Uncovering virulence related secondary metabolites in the human pathogen *Talaromyces marneffei*

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Abstract

Iron is a vital cofactor for a multitude of cellular process in all eukaryotes and some prokaryotes. Due to this, pathogens have had to evolve sophisticated strategies to ensure that they are able to obtain the required amount of iron for survival, including the production of secondary metabolites such as siderophores. Siderophores are defined as "relatively low molecular weight, ferric iron specific chelating agents" that are produced by bacteria and fungi that are growing under low iron conditions. This project aimed to continue the research into targeting non-ribosomal peptide synthetase (NRPS) genes SidD and SidC within the pathogenic fungi *Talaromyces marneffei*, predicted to be involved in siderophore biosynthesis. By doing this, it allowed the further characterisation of the biosynthesis of these siderophores, as well as their effect on *T. marneffei* virulence. Two knockout strains were confirmed for *ΔTmSidC* and three knockout strains were confirmed from *ΔTmSidD*, both from strains previously developed in my honours thesis research. A Cytotoxicity Detection (LDH) assay was completed to test the cytotoxicity of macrophage cells at timepoints selected to use within the macrophage assay testing virulence of the knockout strains. 52.75% cytotoxicity was seen at the 24-hour timepoint, 50.09% cytotoxicity was seen at 48 hours, while 63.44% cytotoxicity was seen at the proposed 72-hour timepoint. A macrophage assay was completed, with no results collected at timepoints used. Elevated levels of cell cytotoxicity seen at all hours measured within the LDH assay suggests that the cells used for the assay were not healthy and may have contributed to the lack of results. The experimentation completed in this scholarship project, while not providing extensive results, will provide a strong base moving forward with further research into this project.

Introduction

Iron is a vital cofactor for a multitude of cellular process in almost all living organisms. Due to this, pathogens have had to evolve sophisticated strategies to ensure that they are able to obtain the required amount of iron for survival, including the production of secondary metabolites such as siderophores. Siderophores are defined as "relatively low molecular weight, ferric iron specific chelating agents" that are produced by bacteria and fungi that are growing under low iron conditions (1). In many microbial pathogens, siderophores have been shown to be important for their virulence (2).

Talaromyces marneffei (synonym *Penicillium marneffei*) is an opportunistic thermal dimorphic fungal pathogen and is the most common cause of mycosis (known as penicilliosis when caused by *T. marneffei*) in AIDS patients in Southeast Asia. Penicilliosis has been reported in HIV negative children and has a high fatality rate (3). Confirmed cases of *T. marneffei* infection were reported in Australia as well, primarily in immunocompromised individuals who had travelled through the endemic regions. As an intracellular pathogen, *T. marneffei* has a unique thermal dimorphic life cycle, which switches from a hyphal from at 25 °C to a yeast stage at 37 °C. The conidia of *T. marneffei* are phagocytosed by pulmonary alveolar macrophages and germinate directly into unicellular yeast cells in the host cells. The ability of *T. marneffei* to switch to yeast form allows the fungus to reside within host macrophages where they are shielded from the rest of the immune system (4). These yeast cells are capable of dividing and killing the macrophages and disseminate to other locations in the host, including bone marrow, liver, spleen, kidneys and lungs in immunocompromised patients (5). Macrophages are known to restrict available iron through production of high-affinity binding proteins such as transferrin and ferritin to prevent intracellular microbial proliferation (2). It was hypothesised that *T. marneffei* produces iron sequestering siderophores that enable them to survive in the iron-limiting intracellular environment. However, the biosynthesis of siderophores and its importance for the virulence of *T. marneffei* is still not well understood.

The genome of *T. marneffei* has been sequenced and it was revealed that its genome encodes two nonribosomal peptide synthetases (NRPS), TmSidC and TmSidD, which shared homology to siderophore-producing NRPSs in other fungal pathogens, such as the model human pathogen *Aspergillus fumigatus* (6). The *A. fumigatus* homologs, AfSidC and AfSidD, are known to responsible for the production of the siderophore fusarinine and ferricrocin, respectively. More detailed bioinformatics analyses, however, revealed that the two *T. marneffei* siderophore-like NRPSs have differences in their conserved domain architecture and surrounding biosynthetic genes compared to *A. fumigatus*. Thus, the actual siderophore products produced by *T. marneffei* TmSidC and TmSidD could be different from those produced by *A. fumigatus*.

