification, texture), acting as antagonists to food pathogens, (i.e. producing hydrogen peroxide and bacteriocins), and imparting health benefits when consumed.

At present within the LAB group there are completed sequenced genomes of *Lactococcus lactis, Streptococcus mutans, Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus pyogenes, Streptococcus thermophilus, Lactobacillus plantarum, Lactobacillus johnsonii, Lactobacillus acidophilus, Lactobacillus gasseri, Lactobacillus brevis, Lactobacillus casei, Lactobacillus reuteri, Leuconostoc mesenteroides, Oenococcus oeni,* and *Pediococcus pentosaceus* (3, 6, 29, 30, 66, 110, 111, 113, 123, 170, 175, 176). With the influx of genomic data there has been an effort to identify new and novel targets (6, 27, 53, 110, 112, 113, 123, 170, 184), which could lead to improved strains for industrial (i.e. metabolic engineering) and medical (i.e. live vaccines, immunotherapeutics) use (17).

In the coming years of LAB research, the cornerstone of advances in the field will stem from genomics. Progression in the area of LAB genomics, will lead to advances within metabolomics, proteomics, taxonomy, ecology, and their application to industry/medicine (17). By advancing in these critical areas, the rapid deployment of LAB based biotechnologies and their implementation in both public and private sectors will accelerate.

1.2 Genus – *Lactobacillus*

This genus is the largest of the LAB group and to date has approximately 80-100 species that occur in many different niches ranging from plants and animals to breads and dairy (25, 83). They occur in all three divisions of LAB, being present in the obligate homo- and heterofermenters (Group A & C lactobacilli) as well in the facultative heterofermenters (Group B lactobacilli), the basis of the divisions being the presence or absence of fructose-1, 6-diphosphate aldolase and phosphoketolase (83, 91, 100). Cells of *Lactobacillus* species are typically straight or curved rods though some may appear as a coccoidal rod (83, 91). Cell shape variation was once the basis for the subgenera of this group of organisms (146). Though phenotype variation is useful, it is no longer used as differential characteristic. Instead, lactobacilli are differentiated based upon tetrad formation, CO_2 release from glucose, environmental growth parameters (e.g., temperature, pH, salt), lactic acid isomers produced and mol% G+C, which has a range of 32-55% (17, 104, 166).

It was once thought that the phylogeny of lactobacilli could be based solely upon the cell shape of the organisms (146). However, this is not possible, nor is it possible to determine relatedness within this genus using identifiers found within the classical definitions of LAB and lactobacilli. Based upon the classical definitions involving cell shape and fermentation patterns lactobacilli were grouped as, *Lactobacillus delbrueckii*, *Lactobacillus-Pediococcus,* and *Leuconostocs* (46, 83, 125, 166). However, with the unrelatedness of cell shape within the *Lactobacillus-Pediococcus* group an alternative measure of relatedness was essential. Utilization of 16S rRNA gene analysis strategies came to define the relatedness of members within this complex genus of organisms, and as a result the genus *Lactobacillus* has been divided into seven different subgroups. Those subgroups consist of *Lactobacillus acidophilus* (*L. acidophilus*) (previously *Lactobacillus delbrueckii*), *Lactobacillus casei* (*L. casei*), *Lactobacillus plantarum* (*L. plantarum*), *Lactobacillus sakei* (*L. sakei*), *Lactobacillus buchneri* (*L. buchneri*), *Lactobacillus salivarius* (*L. salivarius*), *Lactobacillus reuteri* (*L. reuteri*) (25, 83, 125, 166). As predicted, the classical biochemical and phenotypical definitions could not accurately place the lactobacilli within their correct groups, and with the exception of the *L. buchneri, L. reuteri,* and *L. sakei* groups the remaining four groups contain a mixture of groups A, B, and C lactobacilli. The *Pediococci, Weisella, Leuconostoc,* and some unique lactobacilli that have yet to be grouped are still maintained within the family Lactobacillaceae.

Lactobacilli genetics is constantly evolving and it is now possible to perform complementary comparative and functional genomic studies as well as more exacting phylogenetic studies (169). Over the past twenty-plus years that LAB genetics has been studied and prior to the completed sequences of various lactobacilli, the majority of genetic studies were performed in the classical ways, identifying a phenotype and attempting to map its genotype using standard molecular methodologies along with LAB specific techniques. However, with the genome sequences of *Lactobacillus gasseri, Lactobacillus johnsonii, Lactobacillus acidophilus, Lactobacillus plantarum*, *Lactobacillus brevis, Lactobacillus casei, Lactobacillus delbrueckii,* and *Lactobacillus reuteri* available, (6, 110, 113, 123, 155) it has been possible to improve our understanding of lactobacilli.

Barrangou, et al. (20), published a study on fructooligosaccharides (FOS) utilization in *Lactobacillus acidophilus* NCFM, based upon analysis and identification of an FOS transport and catabolic gene cluster present in the genome (6). These compounds have been associated with an increase in bifidobacteria and lactobacilli in the human GI tract, which are beneficial commensals of that environment. Shortly before Barrangou, (20), published their findings, Kleerebezem et al. (113) published the sequenced and annotated genome of *L. plantarum* WCFS1. The release of the genome brought about studies encompassing comparative genome analyses (27) to measuring the influence the dairy environment has upon gene expression (111). Following the publication of the *L. plantarum* WCFS1 genome the genomes of *L. gasseri, L. johnsonii, L. acidophilus, L. brevis, L. casei. L. delbrueckii* and *L. reuteri* (6, 111, 123, 127, 155) in conjunction with the Joint Genome Institute were released. With this release, as with *L. plantarum* WCFS1, came numerous papers that used the genomes for more in-depth investigations of taxonomy, metabolomics, proteomics, host response, nutrition, medicine, industry, ecology and phylogenetics (5, 18-20, 22, 24, 25, 28, 34, 38, 40, 41, 45, 54, 57, 60, 74, 81, 110, 111, 121, 128, 129, 133, 135, 154, 163, 169, 173, 177, 178, 180, 182, 184-186, 201).

1.3 Species-*Lactobacillus plantarum*

Lactobacillus plantarum falls into the Kingdom Bacteria, Division Firmicutes, Class Bacilli, Order Lactobacillales, and Family Lactobacillaceae. It is a Gram-positive facultative heterofermenting rod and part of the *L. plantarum* phylogenetic group. *L. plantarum* is one of the most adaptive of the LAB as well as of the lactobacilli genus and contains one of the largest genomes of the lactic acid bacteria at 3.3 Mb (113, 133). This species is found in plant/vegetable fermentations, meat fermentations and as part of the human/animal microbiota. Having such a wide range of niches suggests that this particular species of *Lactobacillus* is quite flexible at adapting to environmental change. This adaptability/flexibility has brought about development of model *L. plantarum* genetic systems (i.e. vectors, controlled gene expression) (31, 65, 90, 150) and novel probiotic and vaccine delivery applications for human/animal use (51, 77-79, 153).

Other clinical uses of *L. plantarum* include protections against side effects of radiation therapy (124), treatment for irritable bowel disorders (119, 164, 165, 167, 200), immunomodulation (39, 49, 80, 88, 148, 149), and cancer therapy (23, 48, 64, 137, 147, 188). Based upon the wide array of applications involving *L. plantarum*, a greater knowledge of its biochemistry and physiology is essential. However, the physiology/biochemistry of *L. plantarum* is complex, due mainly to its complex nutritional requirements (i.e., plant and/or animal niches) that are adjusted based on the particular environment (32, 170). It also demonstrates auxotrophy for many vitamins and branched-chain amino acids, (52, 136, 170, 178), but this is suspected to be related to the environmental niche that a particular *L. plantarum* strain is isolated from (32). Studies of the *L. plantarum* WCFS1 genome (113) have reinforced that this organism is extremely complex and that there is tremendous variation among strains, particularly with regards to sugar transport/catabolism, plantaricin biosynthesis, nonribosomal peptide biosynthesis, and exopolysaccharide biosynthesis (133). Amino acid biosynthesis in most strains are high energy-demanding systems except for the branched-chain amino acids, which are acquired/transported from the media (27, 170). Areas related to *L. plantarum*'s metabolism that have been studied for some time are focused upon aspects related to its use in industry and health. These previous studies have enabled more directed studies with the availability *L. plantarum*'s genome.

As an industrially utilized microbe, *L. plantarum* is often subjected to high osmolyte concentrations. As a result, strains of *L. plantarum* involved in processes that have high osmolyte concentration (e.g. dairy/vegetable fermentations) are able to accumulate compatible solutes that enable it to survive the resulting osmolyte shifts (71-73). The compatible solutes in *L. plantarum* that function to balance the osmolyte offset are composed of glycine-betaine, proline, glutamate, and alanine (71-73). These amino acids and quaternary ammonium compounds are taken and/or released to and from the surrounding medium through transport systems (e.g., quaternary ammonium transporters, mechanosensitive channels) allowing them to accumulate or dissipate within the cell to combat the upshift or downshift of the turgor pressure (70-73). It is interesting to note that sugars do not induce this type of response (73).

Due to *L. plantarum*'s importance in food and feed fermentations, the ability to utilize a strain that is capable of overproducing essential amino acids that are required in feed and foods for animal nutritional requirements would be important. Since *L. plantarum* is not auxotrophic for lysine (136), the four key enzymes of the L-lysine biosynthetic pathway and gene cluster were mapped, cloned and their regulation investigated (40). Upon close analysis of the key enzymes of this pathway in *L. plantarum*, it was discovered that only aspartokinase was regulated by L-lysine, in contrast to *Escherichia coli* in which each of the four key enzymes involved in L-lysine biosyntheses are repressed by the end product (40). While this presented the possibility of having three potential candidate genes to target, the genome of *L. plantarum* also contained isozymes of these four key enzymes. While, fortunately three of them failed to produce transcript and therefore active protein, the aspartokinase isozyme had low levels of transcript and the protein generated was active (40). Cahyanto et al., (40) did not come up with a way to oversynthesize L-lysine, however the foundation is there for future research in this area.

It was identified that *L. plantarum* can utilize a wide variety of carbohydrates (136) and with the availability of its genome, further elucidation of its carbohydrate utilization pattern has come to light, particularly as it relates to carbohydrate acquisition, binding, and metabolism (28, 169). Kleerebezem et al., (113) found that the genome of *L. plantarum* WCFS1 had 25 predicted and complete PTS sugar transport systems, along with a large genomic region that coded for more than two-hundred extracellular proteins. These regions coding for sugar, mono-, di-, and trisaccharides uptake and extracellular function are all located in a cluster, "lifestyle adaptation region", near the origin of replication and are consistent with being from some exogenous source (113, 170). To validate and classify a portion of these genes predicting extracellular functions, transctriptome profiling was performed resulting in the identification of a highly conserved group of strictly extracellular proteins, cell surface cluster (CSC). This CSC was found not only in *L. plantarum* WCFS1, but also in other Gram-positive bacteria whose environmental niche is associated with plants (169).

Further attempts to clarify this region, that appeared to be involved in carbohydrate acquisition and metabolism, led to the *in silico* analysis of a portion of the "lifestyle adaptation region" termed the "secretome" by Boekhorst et al., (28). This region, like the cell surface cluster operon, contained extracellular proteins responsible for carbohydrate acquisition (28, 113). However, further *in silico* analysis of the predicted "secretome", revealed the presence of not only carbohydrate degradation proteins such as hydrolases and transglycosylases, but also extracellular adherence proteins responsible for host adherence by *L. plantarum* (28). While this is not the first report demonstrating the presence of substrate-specific adhesion proteins (154), this was a first attempt in *L. plantarum* at trying to identify the different classes based on predicted function of extracellular proteins involved in carbohydrate acquisition and metabolism. Genes within the "secretome" encoding both the acquisition and adherence specific proteins could also have been a portion of those that were seen by Bron et al., (33) who identified seventy-two upregulated genes during *Lactobacillus plantarum* WCFS1 passage through the mouse GI tract. While a portion of those genes were involved in stress related functions and a large portion were part of several functionally unrelated pathways, the remainder were involved in sugar uptake and host adherence (33). Identifying and classifying these genes involved in binding and carbohydrate utilization apply directly to probiotic development. Due to the numerous classified and unclassified extracellular protein coding genes present in *L. plantarum* (28, 113, 169), mechanisms of how probiotic strains affect the human host can begin to be worked out. Since *L. plantarum* is one of the few species of lactobacilli that is involved in both food and feed fermentations as well as being a normal commensal within the human GI tract (54) it will be important in furthering probiotic research.

The metabolism of *L. plantarum* was not investigated extensively until the recent publication of it sequenced genome (113). With the publication of its sequenced genome and the availability of its microarrays (133, 151, 173), the ability to advance the knowledge of the physiology of this complex organism is now possible. Teusink, et al., (177) published an *in silico* reconstruction of *L. plantarum* WCFS1 metabolic pathways.

Using a set of pathway reconstruction tools that were utilized to build a similar framework in *Escherichia coli*, EcoCyc, (101-103), Teusink et al., (177) were able to construct a metabolic model of *L. plantarum* WCFS1, LacPlantCyc, that contained 129 pathways. In an effort to validate a portion of the pathways, Teusink et al., (178) tested the vitamin and amino acid requirements of *L. plantarum* WCFS1 and compared them to the *in silico* pathways and found that 32 of the 37 tests performed agreed with the database. Subsequently in an attempt to further the breadth of the metabolic model, Teusink et al., (178) developed a genome-scale metabolic model in an effort to analyze the growth of *L. plantarum* WCFS1 on complex media. From this approach they were able to estimate maximal ATP production and relate it to growth rate, predict amino acid catabolic pathways that were not associated with free-energy metabolism, predict 28 futile cycles, and identify parallel pathways (178), currently the models developed by Teusink et al., (177-178) are the only ones that exist for LAB and Gram-positive microorganisms as a group. To further expand the metabolic knowledge of *L. plantarum* WCFS1, Cohen et al., (45) utilized a proteomic approach to correlate proteins present in different phases of growth to those predicted as gene products with its genome. As a result, of the 200 protein spots that were identifiable, over half of them had been predicted to be involved within *L. plantarum* WCFS1 metabolic pathways (45). Based upon these findings, *L. plantarum* WCFS1 metabolism during log-phase growth is focused upon glucose utilization and energy generation via Embden-Meyerhoff-Parnas pathway, but in stationary-phase the organism uses alternative carbohydrate metabolism pathways that emphasize energy generation and induces proteins responsible for survival under increasingly stressful environment (e.g. lactic acid stress, oxidative stress) (45). While investigating a full metabolic approach is useful in understanding how *L. plantarum* adapts to environmental changes, smaller scale targeted approaches are useful for investigating the unique peculiarities of this organism. Since *L. plantarum* is seen in such a diversity of niches (2, 54, 100, 113), it has been possible with the aid of its sequenced genome to identify and investigate some of those peculiarities that set it apart from other LAB, particularly the metabolism of manganese.

1.4 Manganese in *Lactobacillus plantarum*

Manganese accumulation and its implications in *L. plantarum* have been under investigation for some time. It has been known since the 1940's that *L. plantarum* has a definite requirement for manganese (126). Manganese in *Lactobacillus* sp. serves a prototypical biological role (e.g. structuring and activation of enzymes (e.g. LDH, RNA polymerase, malolactate enzyme, manganese catalase and manganese SOD, chemical detoxification, stabilization) (12, 43, 156, 172). It also serves some very unique functions within the cell (e.g. non-enzymic superoxide radical dismutation) and because of this, *L. plantarum* has served as the LAB and *Lactobacillus* sp. model for investigating Mn(II) uptake, storage, and function (13). As a result of *L. plantarum*'s concentration dependent requirement for manganese, it is able to accumulate manganese to millimolar levels up to 35mM Mn(II) (14-16). Accumulation of manganese not only functions as a mechanism to combat ROS (14, 16), but also as a mechanism to survive radiation (86), and as a key player in production of benzaldehyde via the catabolism of phenylalanine (141, 142). In *L. plantarum*, the apparent oxidation state of manganese is Mn(II) and can be complexed to high and low molecular weight polyphosphate-protein aggregates (15, 16). Noting the difference between the extracellular and intracellular concentrations of manganese in *L. plantarum* (16) and based upon the already observed roles that directly involve manganese in *L. plantarum* (16, 86, 141), Archibald and Duong (13) investigated the uptake of Mn(II) in *L. plantarum* and found it to be regulated by a highly specific, high affinity, high velocity transport system that resulted in the accumulation of up to 35 mM intracellular Mn(II). This system had a preferential affinity for the Cd(II) ion and that affinity was repressed in the presence of citrate or other TCA organic acids, whereas Mn(II) was only taken up in the presence of citrate or other TCA organic acids (13). The authors proposed that this level of accumulation of Mn(II) was either due to numerous Mn(II) transporters or to Mn(II) transporters with a very rapid turnover time (13). To address this question, Hao et al. (84) identified and cloned a high affinity Mn(II)/Cd(II) uptake gene (*mntA*) and a low affinity Cd(II) uptake gene (*cdtB*) from *L. plantarum* that were expressed and functioning in *E. coli*.

They elucidated based upon MntA's amino acid sequence that it belonged to the family of P-type ATPases and was only induced during Mn(II) starvation, while the *cdtB* was constitutively expressed (85). In addition to their findings pertaining to MntA, Hao et al. (84, 85) also found a Mn(II)-containing enolase upstream from *mntA* that was postulated to be involved in the Mn(II) starvation response and that chemically induced mutagenesis resulted in the complete loss of high affinity Mn(II)/Cd(II) uptake. With the influence that Mn(II) undoubtedly has upon the physiology and biochemistry of *L. plantarum* it is no wonder that this system serves as a model for Mn(II) dependent systems in LAB. Not until the genome of *L. plantarum* WCFS1 was made available did a clearer picture of manganese transport and homeostasis begin to emerge. Nierop Groot et al.
(81), described, using an *in silico* approach the presence of five predicted Mn(II) transporters potentially involved in the uptake and maintenance of intracellular Mn(II) in *L. plantarum* WCFS1. A single Mn(II)/Cd(II) P-type ATPase transporter, (MntA), had already been identified in a separate strain of *L. plantarum* (85) and its role in Mn(II) transport and homoeostasis thought to be worked out. While in *L. plantarum* WCFS1 the *mntA* gene was present and expressed, there were also three Nramp transporter homologues (*mntH1, mntH2,* and *mntH3*) and an Mn(II) specific ABC-transporter homologue, (*mtsCBA*), (81). While the *mntH1*, *mntH3*, and *mtsCBA* genes were expressed under Mn(II) limiting conditions, the previously described *mntA* gene (85) was not, and furthermore, phenotype characterization of mutants in each of the five aforementioned genes resulted in no change from the parent strains (81). The data suggested that Mn(II) transport and homeostasis is tightly regulated and could also support the notion that there are additional Mn(II) transport and maintenance mechanisms that have not yet been identified. Given that *L. plantarum* is known for its uniqueness and adaptability (28, 113) and that Mn(II) metabolism plays such a crucial role in this organism's physiology/biochemistry, (12-16, 81, 84-86, 126, 141), it is a wonder that Mn(II) metabolism is not more complex.

1.5 Reactive Oxygen Species (ROS) Generation and Defense in *L. plantarum*

ROS Generation in *L. plantarum*

Species of *Lactobacillus* remove dimolecular oxygen from solution using redox reactions that involve 1, 2, or 4 electron transfers. These transfers result in the production of superoxide (O_2^-), hydrogen peroxide (H_2O_2) or water (H_2O) (47). In *L. plantarum*, when O_2^- is produced it is dismuted to H_2O_2 through high levels of intracellular manganese (Mn) since it lacks superoxide dismutases (SOD's) (14). Also, it should be noted that O_2^- production varies among strains of *L. plantarum* and cannot always be detected (76) since these organisms typically lack the majority of the cellular physiology required to generate O_2^- from O_2 (15, 47, 67). However, the presumption is O_2^- that is generated is most likely an intermediate during H_2O_2 formation (47), which is primarily generated through the activity of NADH oxidase: H_2O_2, pyruvate oxidase, and dihydroorotate oxidase in *L. plantarum* (47, 76, 177); it should be noted here that different strains of *L. plantarum* can harbor a non-H_2O_2 producing NADH oxidase (138). In *L. plantarum*, H_2O_2 can be accumulated into the mM range (12, 138) making it the primary ROS with which these cells have to contend. Since, *L. plantarum* has minimal requirements for iron (Fe) and little iron is found in a *L. plantarum* cell (11), it is evident that it has preferentially developed mechanisms of Mn uptake, previously discussed, over Fe uptake. The preferential uptake and accumulation of Mn for a large number of lactic acid bacteria and *L. plantarum* in particular has coincided with the presence of little to no iron (12). The absence of iron in *L. plantarum*, while an oddity amongst other organisms, it has definite biological advantages. Specifically, the exacerbated effect that would accompany H_2O_2 accumulation through its reaction of Fe to generate OH∞ (12, 58, 95, 192) would be limited. So, while the toxicity of hydroxyl radicals in *L. plantarum* may be minimal, due to its innate ability to accumulate H_2O_2 (12, 138) and since accumulation of H_2O_2 is faster than its removal by its NADH peroxidase (47) the effects of H_2O_2 toxicity through cysteine oxidation and protein carbonylation still remain hazards to the cell.

1.6 Non-Enzymatic and Enzymatic ROS Defense in *L. plantarum*

ROS defense in *L. plantarum* is centered around manganese firstly with regards to its role in non-enzymatic ROS defense as first discovered via the SOD anomaly (12, 67) and secondly as only one strain of this species harbors a non-heme manganese containing catalase (97). Both of these phenomena led to more in depth studies into the exact role and mechanisms that manganese plays in this species of *Lactobacillus*; for a complete review see Archibald (12). Complexes of high and low weight protein-polyphosphate bound manganese, provides the organism with an alternative defense against superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\infty$) (12-16). It is in the complexed Mn(II) state that manganese most readily detoxifies O_2^- , however in the process Mn(II) is oxidized to Mn(III), which can act as an oxidant, but since H_2O_2 is present in the cell during aerobic growth, the Mn(III) form can then be reduced by H_2O_2 to Mn(II) thus becoming available again for O_2^- detoxification (12). This process allows for both the biological scavenging of O_2^- and H_2O_2 simultaneously by Mn(II) and Mn(III) high weight protein-polyphosphates respectively, though the complex of Mn(III) is also able to detoxify O_2^- albeit at a slower rate than that of the Mn(II) complex (12, 16). Manganese, as demonstrated by Archibald & Fridovich (14-16), is required for growth under oxic conditions and the manganese concentration is a growth-limiting factor under these conditions. Thus, the growth limiting effect of Mn(II) is due to its role in non-enzymatic defense against reactive oxygen species (ROS) (12, 14-16). While H_2O_2 often accumulates in *L. plantarum* to millimolar levels and is considered a biologically relevant process with respect to Mn accumulation (12, 138), other mechanisms of H_2O_2 detoxification can also occur non-enzymatically. Organic acids, such as pyruvate are well known in their ability to detoxify H_2O_2, though the efficiency at which it occurs and the generation of pyruvate are limiting, and though these non-enzymatic mechanisms are in place, their efficiency as already mentioned can differ among strains and H_2O_2 can still accumulate and become bacteriostatic and/or bactericidal (12-16,47).

As already shown, Mn in *L. plantarum* is important as seen in the level of its uptake and storage as it relates to the critical functions the metal serves both enzymatically and non-enzymatically (12-16, 81, 84-86, 126, 141). For *L. plantarum*, the most important role that the metal can play is in its ability to provide a detoxification mechanism for H_2O_2, which can accumulate to the millimolar range and become bactericidal to the cell (12, 138). Within this context a significant amount of effort has been put forth towards the investigation of Mn-cofactored catalases, also known as manganese catalases or pseudocatalases.