The aims for this 6-week project is to further investigate the survival of *T. marneffei ΔTmSidC* and *ΔTmSidD* mutants in macrophages, to further observe the morphological changes of *T.*

marneffei over time during intracellular growth and to further investigate the effects of *ΔTmSidC* and *ΔTmSidD* on biosynthesis of siderophores and other metabolites within *T. marneffei*.

Materials and Methods.

1. Cell Lines

Macrophage J774 cells were generously supplied by Dr. Mitali Sarkar-Tyson, School of Biomedical Sciences, UWA. *T. marneffei* fungal cell line *2161ΔligD* was generously supplied by Prof. Alex Andrianopoulos, University of Melbourne.

1.1 Maintenance of fungal cell lines

T. marneffei cell line *2161ΔligD* and knockout mutant strains were maintained on ANM agar plates at 25°C.

1.2 Maintenance of macrophage cell lines

J774 murine macrophage cell line was maintained in DMEM culture media through weekly passaging of cell flasks.

2. DNA Extraction

Liquid culture samples for required DNA extractions were collected and filtered through a vacuum filter and sterile filter paper to remove excess media and liquid. Cell samples were placed into a 1.5mL microfuge tube and tubes were placed into liquid nitrogen to freeze dry samples. Freeze dried samples were then ground with a mortar and pestle until consistency was consistent with broken cell walls. 300µL of 10mM Tris + 10mM EDTA + 1% (w/v) SDS was added to the tubes. 20µL RNaseA at 100ug/mL concentration and incubated at 70 °C for 30 minutes at 400rpm. Once completed, samples were split into two 1.5mL microfuge tubes. To each tube 300µL 2.8M potassium-acetate was added and samples were allowed to cool to room temperature. 300µL chloroform-isoamyl alcohol (24:1) was added to the tubes. Tubes were centrifuged at maximum speed (~16000g) for 10 minutes. Supernatant from centrifuged tubes was transferred to new centrifuge tubes containing 0.7 equivalent amount isopropanol and inverted immediately twenty times to mix thoroughly. Samples were centrifuged at maximum speed for 10 minutes and resulting supernatant was discarded. 400µL 70% (w/v) ethanol was then

added and tubes once again centrifuged for 10 minutes at maximum speed. Ethanol was then poured out of tubes and resulting DNA pellet was allowed to air dry. Pellet was dissolved in 25µL TE buffer which was prewarmed to 50-60°C.

3. Polymerase Chain Reaction

PCR was performed using 10x Activity Buffer, 10mM dNTPs, 10mM primers following manufacturer's protocol for TAQ polymerase enzyme.

4. Agarose Gel Electrophoresis

DNA fragments were loaded onto a 0.2% (w/v) agarose gel with 0.00375% (w/v) GreenView Gel Stain (1.5µl in 40mL) purchased from IORodeo in 1X Tris-acetate-EDTA (TAE) buffer (40mM Tris acetate, 1mM EDTA, pH 8.2). They were separated beside Maestrogen AccuRuler 1 kb DNA RTU Ladder using a Bio-Rad PowerPac Basic. Fragments were run at 90V for 20 minutes. Upon completion, gels were visualised using a blue light transilluminator (IORodeo). Gel images were captured using an iPhone 7 and edited using Microsoft Suite software (cropped, converted to greyscale, contrast +40, brightness -20).

5. Macrophage assay

5.1 Growth of conidiospores

Conidiospores for all strains were grown on SD agar. Strains were plated onto SD agar plates and incubated for ~ 3 weeks until light green tinge to growth was visualized. Conidiospores were collected using sterile spool and added to 1% (w/v) tween solution. Numeration of spore solutions was completed and calculated using a hemocytometer.