As previously defined, lactobacilli are characterized as being catalase negative (17, 91). However, there are documented exceptions to this standard definition, (50, 62, 96, 97, 98, 117, 195, 196), as catalase activity has been detected in a variety of lactobacilli as well as other lactic acid bacteria both in the presence and absence of heme; lactic acid bacteria as a group do not synthesize heme (17) but in some strains exogenously supplied heme may result in catalase activity (140). Of the more than 300 known catalases, heme is a required for more than 275 (44). This leaves ~29 known manganese or non-heme catalases, of which only 1 has been identified, sequenced and characterized within lactic acid bacteria, specifically that found in *L. plantarum* ATCC 14431 and 2 more have merely been suspected, but little molecular evidence exists to validate them as such (62). A single strain, *L. plantarum* ATCC 14431, exhibits pseudocatalase activity, which has been proven to be a Mn-cofactored catalase (94, 98, 117). While there exist another *Lactobacillus* sp. as well as a *Pediococcus* sp. that exhibit pseudocatalase activity, very little work has been done to validate those claims (62). This species of *L. plantarum* has therefore served as the model for manganese catalases within lactic acid bacteria. Pseudocatalase of *L. plantarum* ATCC 14431 at present appears to be of most benefit to the host strain during the stationary phase of growth when H_2O_2 accumulation is most pronounced and the activity of NADH peroxidase is diminished (12, 14, 117). Indeed, there is a significant difference in survival/viability of *L. plantarum* ATCC 14431 versus a manganese catalase negative strain of *L. plantarum* (116).

With the presence of the pseudocatalase and the ability to accumulate manganese, *L. plantarum* ATCC 14431 stands above other lactic acid bacteria with regards to ROS defense versatility by having side-by-side mechanisms to deal with the toxicity of oxygen inherent in its diverse lifestyle.

1.7 Manganese Catalase of *L. plantarum* ATCC 14431

Noting that certain species of *Lactobacillus* exhibited catalase activity, Dacre and Sharp (50) pioneered the investigations into why organisms, which have no means of synthesizing heme, contained in a traditional catalase, were catalase positive. It was later discovered by Whittenbury (195) that the catalase activity present was of two different types; azide/cyanide sensitive and azide/cyanide resistant. This discovery led to the predicted aforementioned function of the manganese catalase of *L. plantarum* ATCC 14431 as being one of survival and viability. As once the cells are in stationary phase, there is an accumulation of H_2O_2 and having the ability to disproportionate H_2O_2 is an advantage for survival, however there appears to be no effect on growth kinetics when compared to a catalase negative *L. plantarum* strain (116).

Partial purification of the manganese catalase, found that there was no iron or heme constituent present and that it was active over a pH range of 4-12, retained 40 % of its activity to a temperature of 75^0 – 80^0 C and was stable to freeze-thaw cycles (98, 117). Manganese catalase has an apparent k_{cat} of $2x10^5$ s^{-1} and a K_m of 250-350 mM for H_2O_2 (117, 168). However, there is no data at present that defines the kinetic constants for the heme catalases that have been isolated in *L. sake* and *L. plantarum* strains (1, 89, 115, 143), which leaves no ability to make interspecies comparisons between the two distinct catalase groups.

Driven further by investigations into multinuclear manganese containing proteins, (i.e. other manganese catalases (4, 7, 99), oxygen evolving complex, ribonucleotide reductase (198), among others (12)), the manganese catalase of *L. plantarum* ATCC 14431 has been of interest with regards to its overall structure and active site. Elucidating its structure and active site have allowed investigators the ability to develop comparisons to other manganese containing catalases, heme catalases, as well as other multinuclear manganese proteins as mentioned previously. Inhibition

studies were used to elucidate the active site structure and mechanism of the manganese catalase demonstrating inhibition by azide (a competitive inhibitor by preventing turnover), cyanide, fluoride and chloride (21, 109, 130, 190), however not at the lower levels, which are used to inhibit heme catalases (44, 171, 191). Early on these studies led to the conclusion that the active site manganese and its catalytic cycle involved the reduction-oxidation of Mn(III) to Mn(V) (59,118). However, there is no evidence that the enzyme contains a Mn(V) valence state, but instead there is a mixed valence superoxidized species, Mn(III)/Mn(IV), that results in an inactive enzyme (189). Enzyme spectra indicated the presence of Mn(III) in the active site, similar to the spectra of the Mn-SOD Mn(III) containing resting enzyme, which indicated the presence of Mn(III) within manganese catalase (106, 187, 191). Utilizing the catalytic cycle models of *Thermus thermophilus* manganese catalase (108, 109), Waldo and Penner-Hahn elucidated that in the manganese catalase of *L. plantarum* ATCC 14431, both of the manganese ions present in a subunit, undergo oxidation and reduction. These ions cycle between the Mn(II) and Mn(III) valencies, both states of which give rise to an active enzyme. Although the superoxidized Mn(III)/Mn(IV) species is present, it is only a minor species among the majority Mn(II)/Mn(II) and Mn(III)/Mn(III) species (21). Solved to a 1.8 **A°** crystal structure, *L. plantarum* manganese catalase is a homohexamer that has a unique complex structure for a hexameric protein. Each monomer of manganese catalase holoenzyme is 28.3-29.7 kDa, contains 2 manganese atoms per monomeric active site and extensive crosslinks between monomers exist that include a β-zipper and crosslinking calcium ions, which are unique to this manganese catalase with respect to the only other crystallized manganese catalase, originating from *Thermus thermophilus* as well as predicted structures of hypothetical proteins in *Bacillus subtilis* (21, 26, 94, 117). Each subunit contains a substrate channel containing charged residues, leading to an active site that has the basic support architecture of a 4-helix bundle, typical of many metallo-proteins (21, 145). With respect to structure and relationship to other 4-helix bundle proteins, the manganese catalase is a dimanganese protein in which the metal centers of each subunit are coordinated by a carboxylate moiety of glutamate and two solvated oxygens, (21).

This in contrast to other proteins of this 4-helix bundle family, which maintain the same basic posture but have a di-iron center and three or more coordinating carboxylate groups (145). Additionally, the enzyme contains a protein overlayer resulting in restricted access to the active site, as well as an active site that is encased within a matrix of hydrogen bonds extending outwards creating an environment suitable for a reduction/oxidation catalytic cycle (21). Apart from an arginine unique to *L. plantarum* manganese catalase, the hydrogen bonding network, its interactions with a phenoxyl group of a conserved tyrosine residue in the outer sphere and a glutamate residue are considered conserved features within manganese catalase structures (194) as identified in the crystallized *Thermus thermophilus* manganese catalase (10, 21) and from homologous manganese catalase sequences (21, 114).

Based upon the basic structure of the active site (21, 194) and the redox mechanism of the metallo-core (190), it was proposed by Barynin that the active site supports a catalytic turnover mechanism that involves a two electron oxidation of bound hydrogen peroxide by Mn(III) in order to release O_2, which is similar to the dissociation of O_2 from oxyhemerythin (21). Furthermore, the two electron transfer is thought to be facilitated by the bridging oxygens of the manganese core, which can be considered analogous to the heme catalase/peroxidase turnover mechanism (152). Furthermore, the use of the tyrosine phenoxyl group as a phenoxyl radical mirrors similar associations within the di-iron redox active site of ribonucleotide reductase (144, 145) and the oxygen evolving complex (OEC) (69, 139, 179), though in the instance of the *L. plantarum* manganese catalase it acts as a safety mechanism to prevent irreversible oxidative damage to the active site, whereas elsewhere it is directly involved in the catalytic cycle, suggesting that these are conserved features both within distantly related families such as the ribonucleotide reductase and within the Mn-cofactored family of proteins (21, 194), despite the difference in functionality.

Using the crystallized manganese catalase of *Thermus thermophilus* (10) as well as mechanisms defined in other di-oxygen evolving Mn-cofactored enzymes it has been possible, as previously mentioned to draw comparisons and build insight to define mechanisms in order to further the mechanistic/structural implications with regards to the manganese catalase of *L. plantarum.*

Additionally, protein sequencing data from both predicted and demonstrable manganese catalases (114) have also allowed us to draw evolutionary relationships and comparisons from this unique set of enzymes. Phylogenetic analysis was performed on this class of catalases by Klotz et al. and based upon 29 known protein sequences it was found they separate into two clades, in which each clade was the result of a single gene duplication event effectively creating two different types of manganese catalase genes (114). In one clade rests the manganese catalase gene common to *Firmicutes*, which include some bacilli, enterococci and *L. plantarum* as well as members of the *Planctomycetes* and Archaea (114). Within the other clade there are members of *Enterobacteriacea, Pseudomoneacea Clostridiaceae*, *Bacillaceae*, and some cyanobacteria. Since the publication of these results the influx of sequencing data has inflated the number of predicted manganese catalases to approximately 80, to include their presence within new bacterial members not represented in the previous work.

1.8 Application of Manganese Catalase in Lactobacilli

Lactic Acid Bacteria (LAB) and lactobacilli in particular are common, well-known commensals of the human gastrointestinal (GI) tract (183) and have been utilized for centuries in the preservation of foods through fermentation processes, particularly within the dairy industry. Important for maintaining a healthy microflora not only within the GI tract but also within the female vagina (63, 183), these microorganisms are undoubtedly a key component of health and well being within humans and animals. There have been several reports of the potential health benefit that lactobacilli administered, often through dairy products, as probiotics ("mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora") (87, 92, 157) can confer upon their host. These include alleviation of diarrheal associated diseases (49, 55, 56, 75, 93, 154, 158), improvement in patients with inflammatory bowel disease, inflammatory bowel syndrome, pouchitis (75, 119, 134, 164, 165), and, though evidence is still forthcoming, there is some suggestion of treatments and prevention of certain cancer types both in animal models and humans (23, 48, 64, 75, 137, 147, 157).

Due to the unique nature of probiotic LAB, a great deal of investigation has commenced to define and identify the characteristics of potential probiotic microorganisms since the type and extent of the effects are strain dependent. The characteristics of probiotic LAB include, antioxidative activity, pathogen antagonism, persistence within the GI, stomach survivability, immunomodulatory effects, and generally recognized as safe (GRAS) (9, 132, 149, 174).

Based upon the current importance of LAB, specifically *Lactobacillus* sp., in health/industry and as one of the defining characteristics of a probiotic is antioxidative activity, it is therefore worthwhile to identify, characterize and exploit antioxidative characteristics found within *Lactobacillus* species. Importance of exploiting antioxidative attributes either within a strain or being able to move a characteristic (i.e. gene) to another strain of health/industrial importance is related to the knowledge that these organisms are obligate fermenters, can accumulate mM levels of H_2O_2 especially when propagated under oxic conditions, and while they have mechanisms to dispose of metabolic H_2O_2 (i.e. NADH peroxidase, NADH oxidase) the accumulation is often faster than the disposition (47). Tolerance of ROS's such as H_2O_2 and oxygen tolerance in general is often strain dependent and is one of the causes that can lead to decreased viability within functional foods, (foods containing or generated by health benefitting microorganisms that elicit a health benefit to the consumer), such as yogurt and mass produced commercially available probiotic formulations decreasing its potency (120, 174). Due to the hazards of ROS, great care and expense must also be taken in the preparation of industrial starter cultures and therapeutic formulations of lactobacilli. As many etiologies of human diseases such as cancer, emphysema, cirrhosis, atherosclerosis, arthritis, and irritable bowel disorders have been linked to ROS (82, 107, 193), the ability to exploit the antioxidative properties of select lactobacilli and utilize them within pharmaceuticals or nutraceuticals could be extremely valuable (105).

There is specific interest in exploiting those LAB that harbor either SOD's or catalases that utilize manganese as their cofactor (8, 35, 36, 42, 94, 161, 162, 181) and evaluating them within heterologous probiotic hosts that could be and are utilized in industrial and therapeutic applications (42, 161, 162).

Particular interest has been directed towards the manganese catalase harboring *L. plantarum* ATCC 14431 as it is one of only 3 LAB that have manganese catalase activity (62) and is the only one that has been characterized from gene to protein (21, 94, 117, 194). While the manganese catalase has been cloned in *Escherichia coli*, the heterologous expression of such formed insoluble inclusion bodies (94) that was related to its complex structure (21). This event required that for practical and more applicable uses that the gene be cloned, expressed, and assayed within heterologous LAB hosts (162, 181). Subsequently the manganese catalase gene was cloned and expressed in *L. casei, L. bulgaricus,* and *Lc. lactis*, however, only one demonstrated an active protein (162, 181), and when utilized as a therapeutic in a colitis mouse model, the cloned manganese catalase had no effect in the enhancement or restoration of the antioxidative capacity within the model. It is suggested by the authors that the level of activity of the manganese catalase in the current host is too low to see any effect (161) even though there are manganese transport homologues of *L. plantarum mtsCBA* system (81), but these transporters may be inherently kinetically different than the ones present within *L. plantarum*. Additionally, it was noted that when *Lc. lactis* was used as a recombinant within the Rochat study it failed to generate any active protein even though manganese catalase protein was detected (162) and there are homologues of the *L. plantarum mtsCBA* system present, again as alluded to by Rochat et al. affinity and ability of the system to bring in manganese can differ. It has been determined that two potential lactobacilli probiotic species, *L. gasseri* NCK 334 and *L. reuteri* NCK 932 are capable of producing high levels and/or high activity manganese SOD from *Streptococcus thermophilus* A054 (35) suggesting that manganese in these species may not be as limited as that seen in those used by Rochat et al. Therefore the body of this work will be to clone and express the manganese catalase of *L. plantarum* CECT 221(ATCC14431) within *L. gasseri* NCK 334 and *L. reuteri* NCK 932 in an effort to improve upon the existing availability of recombinant manganese catalase containing lactobacilli that can be applied to areas of health and industry development.

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Chapter II

Biological Role of the Mn-Catalase of *Lactobacillus plantarum* ATCC 14431

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Abstract

Oxidative stress mechanisms within species of *Lactobacillus* vary widely, encompassing manganese accumulation, peroxidases, and both heme and non-heme (Manganese containing) catalases. While most all species of *Lactobacillus* accumulate manganese, to mM levels, and contain peroxidases, heme and manganese catalases are limited to a select few lactobacilli. Furthermore, manganese catalases are documented in only two *Lactobacillus* species. The current study investigates the construction of a *mnkat* minus strain and resulting physiological effects of the normally catalase positive *Lactobacillus plantarum* ATCC 14431. An insertional inactivation vector was constructed by ligating a 485 bp fragment of the 1449 bp *mnkat* into the vector pLS19. The subsequent construct, pLS485, was transformed into *L. plantarum* ATCC 14431 resulting in a single catalase negative colony under oxic conditions with Em resistance and aberrant colony/cellular morphology. Validation of the strain through biochemical tests and 16S rDNA sequencing confirmed that it was *Lactobacillus plantarum.* Subsequent physiological analysis demonstrated that Mn-catalase, the product of *mnkat*, is essential for normal growth of *L. plantarum* ATCC 14431 under aerobic conditions. Furthermore, Mn-catalase is a critical protein for removing H_2O_2 generated during aerobic growth of *L. plantarum* ATCC 14431 and the inactivation of *mnkat* results in hyper-sensitivity to additional H_2O_2. Additionally, Mn-rich media, (e.g. APT medium), can improve the growth of the *mnkat*$^-$ strain over that seen in Mn-poor media (e.g. MRS medium). Growth of the *mnkat*$^-$ strain under anoxic conditions results in the recovery of normal colony morphology. This is the first known study to genetically inactivate *mnkat* and show that Mn-catalase is an essential part of the ROS defense system within *L. plantarum* ATCC 14431.

2.1 Introduction

Lactic acid bacteria (LAB) are a diverse group of Gram-positive aerotolerant, obligate fermenters that occupy several environmental niches. Lactobacilli comprise the largest genus of the LAB group and occupy habitats ranging from foodstuffs to humans and animals. As with aerotolerant microorganisms, lactobacilli generate the reactive oxygen species (ROS) superoxide (O_2^-) and hydrogen peroxide (H_2O_2) which can accumulate to millimolar levels (4, 16, 20, 42, 51) and therefore require mechanisms to combat their toxicity (29). The presence of NADH oxidases, NADH peroxidases, and the intracellular accumulation of millimolar levels of manganese (4, 6, 16, 51) are common ways by which these organisms protect themselves against the toxic effect of ROS's. In some instances within LAB manganese cofactored superoxide dismutases (SOD's), heme-catalases, and manganese cofactored catalases have been identified in select species (1-3, 7, 17, 18, 27, 31-33, 35, 36, 45, 46, 49, 53). Although, some members of the LAB group contain catalases, examples are rare and the LAB are still generally classified as catalase negative.

The presence of catalases in some lactobacilli is unique, and those that are found to have heme catalases require an exogenous source of heme as LAB are incapable of heme synthesis (22). However, the discovery of a manganese catalase (Mn-catalase) in *L. plantarum* ATCC 14431 led to the cloning of the manganese catalase gene in *Escherichia coli* (*E. coli*) (28), *Lactobacillus* sp. (48), and *Lactococcus lactis* (*Lc. lactis*) (48), determination of the crystallographic structure of the protein (9), and mechanistic studies of the protein based upon site-specific mutagenesis (52). The above studies, including chemical inhibition studies of the enzyme (32, 34, 35) have all centered on just the enzyme or the impact of the enzyme in comparison to other strains of *L. plantarum* that lack catalase activity. However, no investigations known to date have reported creating an isogenic strain that is deficient in making a functional Mn-catalase *in vivo* and investigating the implications of such a mutation upon the host strain. Also, the construction of such a strain could lend itself to further investigations including regulation of the manganese catalase gene, importance of Mn accumulation in a Mn-catalase containing LAB, whose species have long been studied as a model for Mn accumulation in lactobacilli (4-6, 8, 13, 21, 23, 24, 26, 39, 43, 44, 47, 50). To this end, this brief communication describes the inactivation of the manganese

catalase gene (*mnkat*) through insertional mutagenesis and reports the basic physiological behavior of the isogenic derivative.

2.2 Materials and methods

Bacterial strains and media. Bacterial strains and plasmids are outlined in Table 1. Strains of *E. coli* were grown aerobically at 37^0 C in Luria-Bertani broth or agar (1.5%) with Em (150 µg/ml) when needed. *L. plantarum* was cultured in MRS (Mn-poor media; 220 µM) and/or APT (Mn-rich media; 710 µM) broth at 37^0 C from glycerol stocks followed by two isolation transfers to solid MRS and/or APT containing 1.5% agar before every experiment. When appropriate, *L. plantarum* cultures were grown statically in either MRS or APT broth at 37^0 C, or under oxic conditions (150 rpm), or anoxic conditions in a Coy anaerobic chamber (Coy Labs, Grass Lake, MI). In all growth experiments the liquid to flask ratio was 1:5. When required 5 µg/ml Em was added to *L. plantarum* cultures.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source and/or reference [a]
Bacterial strains		
Escherichia coli		
DH5α	pLS19(pUC19E) host	(25, 37)
Lactobacillus plantarum		
NC 1542(ATCC 14431)	Mn-catalase host strain	ATCC
NC 1543	ATCC integrant *mnkat*::pLS485	This study
Plasmids		
pKSKat	1.449-kb *katMn* PCR amplicon from *L. plantarum* CECT 221(ATCC 14431) cloned into pBluescript II KS(+)	J. Bruno-Barcena
pLS19	pUC19 containing *ermC* of pE194	(25, 37)
pLS485	Suicide vector with a 485-bp internal region of *mnkat* cloned into *PstI* sites of pLS19	This study

a NC and NCK, culture collections at North Carolina State University, Raleigh; ATCC, Amercian Type Culture Collection;

Chemicals and enzymes. Lysozyme, proteinase K, 3,3'-diaminobenzidine, horseradish peroxidase, N,N'-tetramethylenediamine(TEMED), and all antibiotics were purchased from Sigma-

Aldrich (St. Louis, MO). All other general use chemicals (i.e. hydrogen peroxide and bacteriological media) were purchased from Fisher Scientific (Pittsburgh, PA). Molecular reagents to include, all cloning enzymes; *PstI,* T4 DNA polymerase, *Taq* polymerase, *Pfu* polymerase, dNTP's, $MgCl_2$, $MgSO_4$ and PCR buffers were purchased from Promega (Madison, WI).

DNA isolation and manipulation. Isolation of total DNA was carried out with QIAGEN's Dneasy Tissue Kit (QIAGEN Valencia, CA). Plasmid DNA from *E. coli* was isolated using QIAGEN's Plasmid DNA Mini-Prep Kit (QIAGEN Valencia, CA). Products generated from cloning enzyme manipulation, with the exception of ligation reactions, and PCR were purified using QIAEX II Gel Extraction Kit (QIAGEN Valencia, CA). Ligation reaction mixes were used directly to transform *E. coli* and/or lactobacilli without further purification.

PCR. Traditional PCR was carried out utilizing Promega's Go*Taq* polymerase, dNTP's, and buffers (Promega Madison, WI). Primers, Sense PstI-KatKO(pLS19) (5'-AAAACTGCAGGTAAA AAAGCAGTTACCCCT-3') and AntiSense PstI-KatKO(pLS19) (5'-AAAAGACGTCATTCTTGT AAGCGTCTTGCC-3'), (Integrated DNA Technologies, Inc., Coralville, IA) were used to amplify ~485 bp internal region of *mnkat*. Primers Lac16SForward (5'-GACGAACGCTGGCGGCGTGCCT-3') and Modified Lac16S Rev (5'-GGTAGCCGTAGGAGAACCTGC-3'), (MWG-BIOTECH Inc., High Point, NC) were used for 16S rDNA amplification and sequencing (MWG-BIOTECH Inc., High Point, NC) to validate *L. plantarum* strains. All amplifications were carried out using Bio-Rad's 96-well iCycler (Bio-Rad, Hercules, CA).

Bacterial transformations. Strains of *E. coli* were transformed using electroporation standard methods (40) with a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA). Transformations of *L. plantarum* were performed using electroporation according to the method of Luchansky et al., (38) with HEPES electroporation buffer and Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA).

Preparation of cell free extracts (CFE's). Following two transfers, cultures of *L. plantarum* with and without the presence of pLS492 were grown under oxic conditions with shaking at 135 rpm and 37^0 C at a 1:5 liquid to flask ratio. Cells were then harvested in exponential growth phase by centrifugation at 3000 X g for 20 min. Pellets were washed three times in an equal volume of phosphate-EDTA buffer (50 mM phosphate, 0.1 mM EDTA buffer, pH 7.8).

After the final wash, the resulting pellet was resuspended in phosphate-EDTA buffer containing 1 mM Phenylmethanesulfonyl fluoride (PMSF), at 1/40th the original culture volume. This suspension was then transferred to a 2 ml gasket sealed screw cap tube containing 1/2 its volume in 0.2 mm silica beads (BioSpec, Inc., Bartlesville, OK) for cellular disruption in a Mini-BeadBeater-8 (BioSpec, Inc., Bartlesville, OK). Cellular suspensions were homogenized for 10x1 min treatments with 3 min rest cycles on ice in between treatments to prevent sample overheating. Following the final homogenization, clarification of the supernatant was achieved by pelleting the cellular debris through centrifugation at 20000 X g and 4^0 C for 30 min. Clarified supernatant was removed from the tube and transferred to 6,000-8,000 MWCO dialysis tubing and dialyzed against two changes of phosphate-EDTA buffer at 4^0 C for 24 hrs.

Biochemical assays. Total protein concentration in CFE's was determined using the Bradford method (12), using bovine serum albumin (BSA) as the standard. Catalase activity gels were performed using 10% non-denaturing native PAGE gels and the staining method of Clare, et al. (14). A screening catalase test was performed by smearing a portion of a colony on a microscope slide followed by a drop of 3% H_2O_2 to each smear and observing the presence or absence of bubbles. Specific catalase activity in cell free extracts (CFE) was determined using a spectrophotometric assay that followed the loss of hydrogen peroxide over time at 240 nm (10). Experiments were performed in biological triplicate. API 20E© (bioMérieux SA, Marcy l"Etoile, France) strips were performed according to manufacturers specifications using both aerobic and anaerobically grown *L. plantarum* cultures.

Western blotting. Cell-free extracts (5-10 μg) were separated on a 10% SDS-PAGE gel using Bio-Rad Mini-Protean II electrophoresis system (Bio-Rad, Inc., Hercules, CA). Electrophoretically separated proteins were then electroblotted to a 0.45 μm nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany) using the Invitrogen XCell II blot module (Invitrogen, Corp., Carlsbad, CA). Verification of complete transfer of the protein bands was performed by Amido Black staining (Bio-Rad, Hercules, CA) of the membrane per the manufacturers instructions. Membranes were then blocked 1 hr in blocking buffer (5% (wt/vol) non-fat milk (Carnation) solubilized in PBS-T (1X phosphate buffered saline pH 7.0, 0.1% Tween 20)). Monoclonal antibodies specific for Mn-

catalase (A gift from J.W. Whittaker, Oregon Health Sciences University) were added to fresh blocking buffer at a 1:10,000 dilution and incubated with gentle agitation for 2 hrs. Membranes were then washed 3X15 min in blocking buffer after which fresh blocking buffer containing 1:10,000 goat anti-rabbit conjugated horseradish peroxidase (Bio-Rad, Inc., Hercules, CA)) was added to the membranes and incubated for 2 hrs. After final incubation, the membranes were washed in fresh blocking buffer 1X15 min followed by a wash in PBS-T for 2X15 min. Signal was detected by incubating the membranes in an equal volume mixture of Western Lightning Chemiluminescence Reagent Plus substrates (Perkin-Elmer, Waltham, MA) for 1 min followed by a 15-30 sec exposure to Kodak BioMax Light film (Perkin-Elmer, Waltham, MA). Experiments were performed in biological triplicate.

Microscopy and images. Cultures were Gram-stained based on standard bacteriological methods (15) and visualized with a Nikon Alphaphot Microscope (Nikon, Inc., USA). Images were taken with a Nikon D40X SLR with 50 mm lens (Nikon, Inc., USA).

H_2O_2 Susceptibility Disk Diffusion Assays. Following two successive transfers in either MRS or APT with and without Em (5μg/ml), *L. plantarum* cultures were used to inoculate 25 ml of either MRS or APT media with or without Em (5μg/ml) to a starting OD_{600nm} ~0.05 and grown 6-9 hr. From overnight cultures, tubes of PBS were inoculated to an OD_{600nm} of 1. From the standardized PBS tubes, 100 μl of inocula was transferred to 5 ml of either MRS or APT top agar (0.75%) tempered to 50^0 C. After gentle vortexing, the suspension was poured onto corresponding bottom agar (1.5%) plates. Once polymerized, sterile 6 mm filter-paper disks were placed on the surface of the top agar for both MRS and APT media. To each disk, 5 μl of a varying of H_2O_2 stock solution, 0-240 mM, was added to the disks. Plates were then incubated at 37^0 C under oxic conditions until zones of inhibition were detected. Disk diffusion assays were performed in three biological replicates and averages of zones of inhibition (mm) were taken per respective concentrations of hydrogen peroxide added to the disks.

Effects of [H_2O_2] on growth (OD_{600nm}). Following two successive transfers in either MRS or APT media with or without Em (5μg/ml), *L. plantarum* cultures were used to inoculate 25 ml of either MRS or APT media with the appropriate antibiotics, to a starting OD_{600nm} of 0.05 and allowed to grow

at 37^0 C, 135 rpm. Exponentially growing cultures of *L. plantarum* were then used to inoculate 100 μl of fresh MRS or APT containing 0.5 μg/ml Em in NUNC-96F microtiter plates (NUNC, ThermoFisher Scientific, Rochester, NY) to a starting OD_{600nm} ~0.08-0.09. Growth at 37^0 C was monitored as a function of time at OD_{600nm} with continuous shaking under oxic conditions in the presence and absence of 1 mM hydrogen peroxide using the FLUOStar OPTIMA plate reader system (BMG LABTECH Inc., Durham, NC). Data were plotted as in OD_{600nm} v. time. Maximum specific growth rate ($\mu_{max} \cdot hr^{-1}$) was calculated from the slope of the line of the exponential phase portion of the growth curve that had an r^2 of 0.99 by fitting the data to a linear regression model using GraphPad Prism 4 for Macintosh (GraphPad Software, San Diego, CA). Data is based upon the average of two biological replicates.

2.3 Results and Discussion

2.3.1 Construction of suicide vector pLS485 for *mnkat* knock-out. An internal fragment of ~485 bp of the ~1.4 kb mnkat gene was amplified from pKSKat using *Pfu* polymerase. The amplified fragment was gel purified and ligated into the *PstI* site of pLS19 (25, 37) that had been treated with T4 DNA polymersase to create a new plasmid, pLS485 (Fig. 1.).

2.3.2 Transformation of *L. plantarum* ATCC 14431 with pLS485. Newly constructed pLS485 was electroporated into *L. plantarum* ATCC 14431 (Fig. 2). A single colony appearing on 5 μg/ml Em containing APT agar was further purified by streaking onto fresh APT agar containing 5 μg/ml Em and allowed to grow overnight at 37^0 C. Six isolated catalase negative colonies exhibiting similar colony characteristics from the APT streak plate, were then streaked onto both APT and MRS agar containing 5 μg/ml Em and allowed to grow overnight at 37^0 C. Isolated colonies from these streak plates were then screened initially for the absence of catalase activity by using a qualitative catalase test, where a drop of 3% H_2O_2 was added to cells smeared on a glass slide. As shown in (Fig. 3.), some showed no effervescence upon addition of 3% H_2O_2, whereas the wild-type *L. plantarum* ATCC 14431 exhibits effervescence resulting from catalase activity. The putative *mnkat*$^-$ colonies were purified by streaking over a series of five transfers on APT and MRS agar containing 5 μg/ml Em.

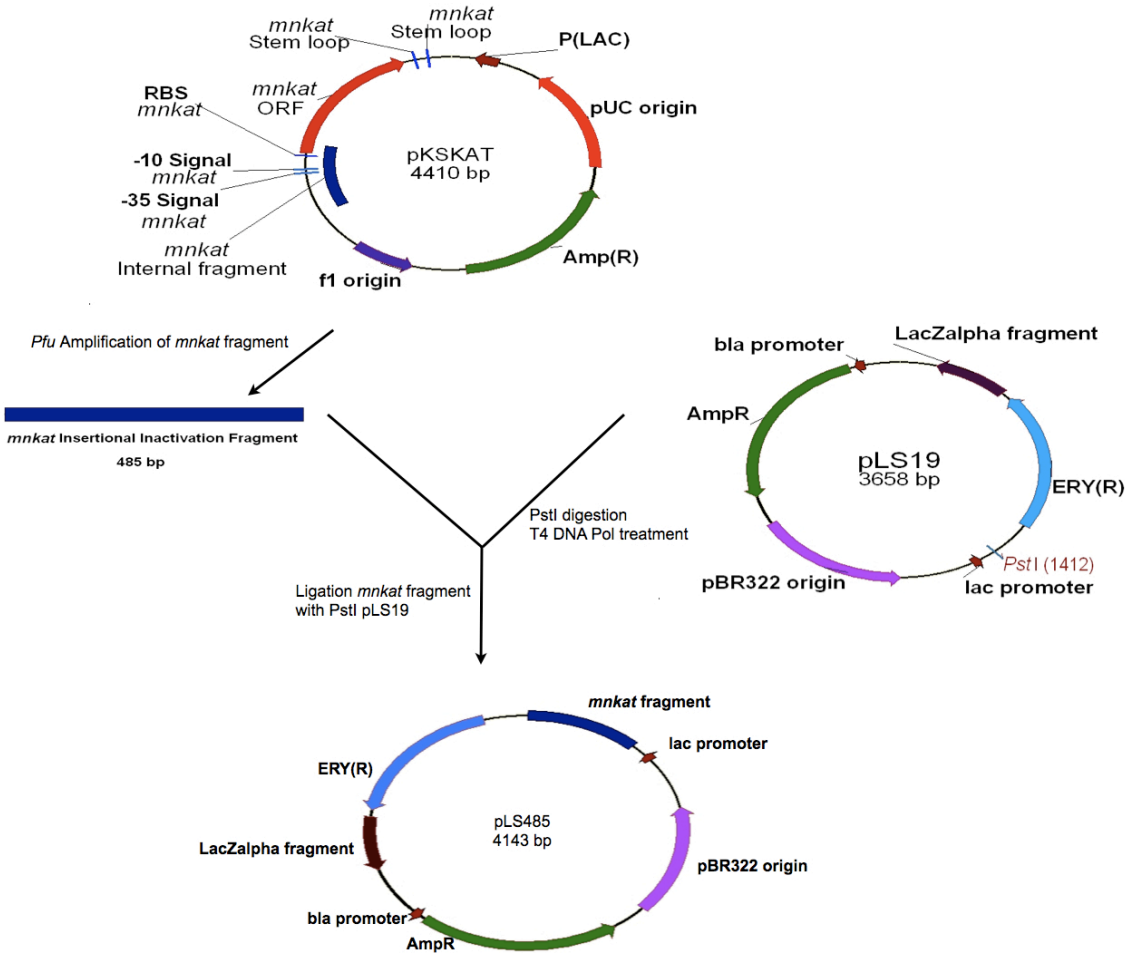

FIG. 1. Construction of the pLS485 plasmid. Plasmid pLS19 was digested with *PstI* and the ends polished with T4 DNA polymerase. This digested plasmid was then ligated to the *Pfu* amplified *mnkat* fragment. This created a new construct termed pLS486.

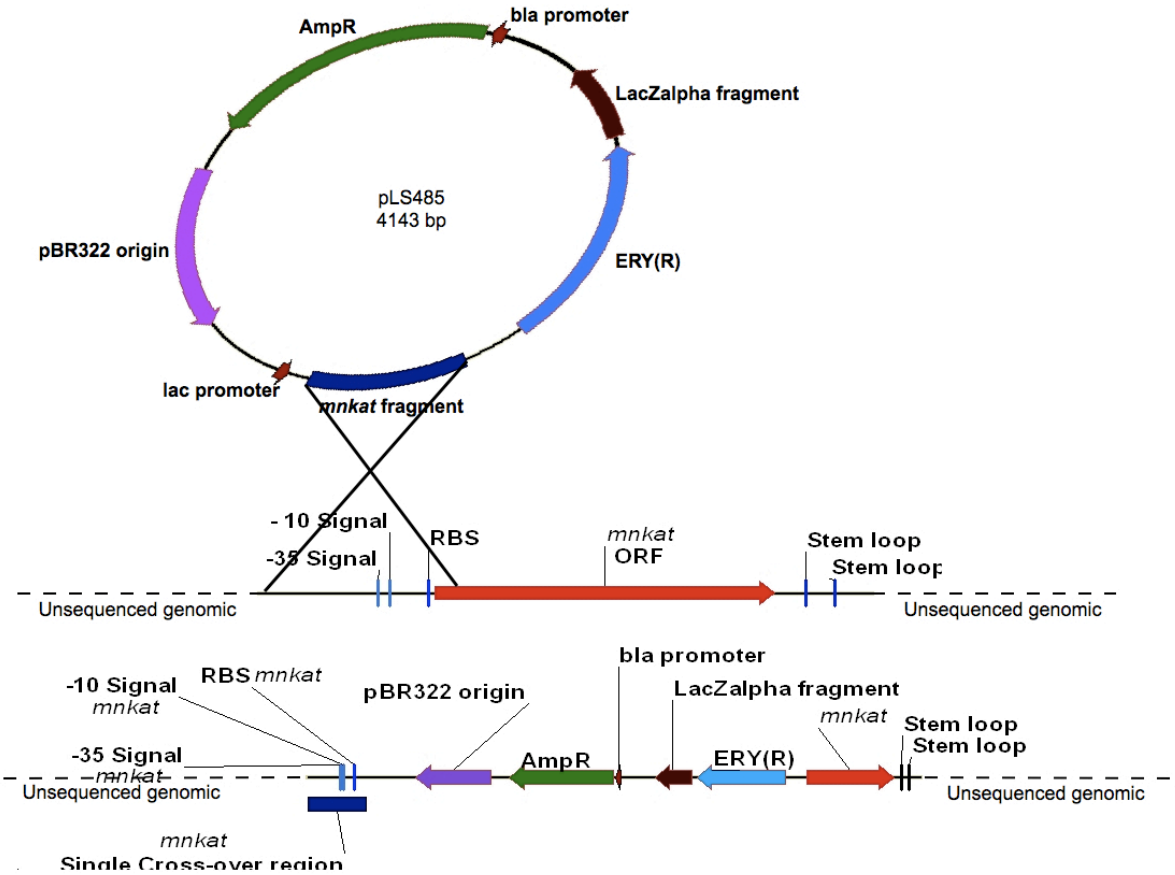

FIG. 2. Schematic of an ideal single-crossover event between the homologous regions of pLS485 and of the genomic *mnkat* (Pseudocatalase, Mn-catalase gene) that results in the insertional inactivation of *mnkat* in *L. plantarum* ATCC 14431.

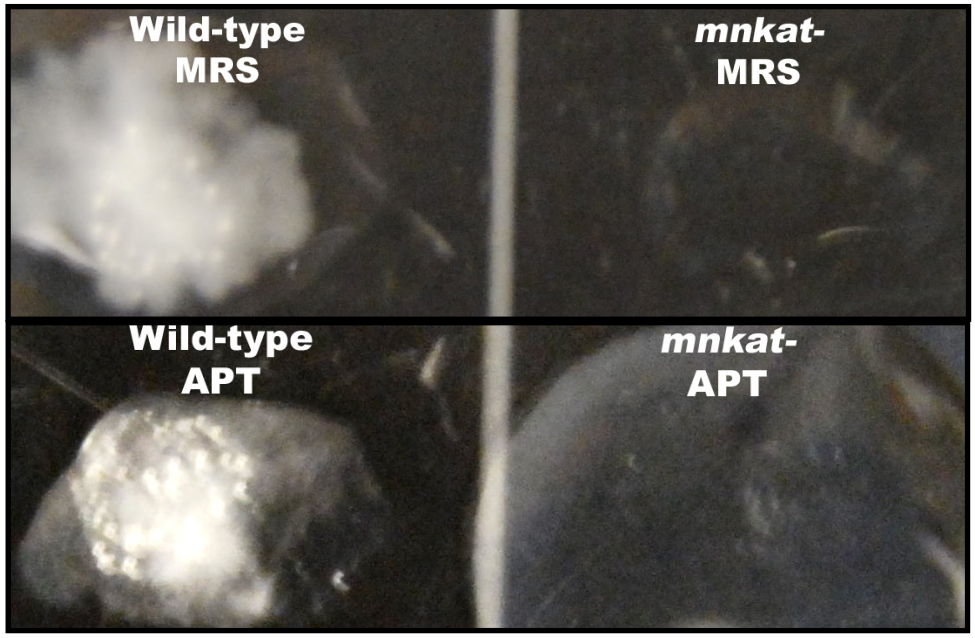

FIG. 3. Catalase activity screening of wild-type (NC1542) and *mnkat*- (NC1543) *L. plantarum* grown on MRS (A) and APT (B).

From a single purified colony that exhibited no catalase activity and was Em resistant, a 10% glycerol stock was made and from this stock the remainder of the experiments were performed.

To validate the inability of *L. plantarum mnkat*- strain to express an intact and functional Mn-catalase, Western blot analysis, catalase activity assay, and visualization of catalase activity bands using non-denaturing PAGE were performed. As seen in Figure 4, the CFE from the wild-type *L. plantarum* ATCC 14431, showed a strong positive antigen-antibody cross reaction of 28 kDa and a weak band at 56 kDa (lane 1). Incontrast, the CFE from the *mnkat*- strain, (NC1543), grown on either MRS or APT with 5 μg/ml Em showed no antigen-antibody cross reaction (Fig. 4, lanes 2 and 3). The presence of a monomeric form (~28 kDa) as well as a dimeric form (~56 kDa) in the CFE for the wild-type strain of *L. plantarum* in SDS-PAGE gel/Western blotting may indicate incomplete denaturation of a dimeric form of the protein, or as previously observed with CuZnSOD, another ROS detoxifying enzyme, could result from oxidative modification/oxidation of a surface cysteine residue (19). Additional validation that the *mnkat* had indeed been inactivated and therefore no longer capable of generating a functionally expressed protein was by visualization of catalase activity in an activity gel, (Fig. 5.).

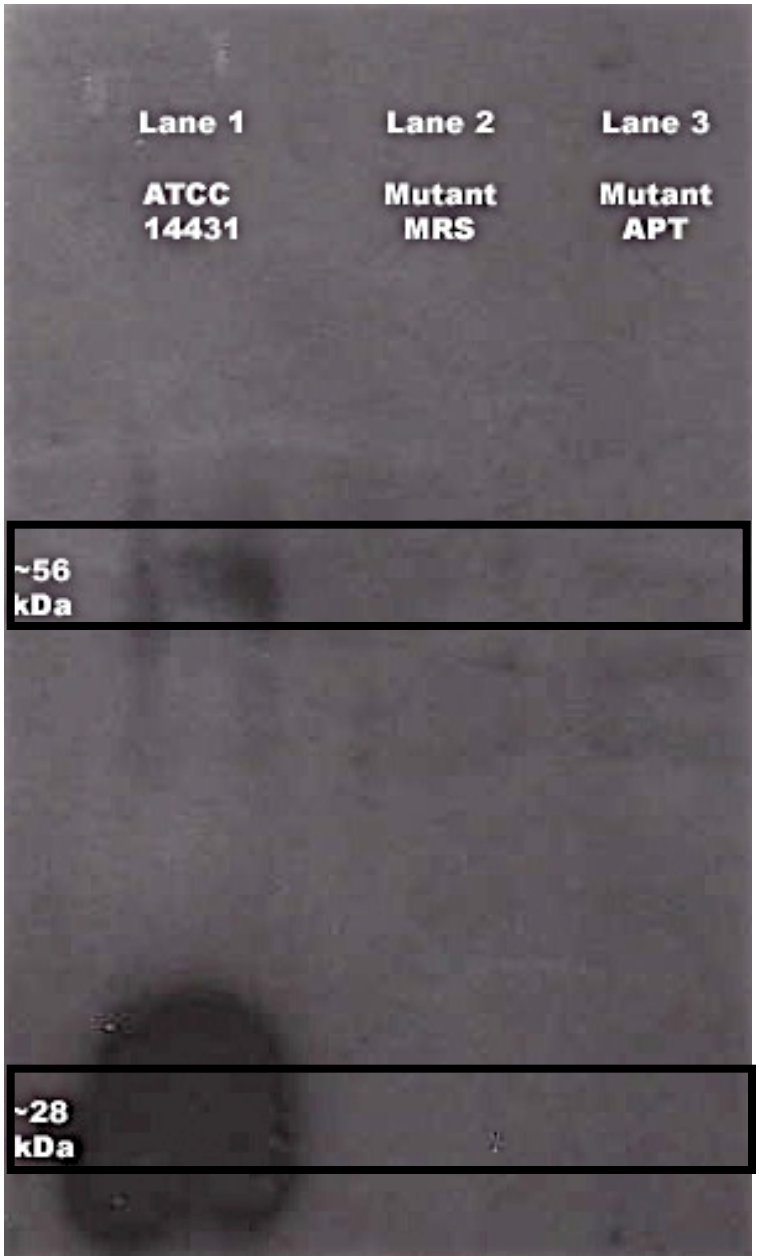

FIG. 4. Western blotting of CFE's of wild-type and *mnkat*- strain (NC 1543) *L. plantarum*. Separation of total proteins, 5 μg/lane, was performed on a 10% denaturing SDS-PAGE gel. Following electroblotting, Mn-catalase was probed for using Mn-catalase monoclonal antibodies. Lane 1. ATCC 14431 wild-type, Lane 2. *mnkat*- strain NC1543 grown in MRS with 5 μg/ml Em, and Lane 3. *mnkat*- strain NC1543 grown in APT with 5 μg/ml Em.

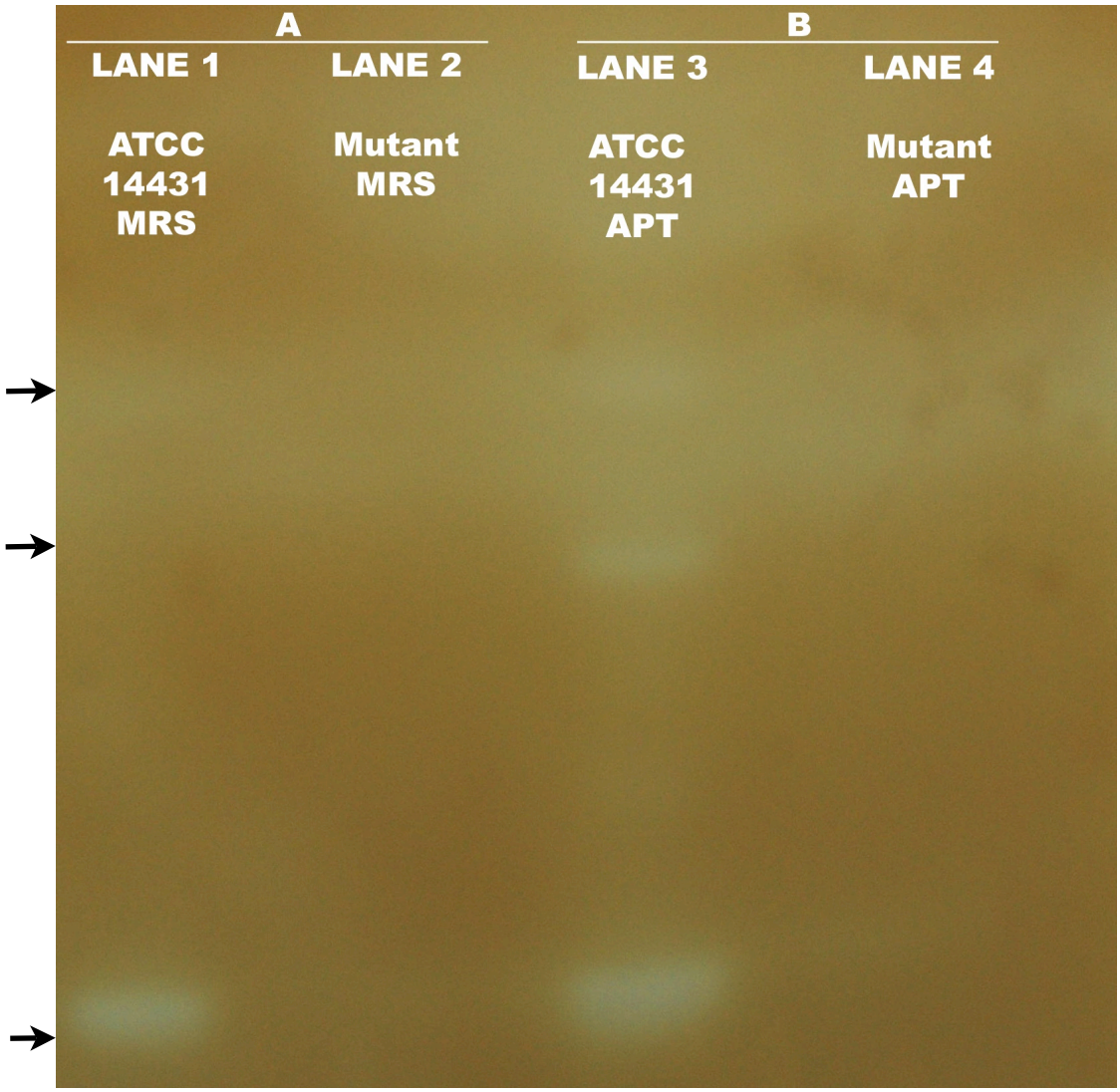

FIG. 5. Catalase activity gels from total protein isolated from cells grown in eitherMRS (A) or APT (B) in the presence of oxygen. Samples of CFE were applied to 10% non-denaturing native PAGE gels based upon total protein (50 µg) and stained using diaminobenzidine stain by Clare et. al. (14) Lane 1-MRS grown cells; ATCC 14431; Lane 2- MRS grown cell; *mnkat*- strain (NC1543). Lane 3-APT grown cells; ATCC 14431; Lane 4-APT grown cells; *mnkat*- strain (NC1543). Arrows highlight the presence of catalase bands.

As shown, the absence of catalase bands in the *mnkat* inactivated *L. plantarum*, confirms both the initial catalase screening, (Fig. 3.), as well as the absence of signal in the Western blot, (Fig. 4.). Additional catalytically active bands present in *L. plantarum* ATCC 14431 (Fig. 5.-Lane 1 and 3) are most likely due to factors associated with non-denaturing conditions (i.e. pI, pH, as well as associated proteins) and polymers of the protein (as seen in the Western blot).