5.2 Macrophage assay method

Seeded flasks of macrophages were collected from incubation. Cell media was removed from flasks and fixed cells were washed with 5mL PBS. 5mL of triple solution was added and incubated at 37°C for 15 minutes. After incubation, cells were scraped from the side of the flask, collected via pipette and added to 15mL tubes. 5mL of DMEM was added to tubes and inverted immediately to mix. Tubes were centrifuged at 1500rpm for 5 minutes at room temperature. Supernatant was removed and remaining pelleted cells were resuspended in 1mL DMEM. Cell number was counted and calculated using a hemocytometer. Cells were diluted in DMEM to 4 x

105 cells/mL. 1mL of cells were added to the number of wells required on each plate. Only centre wells were used while PBS was added to surrounding wells. Well plates with cells were incubated at 37°C in 5% CO2 overnight. Spore

preparations from required strains of fungi were collected and suspended in 10mL 1% (w/v) tween in mqH2O. A 10µL aliquot of spore preparation was taken and counted in the counting chamber to determine CFU. Spore preparation was then diluted down to required concentrations for desired multiplicities of infections. Macrophage seeded well plates incubated overnight were collected and DMEM media present was removed from wells. 1mL of diluted spore preparation was added to macrophage coated wells. Plates were incubated at 37°C for 2 hours for invasion to occur. Wells were then washed 3 times with 1mL PBS. 1mL of L-15 media was added to cells and plates were then incubated at 37°C until relevant timepoint. At each timepoint, 1mL mQH2O was added to wells and the bottom of the wells were scraped with a sterile pipette tip to break away the monolayer of cells. Cells were then pipetted up and down three times to lyse cell walls. Cells were serially diluted to 10–4, taking 100µL to 900µl PBS solution in a 24 well plate. Dilutions and neat solutions were plated out onto labelled SD media plates. Plates were incubated for up to 6 days at 37°C. Once incubated, colonies were counted and recorded.

6. Cytotoxicity Detection (LDH) Assay:

Shortly before use, reaction mixture was created through the combination of the catalyst solution and dye solution provided within the kit at ratio specified in Roche LDH protocol (http://netdocs.roche.com/DDM/Effective/00000000000010040220007 93_000_06_005_Native.pdf). Controls used included high control (complete lysis of cells) and media only absorbance value readings. Background readings of plastic were also taken. 15µl of lysis solution was added to high control cell wells and incubated for 15 minutes. After 15 minutes, 100µl of reaction mixture was added to all wells and incubated for a further 15 minutes. At 15 minutes, absorbance measurements were taken at 490 and 690 nm via an ELISA reader.

Data Analysis

High control data, sample data and media only data was averaged. Media only control measurements were taken from the high control and sample data measurements. Cytotoxicity percentage was determined by sample data/high control*100.

Results

PCR Screening of Mutants

Two colonies from *ΔTmSidC* and four colonies from *ΔTmSidD* were selected for PCR screening confirmation from previously constructed knockout strains. Colonies on selection plates displayed differing morphologies (**Figure 1**), no phenotype was particularly selected for PCR analysis. DNA extraction was performed for all selected colonies.

To determine the correct mutants, diagnostic PCR was performed using P1 or P6 primers that bind to outside of the up- and downstream homology arms paired with a primer that binds to the *bar* marker (BarDownstream-F-seq and BarUpstream-R-seq) (**Figure 2**). For screening of *ΔTmSidC* using the corresponding P1 and bar primer pair, both colonies 19 and 24 displayed correct band size after manipulation of the DNA concentration (**Figure 3**). From this result, these colonies were then used for PCR amplification with P6 primer pair, to determine correct integration of the other homology arm and *bar* gene into the genome. Both colonies displayed correct sized bands (**Figure 4**). For screening of *ΔTmSidD* colonies, amplification of both P1 and P6 primer pairs showed that colonies 12, 13 and 15 displayed correct band sizing for both P1 and P6 amplification (**Figure 5**).

Based on the results above, mutant strains for *ΔTmSidC* colonies 19 and 24, and *ΔTmSidD* colonies 12, 13 and 15 have been successfully constructed and verified.

Cytotoxicity Detection (LDH) Assay

A Cytotoxicity Detection (LDH) assay was completed to test the cytotoxicity of macrophage cells at timepoints selected to use within the macrophage assay testing virulence of the knockout strains. Differences in absorbance values were seen between high control values and samples (**Figure 6**). Sample data of macrophage cells displayed 52.75% cytotoxicity at the 24-hour timepoint, 50.09% cytotoxicity at 48 hours, while 63.44% cytotoxicity was seen at the proposed 72-hour timepoint.