Similarly, multiple catalytically active bands of MnSOD have also been routinely observed during non-denaturing PAGE (personal communication H. Hassan). Furthermore, when assayed for the specific activity of Mn-catalase, there was no observable Mn-catalase acitivity in the CFE's from the *mnkat*$^-$ strain (NC1543) grown on either MRS or APT, while the wild-type *L. plantarum* ATCC 14431 had specific activities of 112.9 U/mg protein and 180.9 U/mg protein of cell-free extracts from cells grown in MRS and APT media, respectively.

2.3.3 Effect of inactivating *mnkat* on the cellular physiology of *L. plantarum*.

(i) Strain validation and colony and cellular morphology. In order to validate the catalase negative strain, it was purified by streaking over a series of five transfers on APT and MRS agar containing 5 μg/ml Em. Following culture isolation, colonies of wild-type and catalase negative strains were photographed. We confirmed that the wild-type and catalase negative cells were Gram-positive through Gram-staining, (Fig. 6 A and B), as well as by streaking onto MacConkey agar, which yielded no growth. Further validation of the *mnkat*$^-$ *L. plantarum* was carried out by comparing its metabolic profile against that of the wild-type *L. plantarum* by performing a series of biochemical tests utilizing API 20E© test strips under both oxic and anoxic conditions (Table 3). Biochemical patterns in both strains were identical except for the level of acetoin production under oxic conditions in the *mnkat*$^-$ strain (NC1543) (Table 3) suggesting an oxidative stress effect in the absence of *mnkat.* Final validation of the strains was accomplished through 16S rDNA sequencing of both the wild-type and the *mnkat*$^-$ *L. plantarum* grown in MRS and APT media. Through sequence BLAST analysis, both strains were confirmed to be *L. plantarum.*

L. plantarum strains grown in MRS appeared to undergo a colony morphology change from round, opaque, convex and entire colonies with a smooth glossy surface to colonies that were pin-point in size round, slightly opaque, raised, and entire with a smooth surface (Fig. 6A). Cells of the *mnkat*$^-$ *L. plantarum* strain (NC1543) grown on MRS appeared to become shorter and more plump in appearance, instead of appearing more elongate and slender as wild-type *L. plantarum* ATCC 14431 (Fig. 6A). Cultures of *mnkat*$^-$ *L. plantarum* grown on APT plate, however showed translucent, slightly raised, irregular colonies with a rough surface as opposed to the wild-type

L. plantarum ATCC 14431 colonies that appear round, opaque, convex and entire with a smooth glossy surface (Fig. 6B). Cellular morphology of the *mnkat*⁻ strain (NC1543) appears slightly shorter and more plump than the wild-type (Fig. 6B), though the wild-type on MRS does appear more elongate and slender than the wild-type on APT (Fig. 6A and B). Morphological changes within bacteria and LAB in particular do occur and these occurrences have been documented as natural events (22), stress induced events (41) and events due to genetic manipulation (11, 30). Interestingly, these cellular and colony morphology changes were not manifested when the wild-type and the mutant were grown in MRS or APT under anaerobic conditions, (data not shown), suggesting that these changes in the *mnkat*⁻ mutant are due to the lack of catalase activity in presence of oxygen, (i.e. oxidative stress).

(ii) Sensitivity to H_2O_2. The wild-type and the *mnkat*⁻ strains were tested for their sensitivity to varying concentrations of H_2O_2 in both MRS and APT. Table 2 summarizes the findings of these assays based upon the measurement of the total diameter (mm) of inhibitory zones.

TABLE 2. Zone of inhibition diameters (mm) of H_2O_2 susceptibility disk diffusion assay. (A) MRS grown cells of *L.plantarum* ATCC 14431 strains. (B) APT grown cells of *L. plantarum* ATCC 14431 strains. Assays were performed in biological triplicate and diameters listed are averages of triplicates.

A. MRS **nmol**

Strain	0	50	100	200	250	500	750	950	1200
ATCC 14431	-	-	-	-	-	8.3	9	9	10
***mnkat*⁻**	-	-	-	7	8	9.8	11	12	13

B. APT **nmol**

Strain	0	50	100	200	250	500	750	950	1200
ATCC 14431	-		-	-	-	7	7	7.2	8.2
***mnkat*⁻**	-	-	-	8	9	11	12	13	13

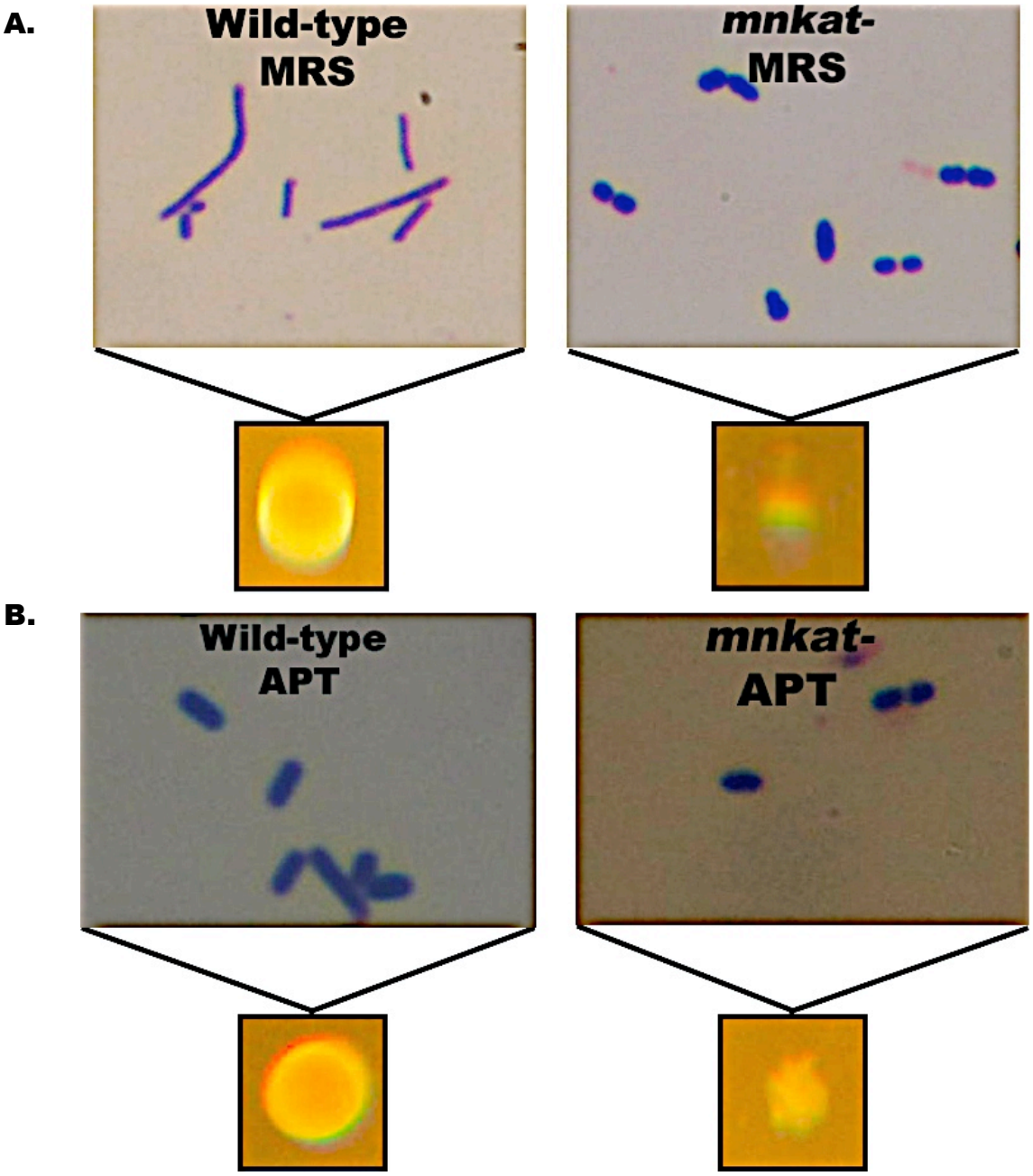

FIG. 6. Gram-stain (top-image) and colony morphology (lower-image) before and after transformation of *L. plantarum* ATCC 14431 with pLS485. (A) Left to Right; WT *L. plantarum* ATCC 14431 on MRS, *L. plantarum mnkat*$^{-}$ strain (NC1543) on MRS. (B) Left to Right; WT *L. plantarum* ATCC 14431 on APT, *L. plantarum mnkat*$^{-}$ strain (NC1543) on APT.

(iii) Effects of Mn-catalase on growth in the presence and absence of H_2O_2. Previous reports have indicated that chemical inhibition of Mn-catalase by H_2O_2 + hydroxylamine(NH_2OH) does impact the viability of the *L. plantarum* ATCC 14431(34). However, the use of chemical inhibition relied upon additional use of protein biosynthesis inhibitors (34), and coupled together may not give the most accurate picture of the impact Mn-catalase may have upon its host, particularly as it pertains to growth kinetics. Inhibitors of protein biosynthesis would undoubtedly affect cellular growth. Therefore, it was of interest to examine the effect that the absence of Mn-catalase would have upon its host, particularly in light of the apparent morphological changes seen in the presence of oxygen (Fig. 6A-B). We examined the growth kinetics of the wild-type *L. plantarum* ATCC 14431 and its isogenic *mnkat*$^-$ mutant in both MRS and APT media in the presence and absence of 1 mM H_2O_2. Data in figures 7A-B indicate that *L. plantarum* ATCC 14431 strains grown in MRS in the absence of H_2O_2 demonstrate marked difference in growth kinetics. The wild-type *L. plantarum* ATCC 14431 was able to sustain growth, $\mu_{max}\bullet hr^{-1}$= 0.76, in the absence of additional H_2O_2, whereas the *mnkat* inactivated strain had a 4-fold reduced growth rate, $\mu_{max}\bullet hr^{-1}$=0.19, and stopped at an OD_{600nm} that was ~8-fold lower than that of the wild-type *L. plantarum* ATCC 14431 (Fig. 7A and Fig. 9A). While the addition of 1 mM H_2O_2 resulted in a slight lag phase and a 1.2-fold reduction in growth rate, $\mu_{max}\bullet hr^{-1}$=0.65, in the wild-type *L. plantarum* (Fig. 7B and Fig. 9A), the same treatment was bactericidal toward the *mnkat*$^-$ strain (NC1543) (Fig. 7B and Fig. 9A). Conversely, growth of the wild-type and *mnkat*$^-$ strains in APT medium in the absence of H_2O_2 resulted in a slower growth rate, $\mu_{max}\bullet hr^{-1}$=0.58, for wild-type, than that observed in MRS, $\mu_{max}\bullet hr^{-1}$= 0.76 (Fig. 9A-B), that is most likely due to the differences in glucose concentration between the two media types, 111 mM and 55.5 mM in MRS and APT respectively. However, the most profound change was that observed in the *mnkat*$^-$ strain (NC1543) of *L. plantarum* in which the growth rate was ~2.8-fold higher in the APT than that in MRS media, $\mu_{max}\bullet hr^{-1}$= 0.54 and $\mu_{max}\bullet hr^{-1}$= 0.19, respectively (Fig. 9A-B). This result could possibly be due to a combined effect of lower glucose levels in conjunction with higher concentrations of phosphate (i.e. 10.4 mM and 26.0 mM) as well as manganese (i.e. 220 µM and 710 µM) in MRS and APT respectively. Furthermore, addition of 1 mM H_2O_2 to the APT growth media of the wild-type strain resulted in comparably the same fold decrease in $\mu_{max}\bullet hr^{-1}$ as that

observed in the MRS media, (i.e.,1.2 and 1.3-fold decreases in μ_{max}•hr^{-1} in MRS and APT respectively) (Fig. 9A-B). However, unlike addition of H_2O_2 to the *mnkat*- strain (NC1543) in MRS media, absence of growth (Fig. 7B and Fig. 9A), growth in APT media in the presence of H_2O_2 resulted only in an ~2.2-fold decrease in μ_{max}•hr^{-1} from that grown in the absence of H_2O_2 (Fig. 8A-B and Fig. 9B). These results could indicate that the generation of H_2O_2 in this strain of *L. plantarum* exhibits the capacity to produce more H_2O_2, (e.g., produces higher levels of H_2O_2 generated by enzymes such as; NADH oxidase:H_2O_2, pyruvate oxidase, dihydroorotate oxidase), than it is capable of removing as mentioned by Condon (16). Other strains of *L. plantarum* lacking catalase are capable of equivalent or faster growth in comparison to *L. plantarum* ATCC 14431 and do not accumulate H_2O_2 until stationary phase, however the H_2O_2 accumulation in *L. plantarum* ATCC 14431 is uncertain as the presence of the Mn-catalase maintains the apparent H_2O_2 concentration at zero (35). Knowing this, the present data suggest that this strain of *L. plantarum* in the absence of Mn-catalase may have a diminished capacity to remove H_2O_2 through other means (i.e. NADH peroxidase, high-molecular weight Mn protein-polyphosphates). Preliminary results in static *L. plantarum* cultures grown in MRS indicate that those cultures grown under oxic conditions have a diminished acetoin production compared to the wild-type, Table 3, which could indicate an alternative use for pyruvate (e.g., H_2O_2 detoxification). As under anoxic conditions the production of acetoin appears to recover to wild-type levels (Table 3).

TABLE 3. API 20E results of wild-type *L. plantarum* ATCC 14431 and mutant (NC1543) grown statically in MRS under both oxic and anoxic conditions.
[a]Acronyms for each reaction/enzyme.
[b]Indicates a weak positive reaction intensity between the two strains.

Tests[a]	Reactions/Enzymes	Aerobic		Anaerobic	
		WT	Mutant	WT	Mutant
ONPG	B-galactosidase	-	-	-	-
ADH	Arginine dehydrolase	-	-	-	-
LDC	Lysine decarboxylase	-	-	-	-
ODC	Ornithine decarboxylase	-	-	-	-
CIT	Citrate utilization	-	-	-	-
H_2S	Hydrogen Sulfide production	-	-	-	-
URE	Urease	-	-	-	-
TDA	Tryptophane deaminase	-	-	-	-
IND	Indole	-	-	-	-
Acetoin Production → VP[b]	Voges-Proskauer	+	weak[b]	+	+
GEL	Gelatin liquefaction	-	-	-	-
GLU	Glucose utilization	+	+	+	+
MAN	Mannitol utilization	+	+	+	+
INO	Inositol utilization	+	+	+	+
SOR	Sorbitol utilization	+	+	+	+
RHA	Rhamnose utilization	+	+	+	+
SAC	Sucrose utilization	+	+	+	+
MEL	Melibiose utilization	+	+	+	+
AMY	Amygdalin utilization	+	+	+	+
ARA	Arabinose utilization	+	+	+	+
NIT RED	Nitrate reductase	+	+	+	+
CAT	Catalase	+	-	+	-
OX	Oxidase	-	-	-	-

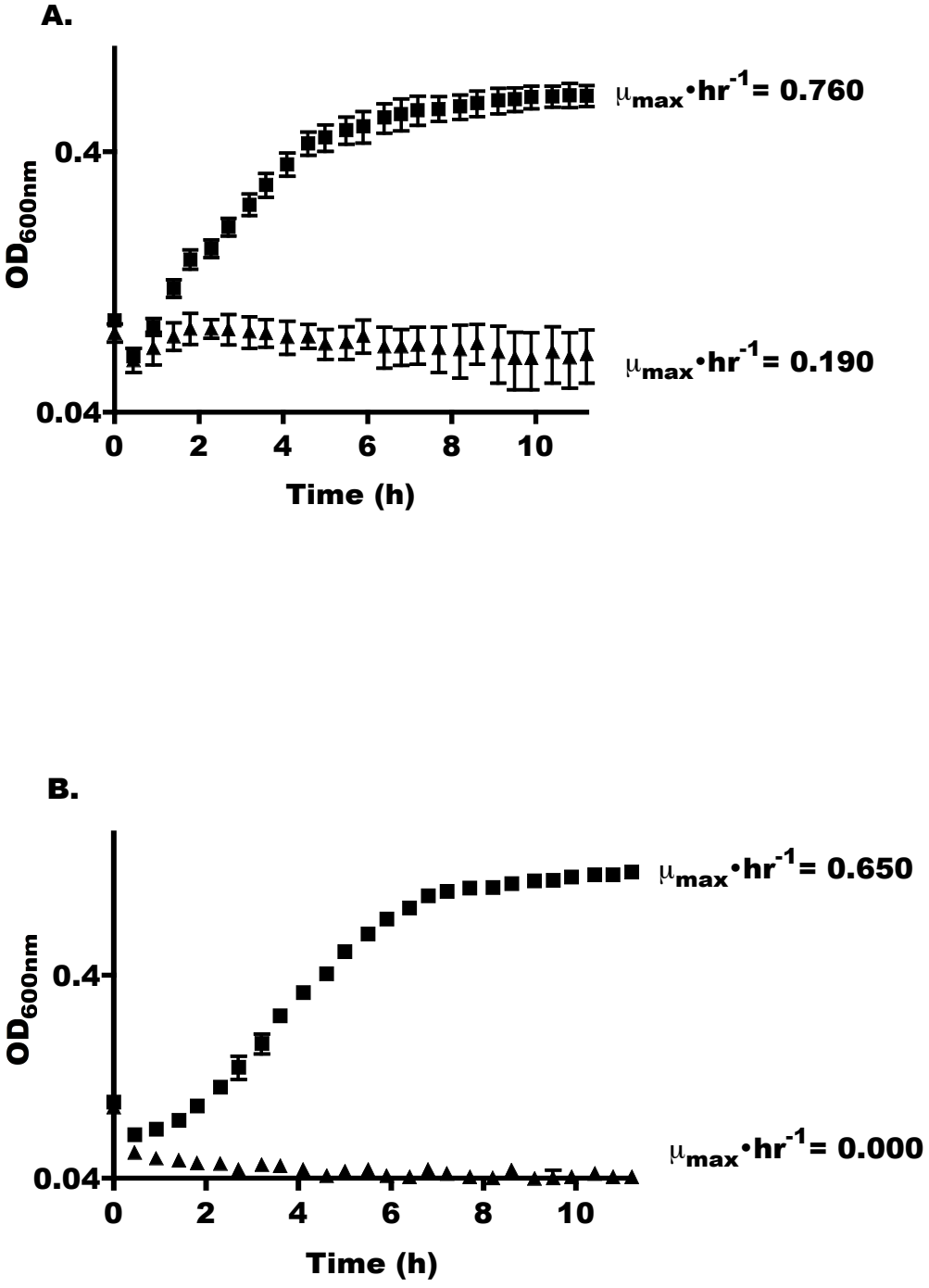

FIG. 7. Growth kinetics of *L. plantarum* ATCC 14431(■) and *mnkat*⁻ (▲) strains in MRS media in the presence and absence of 1 mM H_2O_2. (A) 0 mM H_2O_2. (B) 1 mM H_2O_2. Each point represents an average of biological duplicates and error bars represent SEM.

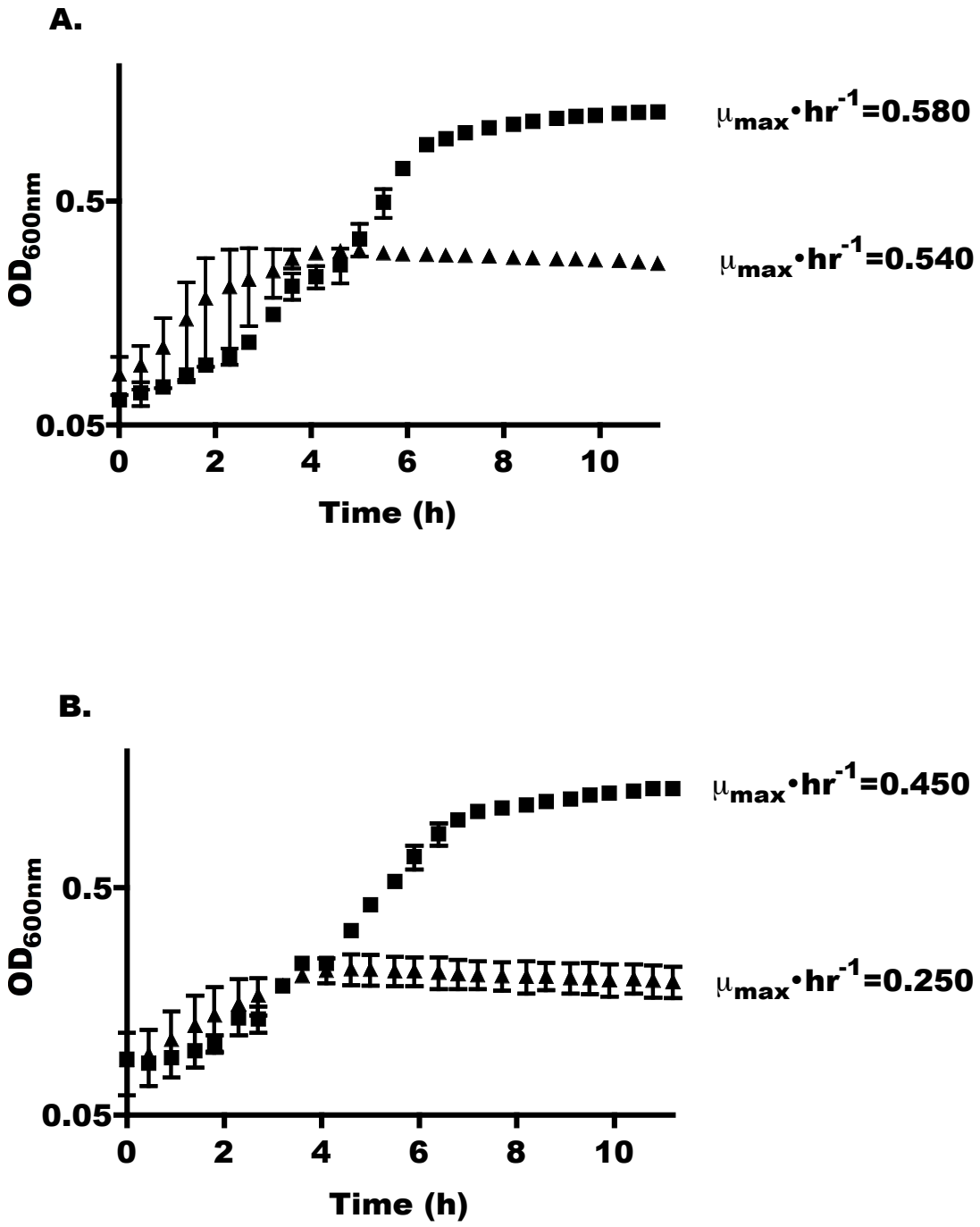

FIG. 8. Growth kinetics of *L. plantarum* ATCC 14431(■) and *mnkat*⁻ (▲) strains in APT media in the presence and absence of 1mM H_2O_2. (A) 0 mM H_2O_2. (B) 1 mM H_2O_2. Each point represents an average of biological duplicates and error bars represent SEM.

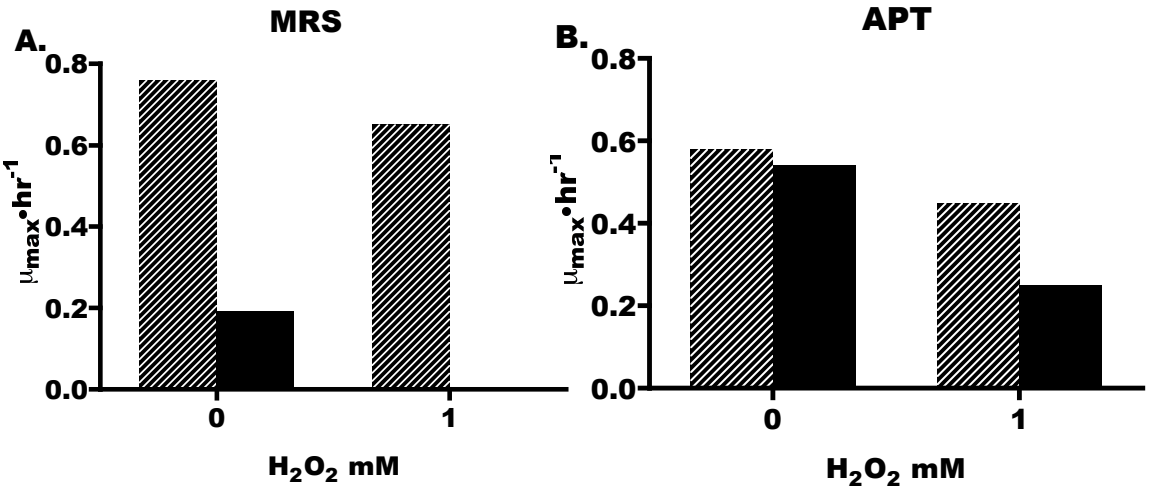

FIG. 9. Specific growth rates (μ_{max}•hr^{-1}) of *L. plantarum* ATCC 14431 (▨) and *mnkat*$^-$(■)strains grown in MRS (A) and APT (B) in the absence and presence of 1 mM H_2O_2.

Discussion

Aerotolerant lactobacilli microorganisms are known to generate superoxide (O_2^-) and hydrogen peroxide (H_2O_2) which can accumulate to millimolar levels (4, 16, 20, 42, 51) and therefore mechanisms to combat the toxicity of ROS are essential for this aerobic survival (29). The presence of the NADH oxidases, NADH peroxidases, and millimolar levels of manganese (4, 6, 16, 51) are common mechanisms by which these organisms defend against the toxicity of ROS's. In addition, some LAB contain manganese cofactored superoxide dismutases (SOD's), heme-catalases, and manganese cofactored catalases (1-3, 7, 17, 18, 27, 31-33, 35, 36, 45, 46, 49, 53). The presence of catalases in some lactobacilli is unique and the discovery of a manganese catalase (Mn-catalase) in *L. plantarum* ATCC 14431 led to in-depth investigations, that resulted in the cloning of the manganese catalase gene in *Escherichia coli*, *Lactobacillus* sp., and *Lactococcus lactis* (28, 48), as well as the determination of the crystallographic structure of the protein (9) and mechanistic studies of the protein based upon site-specific mutagenesis (52). However, there never has been a report on the development of an *L. plantarum* ATCC 14431 strain incapable of expressing Mn-catalase. In this report the development of *mnkat*$^-$ strain of *L. plantarum* ATCC 14431 that is incapable of producing an active Mn-catalase (Fig. 3 and Fig. 5) has been accomplished through the use of a suicide vector (pLS485) targeting *mnkat*.

Our data suggest that its role in H_2O_2 detoxification may have a more profound effect than just long-term cell survival as proposed by Kono and Fridovich (34). This was indicated by the morphological changes seen (Fig. 6A-B) and the ability to reverse those changes when the *mnkat*- strain (NC1543) is grown under anoxic conditions (data not shown). Though growth differences (e.g., growth rate) were observed in the wild-type strain in the presence and absence of exogenous H_2O_2 (Fig. 7-9), fold differences in MRS and APT were similar, ~1.3 and ~1.2 respectively. The most profound difference was in the *mnkat*- strain (NC1543) where growth in MRS appeared to be hindered with and without exogenous H_2O_2 (Fig. 7 and 9A). However, when grown in APT, the *mnkat*- strain (NC1543) appeared to grow better even in the presence of exogenous H_2O_2 (Fig. 8 and 9B) possibly conferring some benefit to the cells. Most likely this additional protective measure is at least in part due to the presence of higher levels of Mn sequestered via high molecular weight protein-polyphosphate molecules (5, 7). As limiting the phosphate reduces the Mn(II) uptake and limited Mn (II) increases *L. plantarum* cells sensitivity to ROS molecules (5, 6). Given the chemistry involved between H_2O_2 and Mn containing protein-polyphosphate molecules, this could be a likely scenario.

Further research is needed to better understand what is happening to *L. plantarum* ATCC 14431 in the absence of Mn-catalase and the exact nature of the genetics of the *mnkat*- strain (NC1543) and the reasons for the morphological changes observed (e.g., oxidation of peptidoglycan, lipid membrane, etc.), when the mutant is grown under aerobic conditions.

2.4 Conclusions

- Inactivation of *mnkat* results in hyper-sensitivity to added H_2O_2.
- Mn-rich media, (e.g. APT medium), can improve the growth of the *mnkat*-strain(NC1543) relative to that seen in Mn-poor media (e.g. MRS medium).
- Mn-catalase, the product of *mnkat*, is essential for normal growth of *L. plantarum* ATCC 14431 under aerobic conditions.
- Mn-catalase is a critical protein for removing H_2O_2 generated during aerobic growth of *L. plantarum* ATCC 14431.

With this knowledge in hand, it will hopefully lead to further investigations into alternative ROS detoxification mechanisms and broaden the scope of the Mn-catalase's role within in *L. plantarum* and those predicted within other members of the LAB group.

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CHAPTER III

Cloning and Heterologous Expression of *Lactobacillus plantarum* CECT 221(ATCC 14431) Mn-catalase within Probiotic Lactobacilli

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Todd-Provided material (pTRK563) and participated in discussions

Hosni-Conceived the idea, directed the research, and contributed to the writing/editing of the MS

Trent-constructed the pMnKat, transformed the lactobacilli, confirmed expression of the Mn-catalase, determined the physiological impact and wrote the MS.

Abstract

Oxidative stress mechanisms within species of *Lactobacillus* vary widely, encompassing manganese accumulation, peroxidases, and both heme and non-heme (Manganese containing) catalases. While most species of *Lactobacillus* accumulate manganese to mM levels and contain peroxidases, heme and manganese catalases are limited to a select few lactobacilli. Furthermore, manganese catalases are documented in only two *Lactobacillus* species. Current research involves the cloning of the 1.449-kb manganese catalase gene from *L. plantarum* CECT 221 (ATCC 14431), containing its native promoter, into the shuttle vector pTRK563. The resulting pMnKat has been transformed into the probiotic lactobacilli; *L. reuteri* NCK 932 and *L. gasseri* NCK 334. Manganese catalase (Mn-catalase) and its resulting activity has been assayed and detected in both species. Furthermore, increases in long-term survival under aerated conditions, increases in growth rate ($\mu_{max} \bullet hr^{-1}$) and an increased resistance to H_2O_2 concentrations up to 10 mM have been shown.

3.1 Introduction

Lactobacilli are members of the Lactic acid bacteria (LAB), a diverse group of low G+C, aerotolerant, Gram-positive obligate fermenters that occupy several environmental niches from foodstuffs to plants to animal/human GI and urogenital systems. *Lactobacillus* is the largest genus of the LAB group and to date has approximately 80-100 species (11, 34). There have been several reports of potential health benefits of lactobacilli, often as probiotics, and delivered through dairy products, (35, 38, 70). Probiotics are ("mono- or mixed cultures of live microorganisms, which when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora") (38). Probiotics have been used in the treatment of diarrheal associated diseases (23, 26, 27, 30, 39, 69, 71); inflammatory bowel disease, inflammatory bowel syndrome, pouchitis (23, 26, 27, 30, 39, 54, 60, 69, 71, 75, 76) and have some promise as a cancer preventative (8, 22, 28, 30, 62, 67, 70). As with aerotolerant microorganisms, lactobacilli generate the reactive oxygen species (ROS) superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which can accumulate to millimolar levels (4, 21, 31, 63, 80). Therefore, these bacteria require mechanisms to combat this toxicity, when growing in oxygen (42). The presence of the NADH oxidases, NADH peroxidases, and millimolar levels of manganese (4, 5, 21, 80) are common ways in which these organisms defend against the toxic effect of ROS. In some instances within the LAB group, manganese cofactored superoxide dismutases (SOD's), heme-catalases, and manganese cofactored catalases have been identified (1-3, 6, 24, 25, 37, 44, 45, 51, 53, 55, 64, 68, 77, 82).

Lactobacilli and LAB as a group are classically defined as catalase negative (34). However, both genomic data and *in situ* experimentation have demonstrated the presence of both heme and non-heme (manganese) catalases in selected species of *Lactobacillus* (1, 37, 41, 44, 45, 51, 53, 64, 82). Therefore, the presence of catalases in lactobacilli represents a unique opportunity to investigate potential advantages associated with having recombinant forms in generally recognized as safe (GRAS) species used as starter cultures in the food industry and as probiotics. Inclusion of antioxidants and antioxidant enzymes within food packages (15, 40, 66), starter cultures (64, 73), dietary adjuncts, and probiotic cultures (13, 14, 16, 72, 73) to inhibit/decrease the deleterious effects

of oxidative damage can significantly improve growth and survival in aerobic environments.

Catalases (E.C. 1.11.1.6) are a class of metalloproteins responsible for catalyzing the disproportionation of H_2O_2 to H_2O and O_2 (17, 36, 49, 78). Currently, there are three types of catalases; the monofunctional heme-cofactored, bifunctional heme-cofactored catalase-peroxidase, and manganese-cofactored catalases (Mn-catalases) that together, constitute several hundred sequences in Bacteria, Eukaryota and Archaea (18). Mn-catalases compose the smallest group (~25) based on *in silico* analyses performed by Chelikani et al. (18) but, recently at this pool has increased to ~67 protein sequences. Mn-catalases to date have not been found outside Bacteria and Archaea (18) and based on phylogenetic analysis, they group within three clades. In one clade rests the Mn-catalases common to *Firmicutes*, which include some bacilli, enterococci and *Lactobacillus plantarum* (*L. plantarum*) as well as members of the *Planctomycetes* and Archaea. Within the other clade, there are members of *Enterobacteriacea, Pseudomoneacea Clostridiaceae*, *Bacillaceae*, and some cyanobacteria (50). The presence of catalases in lactobacilli is unique, considering that lactic acid bacteria (LAB) as a group are classified as catalase negative (34). However, within the genus *Lactobacillus* both heme containing catalases and Mn-catalases have been detected (1, 41, 44, 45, 51, 53, 82), although, they are not widespread and heme catalase activity is dependent upon the presence of exogenous heme (34). The presence of Mn-catalase in *L. plantarum* overcomes its inability to synthesize heme and combat exogenous and endogenous H_2O_2 that is ultimately toxic to the cell. In this respect, the Mn-catalase could ultimately provide a benefit to a host cell that lacks catalase and whose ROS defense mechanisms are lacking, LAB and specifically *Lactobacillus* handle ROS's differently depending upon the species (31, 32, 56, 58, 63, 79).

L. plantarum ATCC 14431 manganese catalase (Mn-catalase) has been cloned in *Escherichia coli* (*E. coli*), *Lactobacillus* sp., and *Lactococcus lactis* (*Lc. lactis*) (41, 73). However, cloning in *E. coli* proved troublesome due to the formation of inclusion bodies (41). While three different species of LAB were transformed with *L. plantarum* Mn-catalase, only one, *Lactobacillus casei* (*L. casei*) demonstrated activity (73). One desired characteristic of a probiotic is to have antioxidative mechanisms. It is also of interest to clone the Mn-catalase into heterologous hosts that

could be utilized in either industrial or therapeutic applications. The objective of this work was to clone and express the Mn-catalase gene from *L. plantarum* in two potential probiotic lactobacilli, *Lactobacillus gasseri* and *Lactobacillus reuteri* for and examine any potential benefits.

3.2 Materials and methods

Bacterial strains and media. Bacterial strains and plasmids are outlined in Table 1. Strains of *E. coli* were grown at 37^0 C, 200 rpm in Luria-Bertani broth or at 37^0 C on Luria-Bertani 1.5% agar medium with either ampicillin (Am, 100 μg/ml) or erythromycin (Em, 150 μg/ml). Species of *Lactobacillus* were cultured at 37^0 C from glycerol stocks first in MRS and/or APT broth and then two subcultures to 1.5% MRS and/or APT agar before every experiment. When appropriate, *Lactobacillus* cultures were grown in either MRS and/or APT broth at 37^0 C and 200 rpm under oxic conditions, or when necessary at 37^0 C without shaking under anoxic conditions in a Coy anaerobic chamber (Coy Labs, Grass Lake, MI). In all growth experiments, the liquid to head space ratio was 1:5. When necessary 5 μg/ml Em was added to *Lactobacillus* cultures.

Sources of chemicals and enzymes. Lysozyme, mutanolysin, proteinase K, phenol, chloroform, 3,3'-diaminobenzidine, horseradish peroxidase, N,N'-tetramethylenediamine(TEMED), and all antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). All other general use chemicals, hydrogen peroxide, and bacteriological media were purchased from Fisher Scientific (Pittsburgh, PA). Molecular reagents to include, all cloning enzymes, *Taq* polymerase, dNTP's, $MgCl_2$, and PCR buffers were purchased from Promega (Madison, WI).

DNA isolation and manipulation. Isolation of total DNA was carried out with QIAGEN's DNeasy Tissue Kit (QIAGEN Valencia, CA). Plasmid DNA from *E. coli* was isolated using QIAGEN's Plasmid DNA Mini-Prep Kit (QIAGEN Valencia, CA), while plasmid DNA from lactobacilli was isolated according to the method of O'Sullivan and Klaenhammer (65). Products generated from cloning manipulations and PCR were purified using QIAEX II Gel Extraction Kit (QIAGEN Valencia, CA).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source and/or reference [a]
Bacterial strains		
Escherichia coli		
DH5α	*rec* cloning strain	Stratagene
Lactobacillus plantarum		
CECT 221(ATCC 14431)	Mn-catalase host strain	J. Bruno-Barcena
Lactobacillus gasseri		
NCK 334	Type strain, human isolate ATCC 33323	T. Klaenhammer (collection stock of NC)
NC 1500	NCK 334 harboring pTRK563	This study
NC 1504	NCK 334 harboring pMnKat	This study
Lactobacillus reuteri		
NCK 932	Type strain, human intestinal isolate, DSM20016, ATCC 23272	T. Klaenhammer (collection stock of NC)
NC 1530	NCK 932 harboring pTRK563	(13)
NC 1535	NCK 932 harboring pMnKat	This study
Plasmids		
pBlueScript II KS(+)		Novagen
pTRK563	Em_r, Δ*cat* derivative of pGK12 with *lacZ* from pBluescript II KS(+)	(74)
pKSKat	1449-bp *mnkat* PCR amplicon from *L. plantarum* CECT 221 (ATCC 14431) into pBluescript II KS(+)	J. Bruno-Barcena
pMnKat	1449-bp *mnkat* from pKSKat cloned into pTRK563	This study

a NC and NCK, culture collections at North Carolina State University, Raleigh; ATCC, Amercian Type Culture Collection; DSM,Deutsche Sammlung von Mikroorganismen; CECT, La Coleción Española De Cultivos Tipo.

Ligation reaction mixes were used directly to transform *E. coli* and/or lactobacilli without further purification.

PCR. Traditional PCR was carried out utilizing Promega's Go*Taq* polymerase, dNTP's, and buffers (Promega Madison, WI). Primers EcoRIKatLpR1 (5'-GATCGAATTCCACCGCCTTAACTTCTC-3') and NdeIKatLpF2 (5'-AGAATTCCATATGTAACGGCAGTCCAG) (Integrated DNA Technologies, Coralville, IO), restriction sites underlined, were used to amplify the 1449-bp Mn-catalase gene from *L. plantarum* CECT 221 (ATCC 14431), including its promoter and terminator elements and the corresponding product was verified by sequencing (Iowa State University DNA Facility, Ames, IA) and BLAST analysis (NCBI).

This gene was also amplified, when necessary, with the above primers from subsequent plasmid constructs in which it was included (Table 1). Primers KatLp Antisense 766bp (5'-TAATATGTGGAATACCACCC-3') and KatLp Sense 766bp (5'-CATACAAGAAAACTGCAATA-3'), (MWG-BIOTECH Inc., High Point, NC) were used to screen for the presence of Mn-catalase plasmid bearing strains by amplifying an ~766-bp fragment of the 815-bp internal structural region of the gene, followed by sequencing validation (MWG-BIOTECH Inc., High Point, NC) and BLAST analysis (NCBI). Primers Lac16SForward (5'-GACGAACGCTGGCGGCGTGCCT-3') and Mod Lac16S Rev (5'-GGTAGCCGTAGGAGAACCTGC-3'), (MWG-BIOTECH Inc., High Point, NC) were used for 16S rDNA amplification and sequencing (MWG-BIOTECH Inc., High Point, NC) to validate *Lactobacillus* sp. All amplifications were carried out using Bio-Rad's 96-well iCycler (Bio-Rad, Hercules, CA).

Bacterial transformations. Strains of *E. coli* were transformed using electroporation standard methods (59) with a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA). Species of *Lactobacillus* were transformed using electroporation according to the method of Luchansky et al., (57) with HEPES electroporation buffer and Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA).

Preparation of cell free extracts (CFE's). Following two transfers, cultures of *Lactobacillus* with and without pMnKat were grown under oxic conditions with shaking at 200 rpm or under anoxic conditions without shaking in a Coy anaerobic chamber (Coy Labs, Grass Lake, MI) at 37^0 C and a 1:5 liquid to head space ratio. Cultures were then harvested in exponential growth phase by pelleting the cells by centrifugation at 3000 X g for 20 min. Resulting pellets were washed three times in an equal volume of phosphate-EDTA buffer (50 mM phosphate, 0.1 mM EDTA buffer, pH 7.8). After the final wash, the resulting pellet was resuspended in phosphate-EDTA buffer containing 1 mM PMSF, at $1/40^{th}$ the original culture volume. This suspension was then transferred to a 2 ml gasket sealed screw cap tube containing 1/2 its volume in 0.2 mm silica beads (BioSpec, Inc., Bartlesville, OK) for cellular disruption in a Mini-BeadBeater-8 (BioSpec, Inc., Bartlesville, OK). Cellular suspensions were homogenized for 10x1 min treatments with 3 min rest cycles on ice in between treatments to prevent sample overheating. Following the final homogenization, clarification of the supernatant was achieved by pelleting the cellular debris through centrifugation at 20000 X g and 4^0 C for 30 min.

Clarified supernatant was removed from the tube and transferred to 6,000-8,000 MWCO dialysis tubing, in which it was dialyzed against two changes of phosphate-EDTA buffer at 4^0 C for 24 hrs. When necessary CFE's were concentrated further using YM-10 Centricon Centrifugal Filter Devices (Millipore).

Biochemical assays. Total protein concentration in CFE's was determined using the Bradford method (12), using bovine serum albumin (BSA) as the standard. Catalase activity gels were performed using 10% non-denaturing native PAGE gels and the staining method of Clare, et al. (19). Screening catalase tests were performed by smearing a portion of a colony on a microscope slide followed by a drop of 3% H_2O_2 to each smear and observing the presence or absence of bubbles. Specific catalase activity in CFE's was determined using a spectrophotometric assay that followed the loss of hydrogen peroxide over time at 240 nm (9). Experiments were performed in biological triplicate.

Western blotting. Total protein extracts of 5-10 μg were separated on a 10% SDS-PAGE gel using a Bio-Rad Mini-Protean II electrophoresis system (Bio-Rad, Inc., Hercules, CA). Separated proteins were then electroblotted to a 0.45 μm nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany) using the Invitrogen XCell II blot module (Invitrogen, Corp., Carlsbad, CA). Verification of transferred protein was performed by Amido Black staining (Bio-Rad, Hercules, CA) of the membrane per the manufacturers instructions. The membrane was blocked for 1 hr in 5% (wt/vol) non-fat milk (Carnation) solubilized in PBS-T (1X phosphate buffered saline pH 7.0, 0.1% Tween 20). Monoclonal antibodies specific for Mn-catalase (J.W. Whittaker, Oregon Health Sciences University) were added to fresh blocking buffer at a 1:10,000 dilution and incubated with gentle agitation for 2 hrs. The membrane was then washed 3 X 15 min in blocking buffer, after which fresh blocking buffer containing 1:10,000 goat anti-rabbit conjugated horseradish peroxidase (Bio-Rad, Inc., Hercules, CA) was added and the membrane and incubated for 2 hrs. After final incubation, the membrane was washed in fresh blocking buffer 1 X 15 min followed by a wash in PBS-T for 2 X 15 min. Signal was detected by incubating the membrane in an equal volume mixture of Western Lightning Chemiluminescence Reagent Plus substrates (Perkin-Elmer, Waltham, MA) for 1 min

followed by a 15-30 sec exposure to Kodak BioMax Light film (Perkin-Elmer, Waltham, MA). Experiments were performed in biological triplicate.

Microscopy and images. Cultures were Gram-stained based on standard bacteriological methods (20) and visualized with a Nikon Alphaphot Microscope (Nikon, Inc., USA). Images were taken with a Nikon D40X SLR with 50 mm lens (Nikon, Inc., USA).

Cell viability. Cultures were transferred twice in either liquid MRS or APT media with Em (5 μg/ml), The cultures were then used to inoculate 25 ml of either MRS or APT media without Em to a starting OD_{600nm}~0.05 and grown to exponential phase at 37^0 C with shaking at 200 rpm. From the exponentially growing cultures, 50 ml of MRS or APT media without Em was inoculated to a starting OD_{600nm}~0.05 and allowed to grow for 96 hrs at 37^0 C with shaking at 200 rpm under oxic conditions. Samples were removed from each culture at 6, 12, 24, 36, 48, 72, and 96 hr time points, serially diluted in phosphate buffered saline (PBS) pH 7.0 and 10 μl were spot plated on either MRS or APT agar without Em. Plates were incubated at 37^0 C for 24-48 hrs and colonies enumerated. Survival plots were plotted as a function of time using GraphPad Prism 4 for Macintosh (GraphPad Software, San Diego, CA). Experiments were performed in biological triplicate.

H_2O_2 Susceptibility Disk Diffusion Assays. Following two successive transfers in either MRS or APT with Em (5 μg/ml), *Lactobacillus* cultures were used to inoculate 25 ml of either MRS or APT media with Em (5 μg/ml) to a starting OD_{600nm} ~0.05 and grown overnight (9-12 hr). From overnight cultures, tubes of PBS were inoculated to an OD_{600nm} of 1. From the standardized PBS tubes, 100 μl of inocula was transferred to 5 ml of either MRS or APT top agar (0.75%) tempered to 50^0 C. After gentle vortexing, the suspension was poured onto corresponding bottom agar (1.5%). Once polymerized, sterile 6 mm disks were placed onto the surface of the top agar according to varying stock concentrations of hydrogen peroxide, 0-190 mM in 25 mM increments for MRS and 0-250 mM in 50 mM increments for APT media, respectively. To each disk, 5 μl of a corresponding stock concentration was added and plates were then incubated under oxic conditions at 37^0 C until zones were visualized. Diffusion assays were performed in three biological replicates and averages

of zones of inhibition (mm) were taken per respective concentrations of hydrogen peroxide added to the disks.

Effects of [H_2O_2] on growth (OD_{600nm}). Following two successive transfers in either MRS or APT media with Em (5 μg/ml), *Lactobacillus* cultures were used to inoculate 25 ml of either MRS or APT media with Em (5 μg/ml) to a starting OD_{600nm}~0.05 and grown to exponential phase at 37^0 C, 200 rpm. Exponentially growing cultures of lactobacilli were then used to inoculate fresh MRS or APT (100 μl) containing 0.5 μg/ml Em in NUNC-96F microtiter plates (NUNC, ThermoFisher Scientific, Rochester, NY) to a starting OD_{600nm} ~0.1. Growth at 37^0 C was monitored at OD_{600nm} with continuous shaking under oxic conditions in the presence and absence of 1, 5, and 10 mM hydrogen peroxide over time using the FLUOStar OPTIMA plate reader system (BMG LABTECH Inc., Durham, NC). Maximum specific growth rate ($\mu_{max} \cdot hr^{-1}$) was calculated from the slope of the line of the exponential phase portion of the growth curve that had an r^2 of 0.99 by fitting the data to a linear regression model using GraphPad Prism 4 for Macintosh (GraphPad Software, San Diego, CA). Data is based upon the average of three biological replicates.