Macrophage Assay

A macrophage assay was completed using timepoints 0-hour, 6-hour, 24-hour and 48 hours. No infection was seen at timepoint 0-hour. Following this lack of result, no successful growth of *T. marneffei* yeast colonies was seen at the following further timepoints.

Discussion

Many pathogenic fungal species have developed various strategies to acquire iron, which is essential for most organisms' survival as it is a requirement for many cellular processes. One of these strategies is the production of siderophores. Siderophores are a known secondary metabolite virulence factor contributing to the survival of several different pathogenic fungal and bacterial species (7-11) in both plant and animal host systems. Siderophores can be both intracellular and extracellular, and sequester iron from the host environment for use by the pathogen in cellular processes and survival.

From randomly selected fungal transformants growing on glufosinate for each gene transformation, PCR screening confirmed the successful knockout of *TmSidC* in two colonies and *TmSidD* in three colonies. Optimisation of screening protocol was required to obtain results for the PCR screening protocol. Manipulation of annealing temperature and DNA quantity used within the protocol allowed for the right conditions for the screening to be successful. The confirmation of correct integration of the knockout cassette within these mutants provides biological replicates for further characterisation. However, due to time constraints of this project, the knockouts successfully screened were unable to be further characterized.

The Cytotoxicity Detection (LDH) assay was completed to determine the cell cytotoxicity of macrophage cells at timepoints within the macrophage assay before *T. marneffei* was added. To begin, timepoints proposed for the macrophage assay were 0 hours, 6 hours, 24 hours, 48 hours and 72 hours, to extend on previous data collected up until 48 hours. The cells used for the LDH assay were the same passage of cells used within the macrophage assay run in this project. High levels of cytotoxicity were seen for all timepoints tested, as previous data collected within the Sarkar-Tyson laboratory using the same protocol would suggest that usual cytotoxicity for J774 macrophages in L-15 media range from 12-15% cytotoxicity (unpublished data). High levels of

cytotoxicity in these macrophage cells used suggest unhealthy cells to begin with, and may suggest a cause for the lack of colonies seen from the macrophage assay completed.

Further LDH assays completed with the macrophage cell line used would be useful in increasing understanding into the cell 'health' in media used. For this experimentation, cells were unable to be stored in a CO_2 incubator and were required to use L-15 CO_2-releasing media to provide suitable prerequisites for cell survival. Further experimentation with media used may provide information into suitable media to maintain cell health.

Unfortunately, no results were collected from the macrophage assay completed in this project. Time constraints did not allow for further macrophage assays to be completed within this project. The proposed 72-hour timepoint in this assay was not completed due to the high cytotoxicity seen in the LDH results of 63.44%. This high cytotoxicity would likely lack to produce any reliable data on the survival and replication of *T. marneffei* within macrophages. The LDH assay results suggested a possible cause for the lack of results pertaining from this experiment, however due to the multi-step complexity of the macrophage assay, there are several influencing factors that could cause the result obtained. The conidiospores used for this macrophage assay were grown over a period of three to five weeks, and it is unclear whether these spores were viable for use. Further testing of these spores and their viability would be useful for further macrophage assay experimentation completed.

Due to time constraints of this project, further LCMS analysis of metabolite extractions from *T. marneffei* mutants was not completed. Subsequent experimentation will proceed outside the scope of this project, to further analyse the siderophores produced by *T. marneffei*.

Experimentation completed in this project has provided a continuation to experimentation completed in the honours year project, and continues to provide an established base to further research into the role of siderophores in the pathogenic fungal response to the immune system response. The optimisation of the macrophage assay protocol through testing of cytotoxicity in macrophage cells provides the basis for further research into the survival of *T. marneffei* within macrophages, which can be extended further to include THP-1 human macrophage lines, as well as primary cell lines and further on from this, verification within an animal infection model such as mice. If further research displays a strong correlation between lack of siderophore presence and decreased pathogenicity of disease, this could provide contributing data to the development

of strategies to treat *T. marneffei* infection through the inhibition of such siderophore biosynthesis.