3.3 Results and Discussion

3.3.1 Construction of pMnKat. From genomic DNA of *L. plantarum* CECT 221, *mnkat* was amplified with the primer set EcoRIKatLpR1 and NdeIKatLpF2 and cloned into pBlueScript II KS(+) creating the pKSKat construct. Following an *EcoRI* digest, of pKSKat, *mnkat* was isolated from a gel as a predicted 1.4-kb band. This purified 1.4-kb fragment was then ligated to *EcoRI* digested pTRK563 shuttle vector (74) to create a new plasmid, pMnKat (Fig. 1.).

3.3.2 Transformation of *Lactobacillus* spp. with pMnKat. Newly constructed pMnKat was electroporated into *L. gasseri* NCK 334 and *L. reuteri* NCK 932. Positive transformant colonies were detected initially by using a qualitative catalase test, where a drop of 3% H_2O_2 was added to cells smeared on a glass slide. As shown in (Fig. 2.), positive colonies demonstrate effervesence, while wild-type colonies lacked effervescence activity.

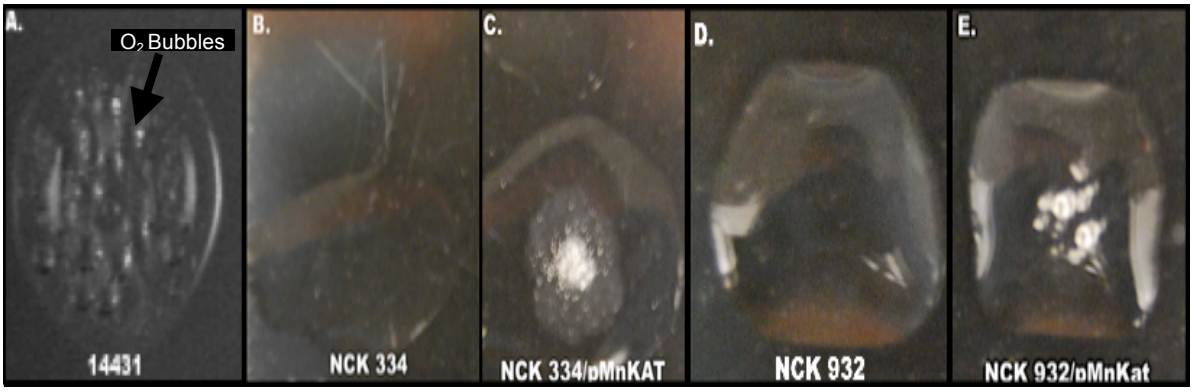

FIG. 2. Catalase activity screening of wild-type and recombinant lactobacilli with and without Mn-catalase. **A.** ATCC 14431, **B.** *L. gasseri* NCK334, **C.** *L. gasseri* NCK 334 pMnKat, **D.** *L. reuteri* NCK 932, and **E.** *L. reuteri* NCK 932 pMnKat. Wild-type lactobacilli with pTRK563 were also tested and lacked catalase activity (data not shown).

Following qualitative catalase screening, colonies were purified by streaking over a series of five transfers on APT agar containing 5 μg/ml Em. From a single purified colony that exhibited catalase activity and Em resistance. A 10% glycerol stock was made and from this stock the remainder of the experiments were performed.

To further validate the presence of an intact and functional Mn-catalase, Western blotting with Mn-catalase specific antibodies, spectrophotometric catalase activity assay, and catalase activity gels were performed. As seen in Figure 3 Panel A and B the expression level of the recombinant Mn-catalase out of the total protein appears to be less than that of the wild-type host *Lactobacillus* spp based upon same total protein loads. This occurrence is most likely due to differences in expression of MnKat by *L. gasseri* and *L. reuteri,* this phenomenon was also seen in the expression of MnSOD (13). Additionally, in the recombinant species there also appears to be multimeric (polymeric) forms of the MnKat protein detected (Fig. 3. A and B). These are possibly multimers of the protein as the molecular weight correlated well to a trimeric form of a ~84 kDa protein. These multimers are not seen in the in the negative controls of the same species with and without the empty shuttle vector, (Fig. 3. A and B). Multimeric forms of the protein are also suggested in the wild-type *L. plantarum* Mn-catalase host. as the detected bands correlate well with a dimerized form of the protein.

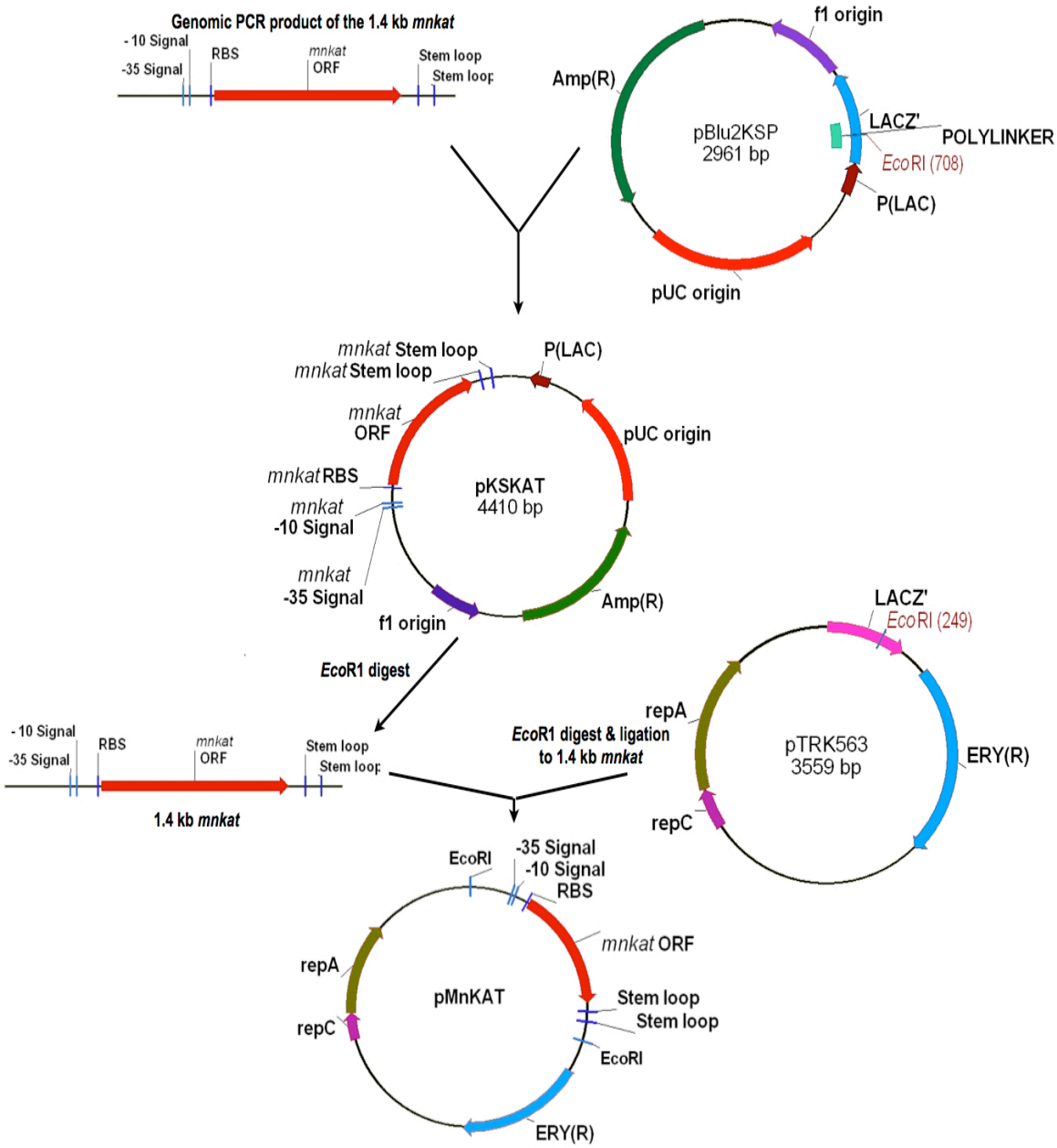

FIG. 1. Construction of the pMnKat plasmid. BlueScript vector, pBlueScript KSII (+), containing *mnkat* along with its promoter and terminator elements was digested with *EcoRI*. This digestion led to a 1.4-kb fragment that was gel purified and ligated into an *EcoRI* and CIP treated shuttle vector, pTRK563. This created a new construct termed pMnKat.

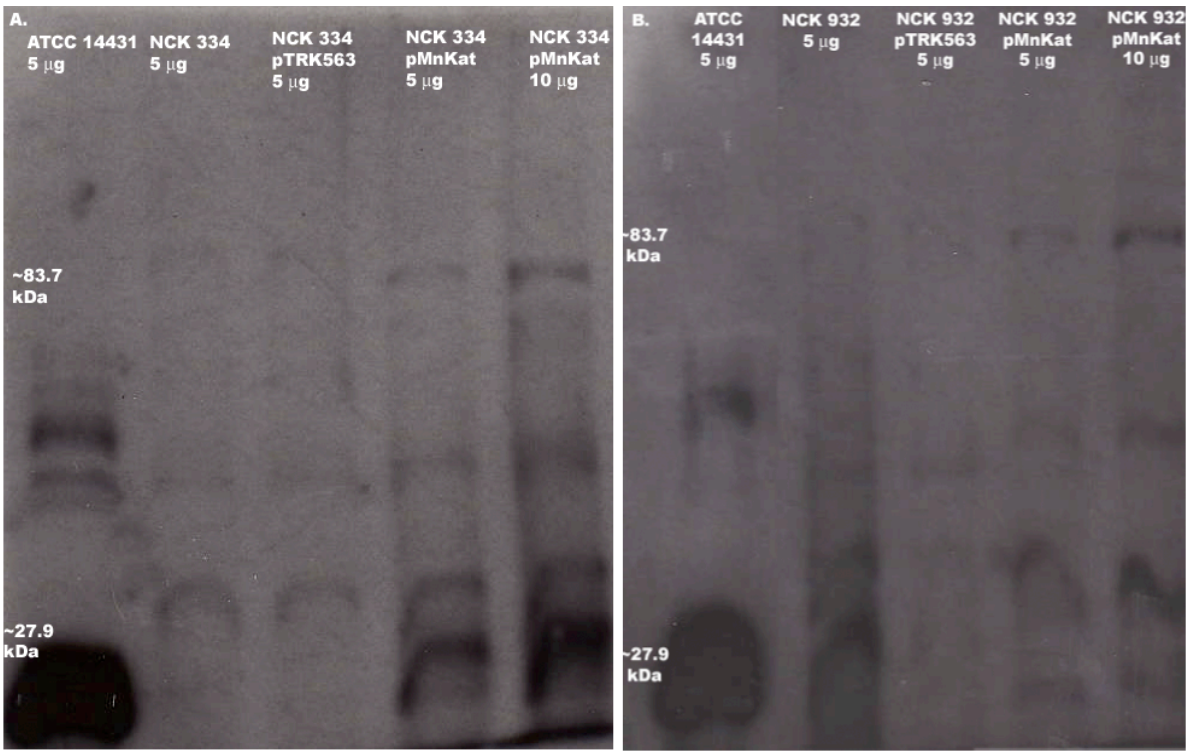

FIG. 3. Western blotting of CFE's of *L. gasseri* NCK 334 strains and *L. reuteri* NCK 932 strains expressing and not expressing Mn-catalase. Separation of total proteins, 5 to 10 μg, was performed on a 10% denaturing SDS-PAGE gel. Following electroblotting, Mn-catalase was probed for using Mn-catalase monoclonal antibodies. Panel A. ATCC 14431, *L. gasseri* NCK 334, *L. gasseri* NCK 334 pTRK563, *L. gasseri* NCK 334 pMnKat (5 μg), and *L. gasseri* NCK 334 pMnKat (10 μg). Panel B. ATCC 14431, *L. reuteri* NCK 932, *L. reuteri* NCK 932 pTRK563, *L. reuteri* NCK 932 pMnKat (5 μg), and *L. reuteri* NCK 932 (10 μg).

Polymers of other ROS detoxifying enzymes have been reported before in Western blots (29). Not only was the monomeric form detected, but polymeric forms were also detected, as a result of hydrogen peroxide mediated oxidation of specific surface amino acids as a result of H_2O_2 generation from SOD1 (29). Therefore, the presence of detectable multimeric forms of this and other ROS detoxifying proteins may not be that uncommon due to their role in oxidative stress. Additional validation that *mnkat* was indeed coding for a catalase was visualized in activity gels, (Fig. 4. A and B). As observed in the Western blot, multiple catalytically active bands of Mn-catalase were also detected in recombinant *L. gasseri* NCK 334 (Fig. 4 A and B) as well as in *L. plantarum* ATCC 14431 (data not shown). Only a single band of activity was ever observed in recombinant *L. reuteri* NCK 932, (Fig. 4 A and B), although polymeric forms of the Mn-catalase protein were detected in the Western blot, (Fig. 3B).

Additionally, only a single band of activity was detected in *L. plantarum* ATCC 14431 (not shown) as well *L. gasseri* NCK 334 and *L. reuteri* NCK 932 expressing Mn-catalase under anoxic conditions. However, due to non-denaturing conditions, other factors influencing the way the catalase bands appear on the native gel including; pI, pH, associated proteins, and potential differences in host protein handling (i.e. post-translational modifications) all can influence the appearance of catalytically active bands on an activity gel. Similarly, multiple catalytically active bands of MnSOD have also been observed during non-denaturing PAGE (personal communication H. Hassan).

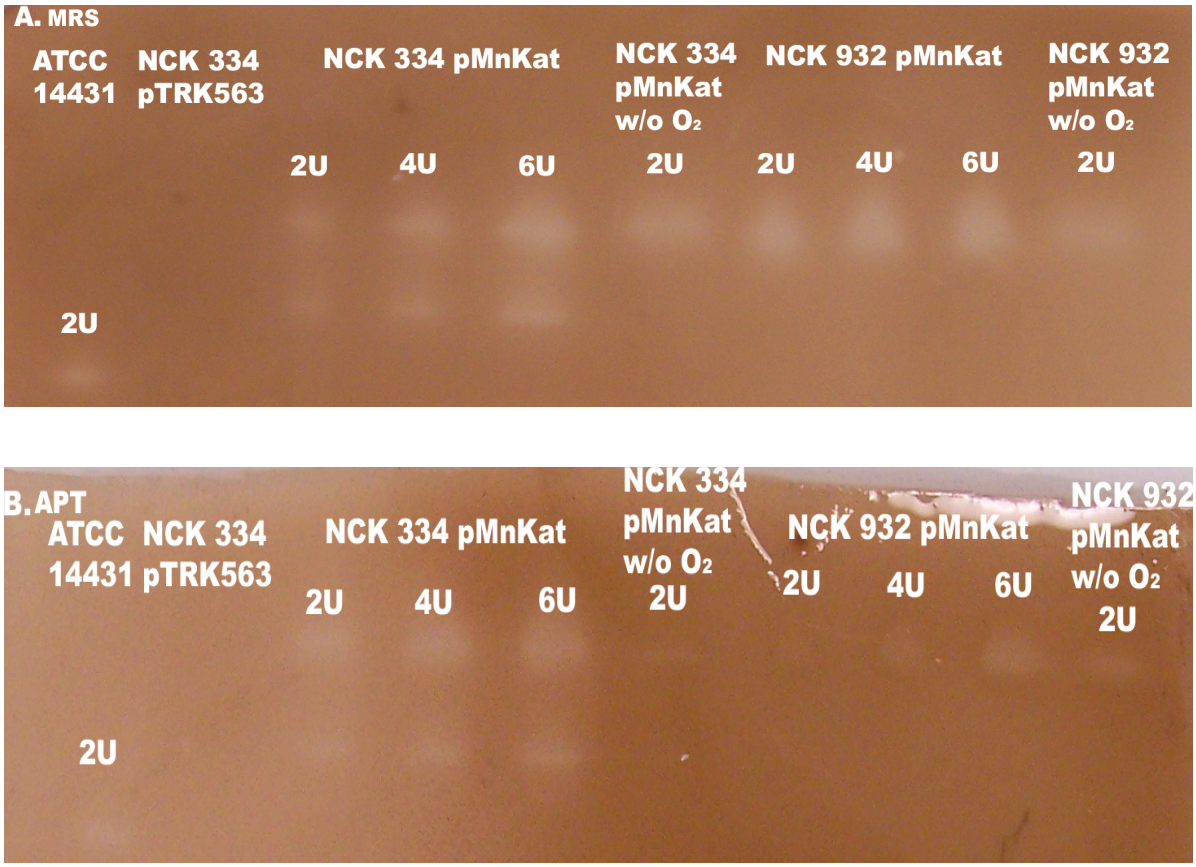

FIG. 4. Catalase activity gels from total protein isolated from cells grown in either MRS (A) or APT (B) and in the presence and absence of oxygen. Samples were applied to 10% non-denaturing native PAGE gels based upon constant units of activity (U) and stained using diaminobenzidine stain by Clare et. al. (A) MRS grown cells; ATCC 14431; NCK 334 pTRK563 (Neg Control, 100 μg protein); NCK 334 pMnKat-2U, 4U, and 6U; NCK 334 pMnKat w/o O_2-2U; NCK 932 pMnKat-2U, 4U, and 6U; NCK 932 pMnKat w/o O_2-2U. (B) APT grown cells; ATCC 14431; NCK 334 pTRK563 (Neg Control, 100 μg protein); NCK 334 pMnKat-2U, 4U, and 6U; NCK 334 pMnKat w/o O_2-2U; NCK 932 pMnKat-2U, 4U, and 6U; NCK 932 pMnKat w/o O_2-2U.

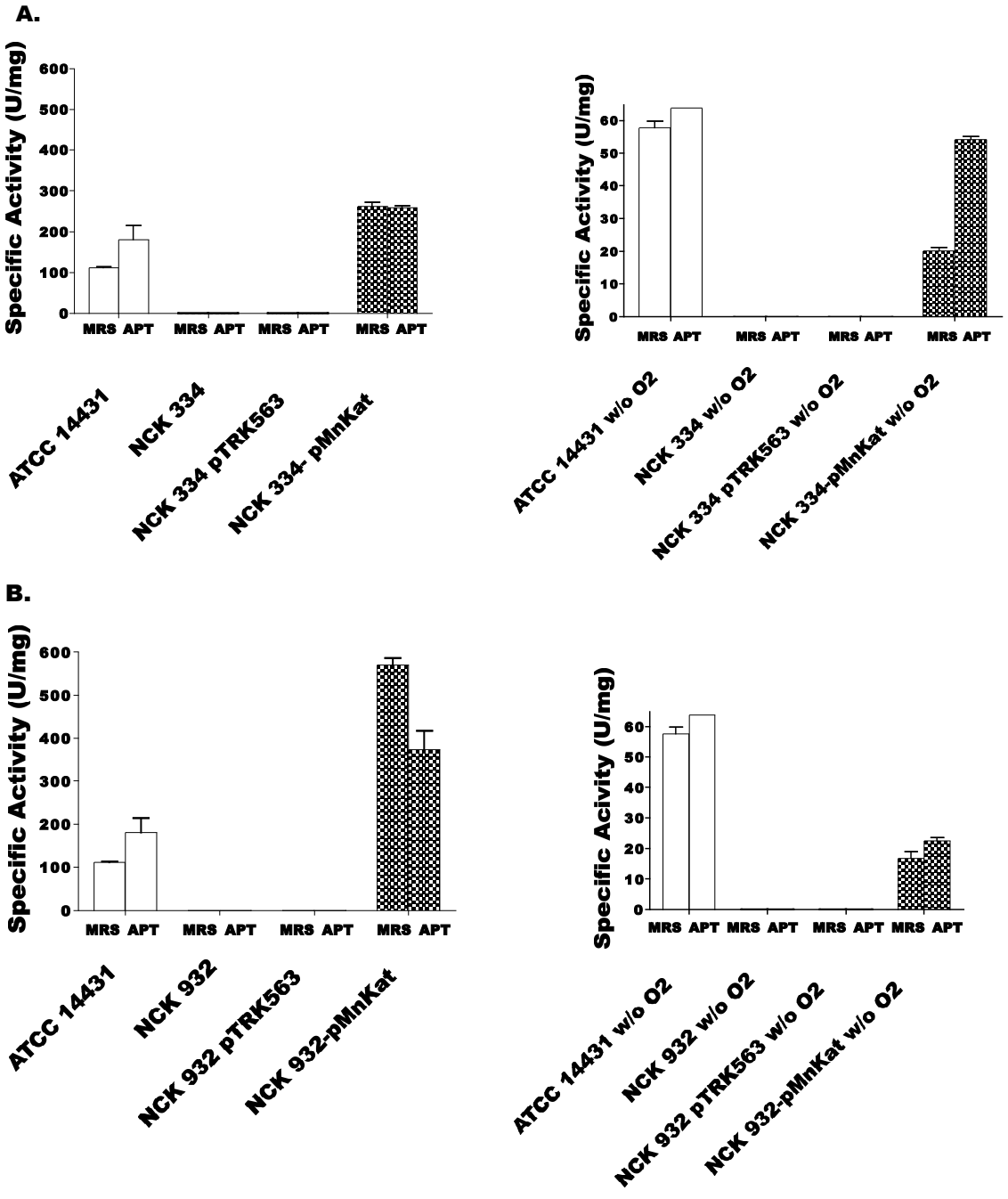

FIG. 5. Catalase activity assays (A and B) performed using CFE's of MRS and APT grown cells in the presence and absence of oxygen. Specific activities (U/mg) of Mn-catalase was measured according to the method of Beers and Sizer. (A) Specific activities (U/mg) of MRS and APT grown cells of *L. plantarum* ATCC 14431 and *L. gasseri* NCK 334 strains in the presence and absence of oxygen. (B) Specific activities (U/mg) of MRS and APT grown cells of *L. plantarum* ATCC 14431 and *L. reuteri* NCK 932 strains in the presence and absence of oxygen. Experiments were performed in biological triplicate under oxic conditions and biological duplicate under anoxic conditions.

Activity assays of the *Lactobacillus* strains transformed with pMnKat demonstrated that catalase activity was expressed in both *L. gasseri* NCK 334 and *L. reuteri* NCK 932 (Fig. 5 A and B). Additionally, both *L. gasseri* NCK 334 and *L. reuteri* NCK 932 harboring the pMnKat exhibited higher levels of Mn-catalase activity under oxygenated conditions in both MRS and APT, while *L. plantarum* ATCC 14431 expressed higher levels of Mn-catalase activity under anoxic conditions in the same media types (Fig. 5 A and B). While this is the first report to document the activities of recombinant the Mn-catalases in *L. gasseri* and *L. reuteri* in MRS and APT media under anoxic conditions, Rochat et al. (73) demonstrated that *L. casei*, expressing Mn-catalase exhibits low activity under oxygenated conditions in MRS media when compared to *L. plantarum* ATCC 14431 (73). This is in contrast to the present findings in which both of the recombinant *L. gasseri* NCK 334 and *L. reuteri* NCK 932 expressed Mn-catalase activity at 2.5-times and 5-times higher, respectively, than observed in *L. plantarum* ATCC 14431 (Fig. 5 A and B). Whereas the detected protein of Mn-catalase appeared to mirror the activity in *L. casei* (73), we found that the expression of Mn-catalase in *L. gasseri* NCK 334 and *L. reuteri* NCK 932 appears to be lower in comparison to *L. plantarum* ATCC 14431 for the same amount of total protein (Fig. 3 A and B). Additionally, growth of the recombinant strains in MRS or APT media under oxic conditions resulted in a higher Mn-catalase activity in *L. reuteri* NCK 932 than in *L. gasseri* NCK 334 (i.e. 2-times and 1.5-times higher in MRS and APT, respectively) (Fig. 5 A and B). Though the reasons for the difference in the activity of Mn-catalase in *L. reuteri* cells grown in MRS and APT are not fully clear at this time, one possible explanation could be linked to composition differences between MRS and APT with respect to phosphate content, (i.e.10.4 mM and 26.0 mM, respectively). *L. reuteri* NCK 932 may form polyphosphate granules that could potentially store up free Mn(II) that would be utilized to cofactor the Mn-catalase. For a complete review on polyphospate and Mn(II) in LAB refer to Archibald and his work on manganese accumulation in LAB (4). Differences between the two recombinant strains of lactobacilli are also seen in the overall expression of the Mn-catalase. While *L. gasseri* NCK 334 demonstrated lower enzymatic activity, it appears to express overall more Mn-catalase protein than that of *L. reuteri* NCK 932, Fig. 3, Fig 5. A and B. This finding of low Mn-catalase protein expression with higher enzymatic activity and its inverse has also been

reported for the MnSOD of *S. thermophilus* AO54 (13). This effect could be due to differences in the Mn concentration inside the respective cells as well as differences in abilities to express the protein.

3.3.3 Effect of expressing Mn-catalase on the cellular physiology of *L. gasseri* NCK 334 and *L. reuteri* NCK 932.

(i) Colony and cellular morphology. Upon transformation of the both *L. gasseri* NCK 334 and *L. reuteri* NCK 932 with the plasmid pMnKat, the cellular and colony morphologies underwent morphological shift.

In order to validate our strains, colony isolates from the transformation were isolation streaked for 5 transfers in order to verify the purity of the culture. Following culture isolation, colonies of wild-type and strains harboring pTRK563 and strains of both species were photographed. We confirmed that wild-type cells and cells harboring pTRK563 or pMnKat from both species were Gram-positive, (Fig. 6 A and B). The different isolates were also streaked onto MacConkey agar (validate Gram reaction), and total DNA was extracted from each strain of both species and 16S rDNA sequencing was performed. Subsequent sequence BLAST analysis confirmed the corresponding wild-type to its pMnKat recombinant counterpart. While these tests were performed on isolates growing in both MRS and APT media, the results were the same. Therefore, images shown are from cultures propagated using MRS. *L. gasseri* NCK 334 underwent the most obvious colony morphology change; where it changed from translucent, slightly raised, irregular colonies with a rough surface to round, opaque, convex and entire colonies with a smooth glossy surface (Fig. 6A). On the other hand, *L. reuteri* NCK 932 had very little change (Fig. 6B). Furthermore, both species underwent a cellular morphology shift from distinct rods to coccoid bacilli (Fig. 6 A and B). Upon curing of the plasmid, the cells as well as the colonies reverted back to the original wild-type appearance and lost catalase activity (data not shown). It is clear that the morphological changes seen are related to the expression of Mn-catalase. This effect was not seen when the MnSOD gene was expressed in these strains (13). At the present time, we do not have a definite explanation for these changes; however, morphological changes within bacteria and LAB in particular do occur and these occurrences have been documented as natural events (34), stress induced events (61) and events due to genetic manipulation (10,43).

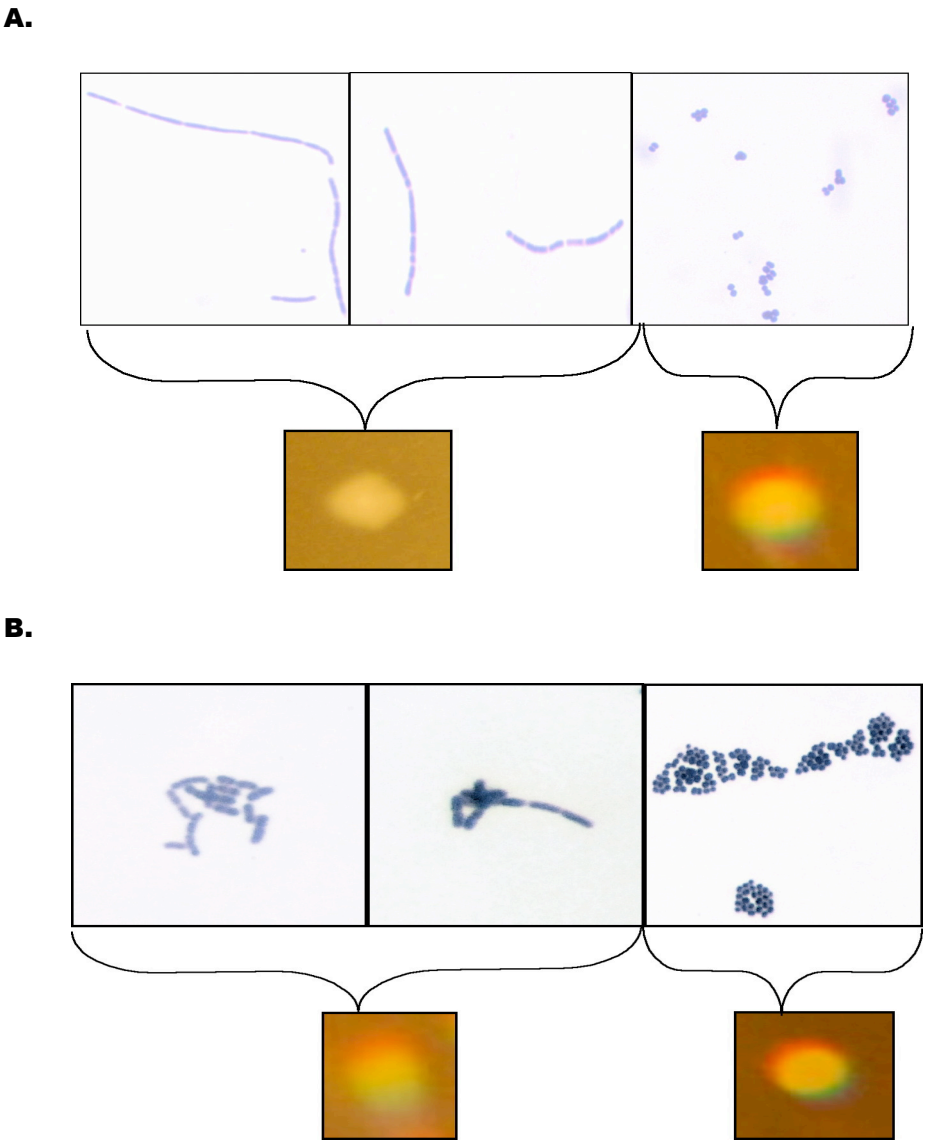

FIG. 6. Gram-stain and colony morphology before and after transformation images of pMnKat transformed *L. gasseri* NCK 334 and *L. reuteri* NCK 932 strains. (A) Left to Right; WT *L. gasseri* NCK 334, *L. gasseri* NCK 334 pTRK563, and *L. gasseri* NCK 334 pMnKat. (B) Left to Right; WT *L. reuteri* NCK 932, *L. reuteri* NCK 932 pTRK932, and *L. reuteri* NCK 932 pMnKat.

(ii) Long term survival of the transformants in aerated cultures. Previous studies have indicated that the functional significance of the Mn-catalase of *L. plantarum* ATCC 14431 is to increase the viability of the organism during the stationary phase of growth (52) by removing the intracellular hydrogen peroxide generated by the cells (63). Therefore, it was of interest to ascertain if this enzyme could also improve the viability of the recombinant strains of *L. gasseri* NCK 334 and *L. reuteri* NCK 932.

We tested the survival of the different strains and their controls under oxic conditions with shaking in both MRS and APT media. The data showed that survival of both species was indeed extended from that of the wild-type and cells harboring the empty vector pTRK563, (Fig. 7 A-D). Survival of *L. gasseri* NCK 334 cells expressing the Mn-catalase was extended in both media types (i.e. MRS and APT) with growth in the APT media being slightly more beneficial with regards to onset of cell death, though survival of cells extended to the same time point (Fig. 7A-B). Cultures of *L. reuteri* NCK 932 expressing Mn-catalase were found to be more robust with regards to cell survivability in both MRS and APT cultures, Fig. 7 C and D respectively, in comparison to *L. gasseri* NCK 334 strains (Fig. 6A and B). However, survival of both recombinant species grown in MRS was extended to 72 hrs. Cultures of *L. reuteri* NCK 932 pMnKat grown in APT media was able to maintain high viable cell numbers over a period of 36 hrs before losing significant viability, though cells survived to 96 hrs (Fig. 7C). These data indicate that expression of the Mn-catalase of *L. plantarum* ATCC 14431 can also serve a similar functional role in these recombinant species as it does in its native strain under aerated conditions, in agreement with previous reports (73).

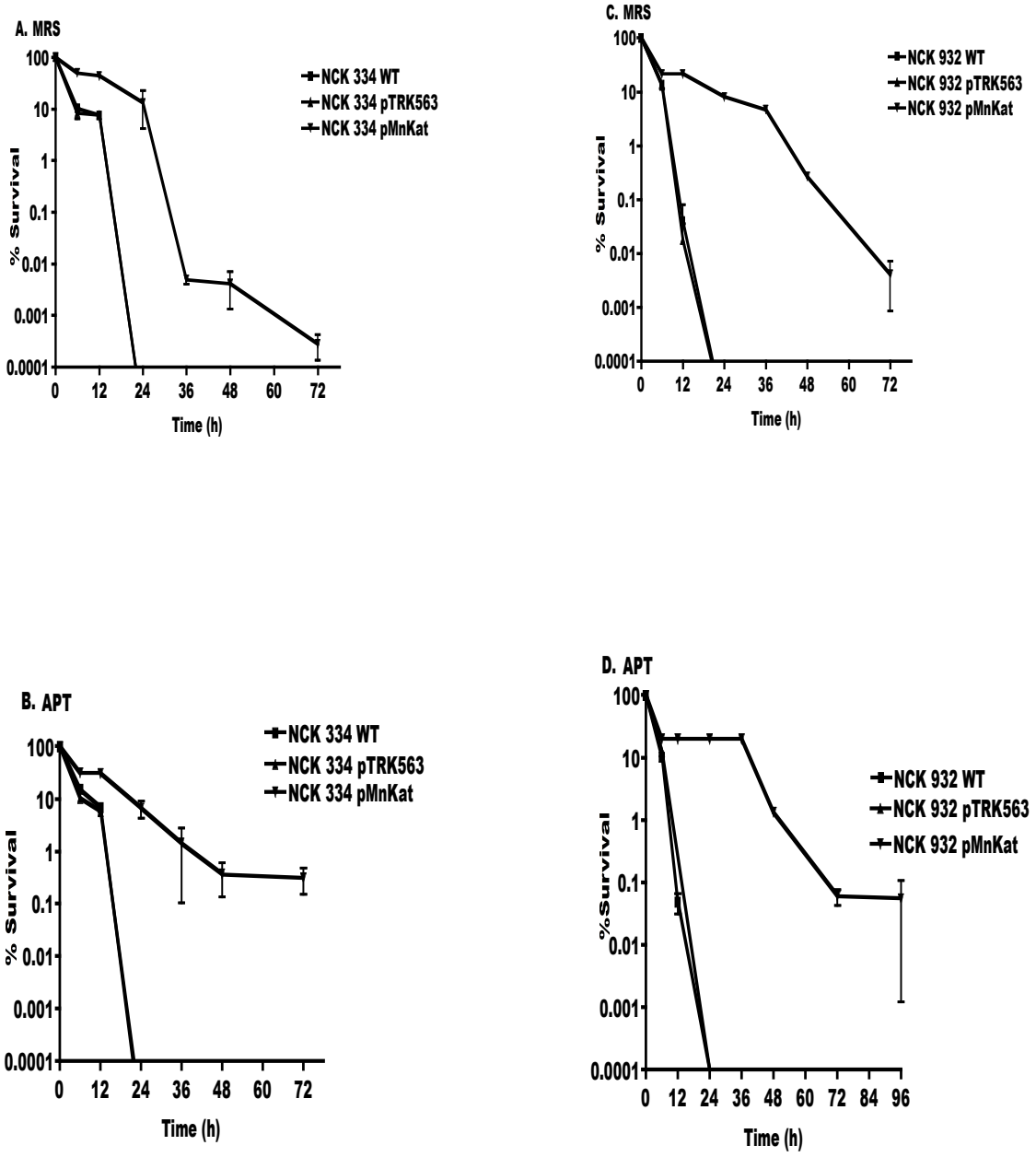

FIG. 7. Survival of aerated cultures during long-term growth, 200 rpm and 37^0 C, grown in and plated on MRS and APT media. (A) Cultures of *L. gasseri* NCK 334 WT, *L. gasseri* NCK 334 pTRK563, and *L. gasseri* NCK 334 pMnKat grown in MRS. (B) Cultures of *L. gasseri* NCK 334 WT, *L. gasseri* NCK 334 pTRK563, and *L. gasseri* NCK 334 pMnKat grown in APT. (C) Cultures of *L. reuteri* NCK 932 WT, *L. reuteri* NCK 932 pTRK563, and *L. reuteri* NCK 932 pMnKat grown in MRS. (D) Cultures of *L. reuteri* NCK 932 WT, *L. reuteri* NCK 932 pTRK563, and *L. reuteri* NCK 932 pMnKat grown in APT. Each point represents the average of three biological replicates and the error bars represent Standard Error of the Mean (SEM).

(iii) Sensitivity to H_2O_2. All lactobacilli strains utilized in this study were tested for their sensitivity to varying concentrations of H_2O_2 in both MRS and APT as outlined in materials and methods. Table 2. summarizes the findings of these assays based upon the measurement of the total diameter (mm) of inhibitory zones.

TABLE 2. Zone of inhibition diameters (mm) of H_2O_2 susceptibility disk diffusion assay. (A) and (B) MRS grown cells of *L. gasseri* NCK 334 strains and *L. reuteri* NCK 932, strains respectively. (C) and (D) APT grown cells of *L. gasseri* NCK 334 strains and *L. reuteri* NCK 932 strains, respectively. Differences in some H_2O_2 concentrations are based upon observed differences between species in a particular media. Assays were performed in biological triplicate and diameters listed are averages of triplicates.

A. MRS **nmol**

Strain	0	125	250	375	500	625	750	875	950
334 WT	-	-	-	9	9.2	9.6	10	10	11
334 pTRK563	-	-	-	8	9	9	10	10	11
334 pMnKat	-	-	-	-	-	8	8	9.3	9.7

B. MRS **nmol**

Strain	0	125	250	375	500	550	625	700	775
932 WT	-	-	6	8	9.3	9.7	10	11	11
932 pTRK563	-	-	6	8	9	9	9.7	10	10
932 pMnKat	-	-	-	-	9	9	9	9	9

C. APT **nmol**

Strain	0	125	250	375	500	625	750	875	950
334 WT	-	-	-	-	-	9	9.3	10	11
334 pTRK563	-	-	-	-	-	9	10	10	11
334 pMnKat	-	-	-	-	-	7	7.5	8	8

D. APT **nmol**

Strain	0	125	250	500	750	1000	1125	1250
932 WT	-	-	-	-	8	9	11	11
932 pTRK563	-	-	-	-	8	9	11	11
932 pMnKat	-	-	-	-	-	-	-	-

(iv) Expression of Mn-catalase enhances the growth of *L. gasseri* and *L. reuteri* in the presence and absence of H_2O_2. A previous study (73) indicated that a recombinant *L. casei* expressing Mn-catalase was capable of short term survival in the presence of exogenously added hydrogen peroxide at levels up to 10mM. However, in that report, the authors did not indicate how growth kinetics and the maximum specific growth rate (μ_{max}•hr^{-1}), of the recombinant organism were affected. Therefore, it was of interest to examine the effect that the expression of *mnkat* would have upon the host strains, especially in light of the cellular changes that have been noted (Fig. 6A-B). A previous report from our laboratory (13) noted that expression of a heterologous *sodA*, encoding the Mn-containing superoxide dismutase, improved the growth profile of some recombinant strains. Specifically, the expression of MnSOD resulted in a significant decrease in the specific growth rate (μ_{max}•hr^{-1}) of *L. gasseri* NCK 334, while it had no effect on the μ_{max}•hr^{-1} of *L. reuteri* NCK 932 (13).

In the present study, we examined the effects of the expression of the heterologous *mnkat* from *L. plantarum* on the specific growth rates of *L. gasseri* NCK 334 and *L. reuteri* NCK 932 growing in MRS and APT media in the absence and presence of different concentrations of hydrogen peroxide. Data in figure 10A-B indicated that the specific growth rate of the recombinant *L. gasseri* NCK 334/pMnKat strain, growing in either MRS or APT in the absence of H_2O_2 was ~2-fold greater than that of the wild-type strain or the wild-type strain harboring the empty vector pTRK563. In addition the specific growth rates in the presence of added hydrogen peroxide were significantly improved over the controls that lacked the pMnKat (i.e., not possessing Mn-catalase acitivity) (Fig. 10A-B). Thus, in MRS media the recombinant *L. gasseri* NCK 334/pMnKat strain continued to grow steadily in the presence of 1-10 mM H_2O_2 (Figs. 8B-D and 10A), while the wild-type and the strain harboring pTRK563 grew only at 1 mM H_2O_2 at a significantly reduced rate (Figs. 8B-D & 10A). Similar results were seen when APT media were used except that the specific growth rate of the recombinant *L. gasseri* NCK 334/pMnKat strain was reduced by 40% (i.e., from 1.3 h^{-1} and 0.71 h^{-1}) when exposed to 10 mM H_2O_2 (Fig. 10B). Also, in APT media and in presence of 10 mM H_2O_2, the recombinant *L. gasseri* NCK 334/pMnKat strain experienced a lag or a slow growth for a period of four hours before logarithmic growth was established (Fig. 9D). However, the μ_{max}•hr^{-1} of the wild-type

and pTRK563 harboring strains declined by 20% & 62% or by 10% & 28% when grown in the presence of 1 mM H_2O_2 in MRS or APT media, respectively. The decrease in the μ_{max}•hr^{-1} of the strain harboring pTRK563 grown in APT is in agreement with that reported previously (13).

On the other hand, in the absence of added hydrogen peroxide, cultures of the recombinant *L. reuteri* NCK932/pMnKat strain, showed only a slight increase in the specific growth rate when grown in MRS (Fig. 11A), and an ~1.6-fold increase in μ_{max}•hr^{-1} when grown in APT media, relative to that seen with the wild-type and the strain harboring pTRK563 (Fig. 12A). In the absence of H_2O_2, the *L. reuteri* NCK 932 cultures without *mnkat* grown in MRS, demonstrated similar growth characteristics to *L. gasseri* NCK 334 cultures without *mnkat* (i.e., have similar μ_{max}•hr^{-1} values at 0 mM H_2O_2) (Fig. 8A, 11A). When grown in MRS and in the presence of 1 mM H_2O_2, there was insignificant change in the growth characteristics, including μ_{max}•hr^{-1}, in the pMnKat harboring *L. reuteri* NCK 932 (Fig. 11B), which is similar to that seen in *L. gasseri* NCK 334 harboring pMnKat (Fig. 8B). However, unlike *L. gasseri* NCK 334 in MRS with 5 and 10 mM H_2O_2, *L. reuteri* NCK 932 strain harboring pMnKat exhibited a lengthened lag phase at 5 mM H_2O_2 then entered exponential growth phase with no apparent change in the μ_{max}•hr^{-1} (Fig. 11C), while the presence of 10 mM H_2O_2 completely inhibited the growth (Fig. 11D). Data in Figures 10 & 13 summarize the effects of different concentrations of hydrogen peroxide on the specific growth rates of the different constructs and their controls. Clearly, strains expressing the Mn-catalase have greater growth advantages in presence and in absence of H_2O_2.

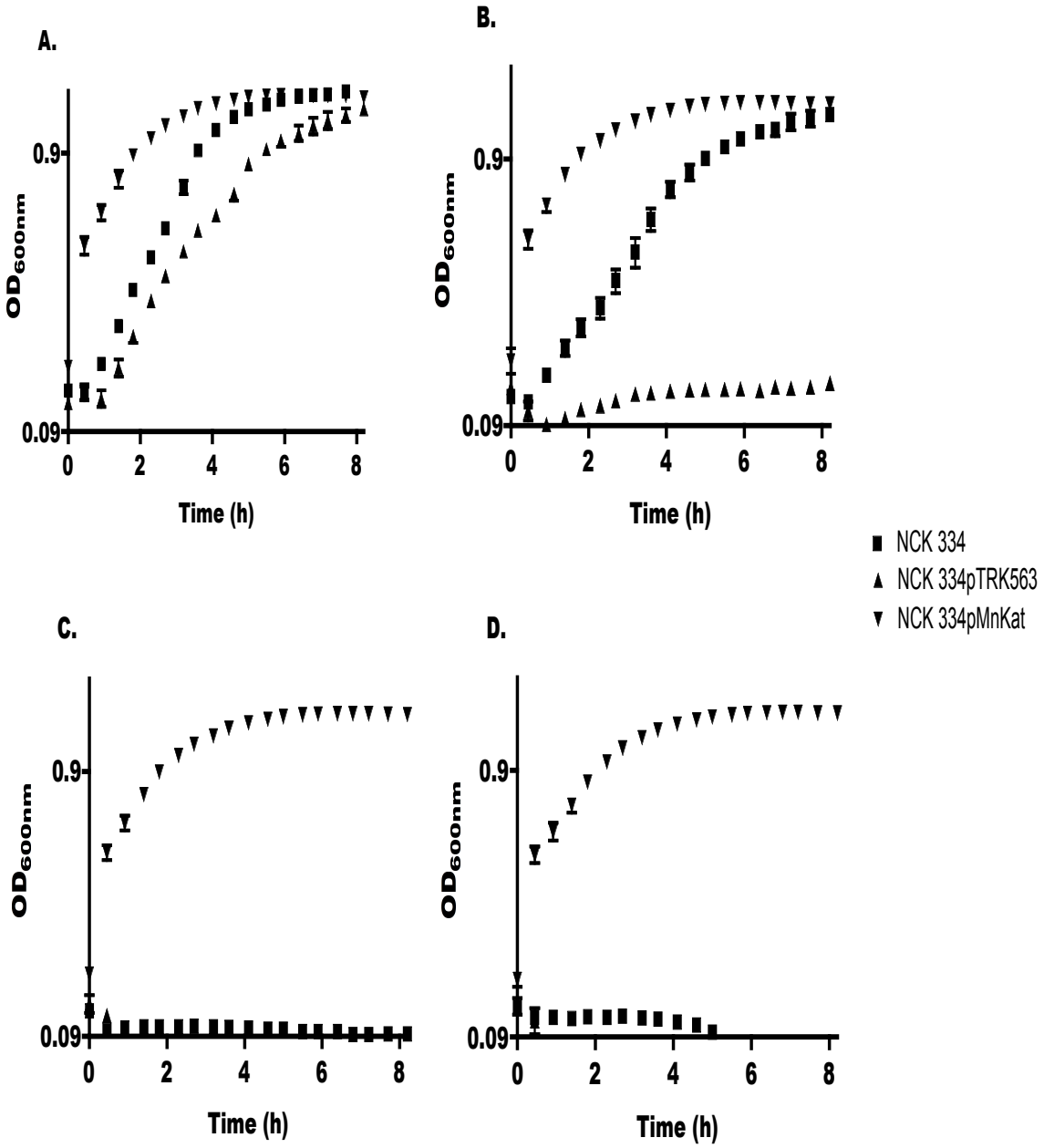

FIG. 8. Growth kinetics of MRS grown *L. gasseri* NCK 334 strains in the presence and absence of H_2O_2. (A) 0 mM H_2O_2. (B) 1 mM H_2O_2. (C) 5 mM H_2O_2. (D) 10 mM H_2O_2. Each point represents an average of biological triplicates and error bars represent SEM.

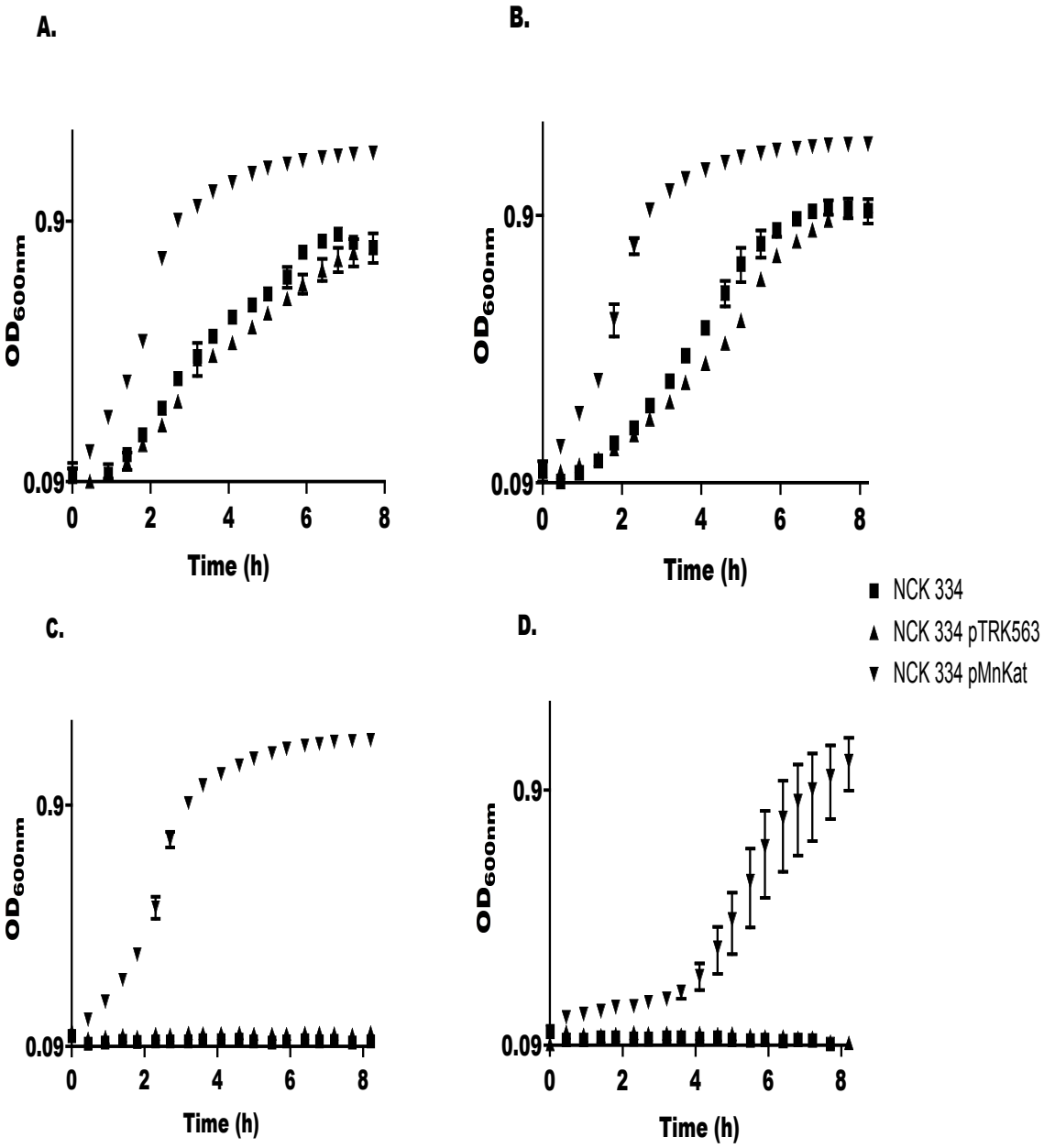

FIG. 9. Growth kinetics of APT grown *L. gasseri* NCK 334 strains in the presence and absence of H_2O_2. (A) 0 mM H_2O_2. (B) 1 mM H_2O_2. (C) 5 mM H_2O_2. (D) 10 mM H_2O_2. Each point represents an average of biological triplicates and error bars represent SEM.

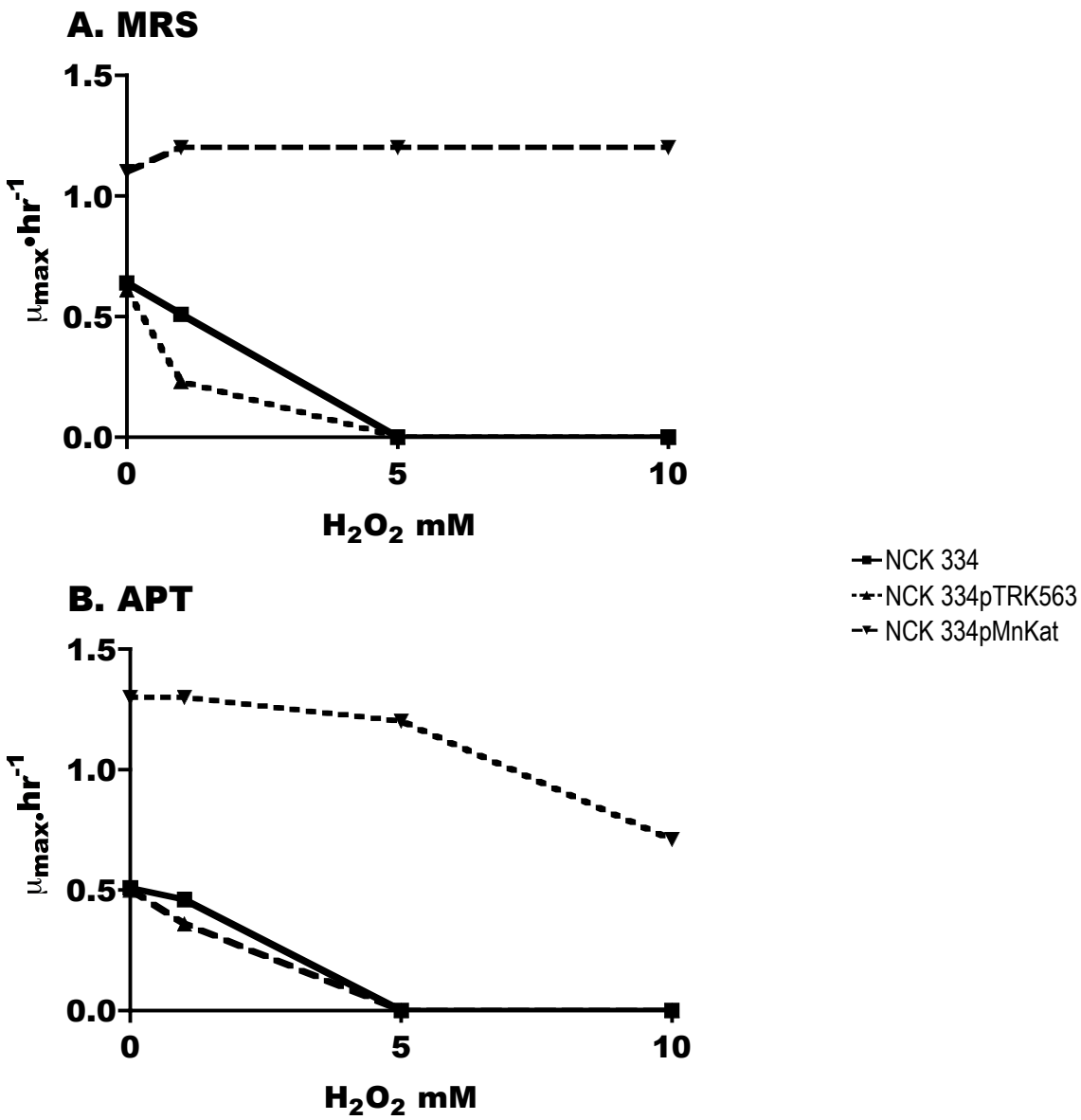

FIG. 10. Specific growth rates of MRS(A) and APT(B) grown *L. gasseri* NCK 334 strains in the presence and absence of H_2O_2.

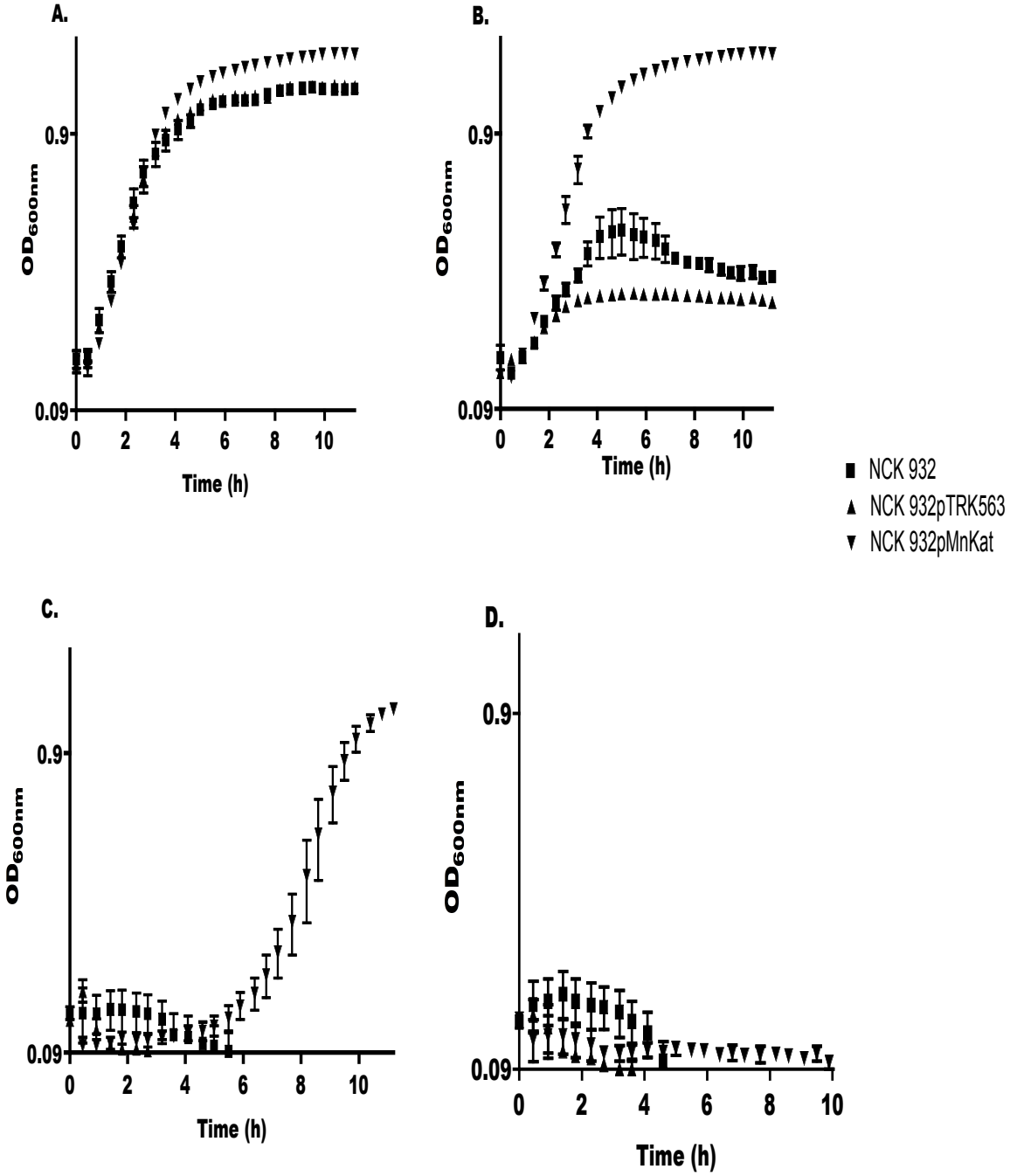

FIG. 11. Growth kinetics of MRS grown *L. reuteri* NCK 932 strains in the presence and absence of H_2O_2. (A) 0 mM H_2O_2. (B) 1 mM H_2O_2. (C) 5 mM H_2O_2. (D) 10 mM H_2O_2. Each point represents an average of biological triplicates and error bars represent SEM.

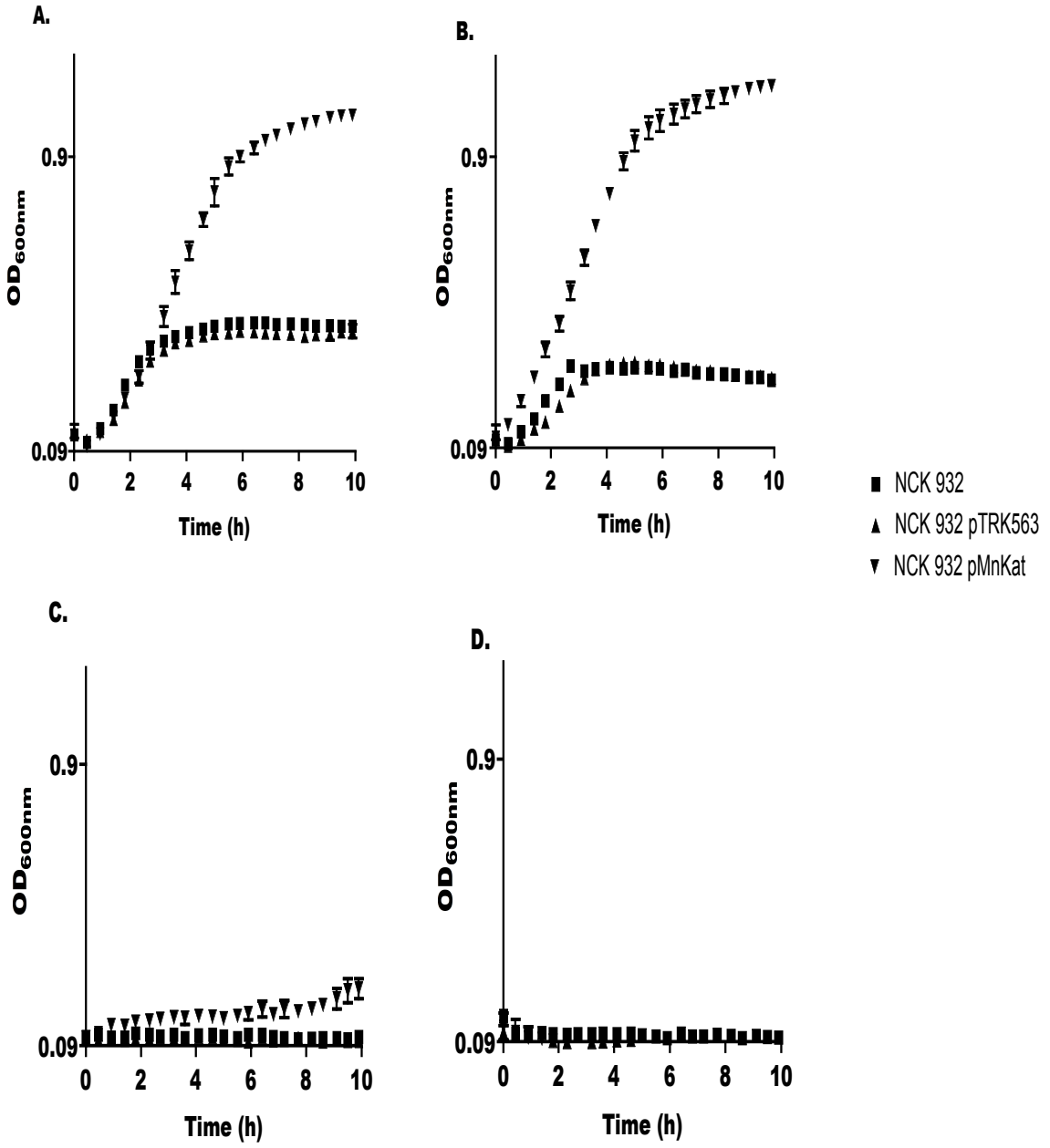

FIG. 12. Growth kinetics of APT grown *L. reuteri* NCK 932 strains in the presence and absence of H_2O_2. (A) 0 mM H_2O_2. (B) 1 mM H_2O_2. (C) 5 mM H_2O_2. (D) 10 mM H_2O_2. Each point represents an average of biological triplicates and error bars represent SEM.

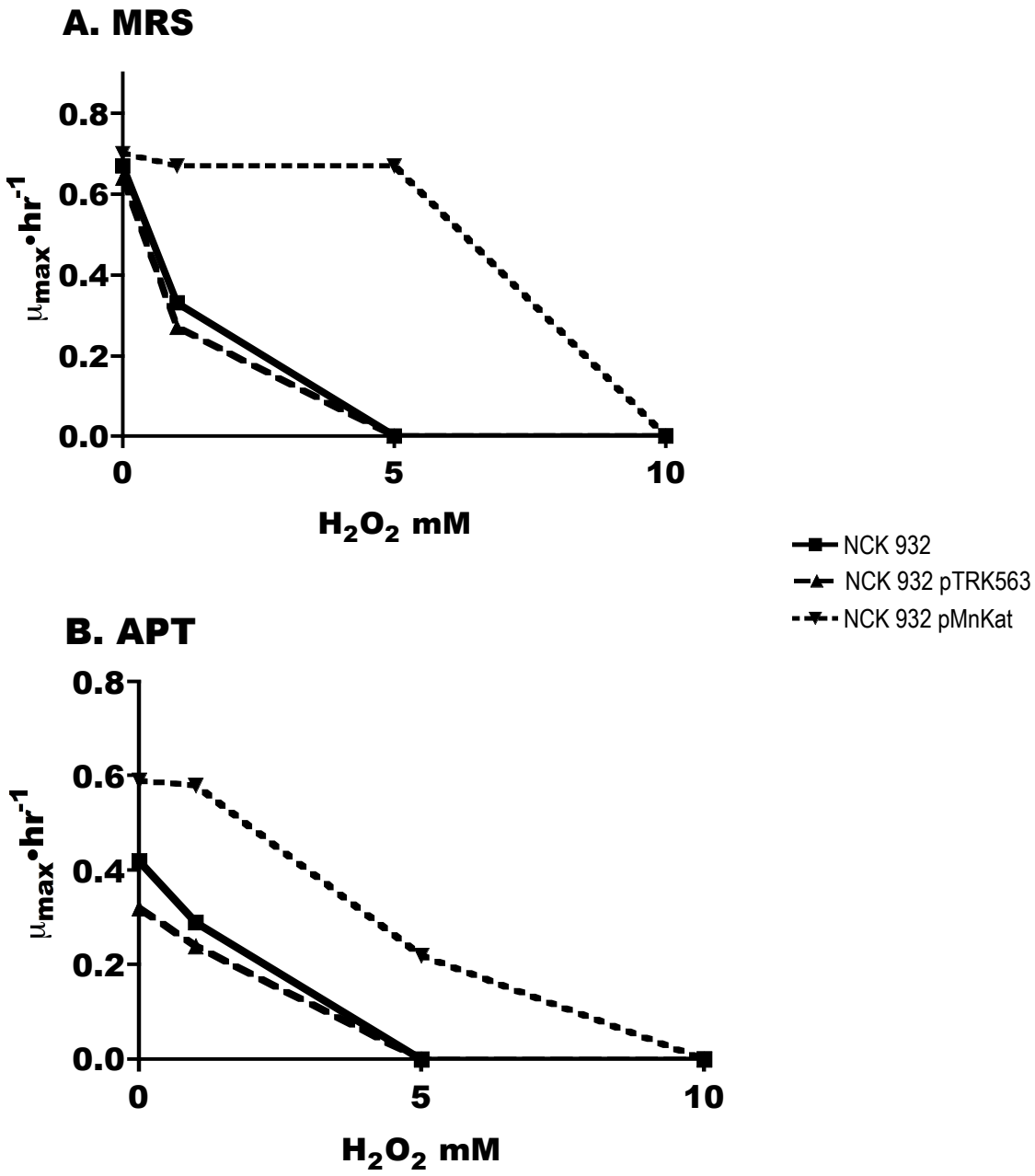

FIG. 13. Specific growth rates of MRS(A) and APT(B) grown *L. reuteri* NCK 932 strains in the presence and absence of H_2O_2.

3.4. Conclusions

LAB are a heterogeneous mixture of twenty different generate that are grouped into three different categories; obligate homofermenters, obligate heterofermenters, and facultative heterofermenters (7, 46) based upon individual organisms primary sugar fermentation pathways (7). Due to the heterogeneity of the group in both physiology and ecology, they must endure a variety of stressors (i.e. acid, osmotic, cold, salt/bile-salt, oxygen, heat, starvation) based upon the different niches that they inhabit. One of the common stressors that they encounter is oxygen toxicity, particulary H_2O_2 accumulation, and while they have varying mechanisms and susceptibility thresholds to combat it, often the accumulation is faster than the disposition (21). Therefore, in an attempt to improve upon the antioxidative capacity of lactobacilli, the Mn-catalase of *L. plantarum* ATCC 14431 was cloned and functionally expressed in two human lactobacilli isolates; *L. gasseri* NCK 334 and *L. reuteri* NCK 932. As there has been only one other report pertaining to a functionally expressed manganese containing catalase in a single lactobacilli (73), it was of interest to examine the impact that Mn-catalase could have upon other species of lactobacilli, considering the heterogeneity of the group. Expression of the plasmid based Mn-catalase increased the μ_{max}•hr^{-1} of *L. gasseri* NCK 334 by ~2-fold in both MRS and APT media, as well as afford it a significant level of protection against the presence of exogenous H_2O_2 up to 10 mM (Fig. 8-10, Table 2A and C) as compared to the wild-type and pTRK563 harboring strain controls. Alternatively, *L. reuteri* NCK 932 exhibited only a slight increase in μ_{max}•hr^{-1} in MRS media and a ~1.6-fold increase in APT media. Again, as in *L. gasseri* NCK 334, the presence of the Mn-catalase afforded an increase in protection towards exogenous H_2O_2 (Fig. 11-13 and Table 2B and D). Although the protection afforded was not to the extent that *L. gasseri* NCK 334 exhibited (Fig. 8-10, Table 2A and C) and though Mn-catalase expressing *L. reuteri* NCK 932 demonstrated higher activity in both MRS and APT media than Mn-catalase expressing *L. gasseri* NCK 334 (Fig. 5A and B), there was most likely other reasons for H_2O_2 resistance in *L. gasseri* NCK 334 (i.e. NADH peroxidase, NADH oxidase) and the levels of their expression and activity are not at the current time known, but coupled with the Mn-catalase could help explain some of the differences between the species with respect to H_2O_2 resistance at concentrations of 5-10 mM.

Also, the increases in specific growth rates within the species utilized in this study are peculiar as the same strains were utilized in the cloning of the MnSOD of *S. thermophilus* AO54, but reported μ_{max}•hr^{-1} were slightly decreased or remained unchanged in comparison to the controls (13). This increase in μ_{max}•hr^{-1} particularly in *L. gasseri* NCK 334, as well as that observed in *L. reuteri* NCK 932 will need to be investigated further. However, from what has been ascertained thus far, particularly from the cellular morphology shift, it is apparent that the truncated nature of the cells represents a condition that allows them to divide faster without apparent deleterious effect. This could represent an apparent increase in NADH turnover, allowing the cells to utilize the most out of their growth substrates, MRS and APT are considered complex media, improving the efficiency of their energy generation as implied by Condon, in is review on responses to LAB to oxygen (21).

As this study has demonstrated that Mn-catalase gene of *L. plantarum* ATCC 14431 can be functionally expressed in other lactobacilli, it brings to light the applications that the example shown herein could afford. By improving the ROS defense capacity in certain beneficial lactobacilli associated with functional foods, (foods containing or generated by health benefitting microorganisms that elicit a health benefit to the consumer), such as yogurt and mass produced commercially available probiotic formulations (55,79) it could inherently reduce the associated hazards of ROS's in the preparation of industrial starter cultures and therapeutic formulations of lactobacilli. Additionally, improved ROS defense capacity could prove useful in therapeutic applications (47) for diseases that have ROS linked etiologies (33, 48, 81).

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3.5 References

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APPENDICES

Appendix A

MRS Agar for Isolating and Propagating Lactobacilli
(de Man et al., 1960)

Component	**Mass**
Oxoid peptone	10.00 g
Meat extract	10.00 g
Yeast extract	5.00 g
K_2HPO_4	2.00 g
Diammonium citrate	2.00 g
Glucose	20.00 g
Tween 80	1.00 ml
Na acetate	5.00 g
$MgSO_4 \cdot 7H_2O$	0.58 g
$MnSO_2 \cdot 4H_2O$	0.05 g

APT Medium for Isolating and Propagating Lactobacilli (Evans and Niven, 1951)

Component	**Mass**
Tryptone	10.00 g
Yeast extract	5.00 g
K_2HPO_4	5.00 g
Na citrate	5.00 g
NaCl	5.00 g
Glucose	10.00 g
Tween 80	1.00 ml
$MgSO_4 \cdot 7H_2O$	0.80 g
$MnCl_2 \cdot 4H_2O$	0.14 g
$FeSO_4 \cdot 7H_2O$	0.04 g

A1. Lactobacilli growth medium constituents and amounts per liter of prepared media utilized during this study.