Figures

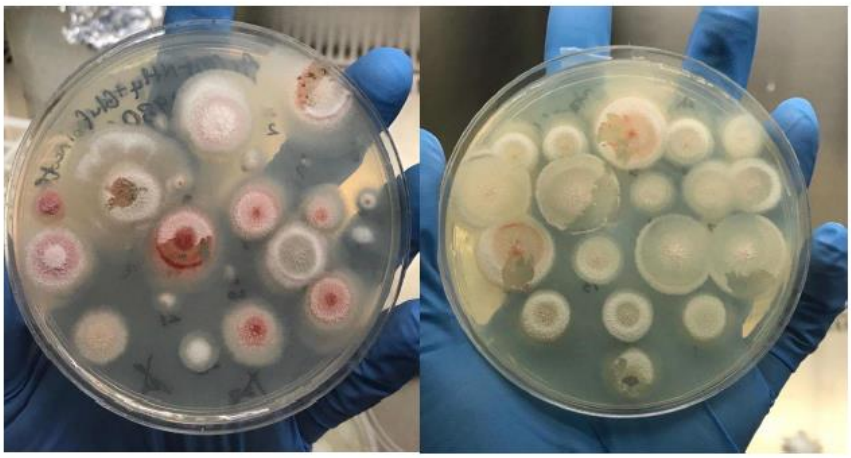

Figure 1: Glufosinate-containing ANM agar plates containing *ΔTmSidD* mutants (left) and *ΔTmSidC* mutants (right).

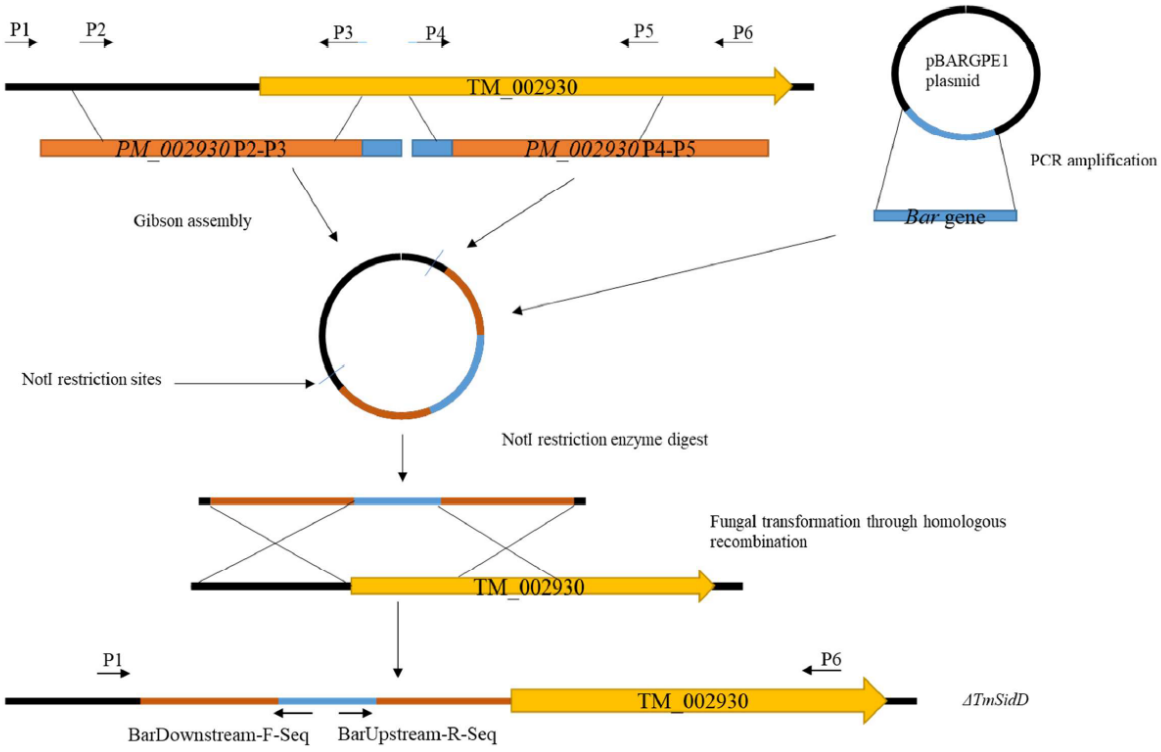

Figure 2: Molecular strategy used to construct knockout cassette used in transformation of *T. marneffei ΔTmSidD*. The same method was used for *ΔTmSidC* knockout construction. Primers used in both construction and screening displayed through use of short arrows along gene structure.

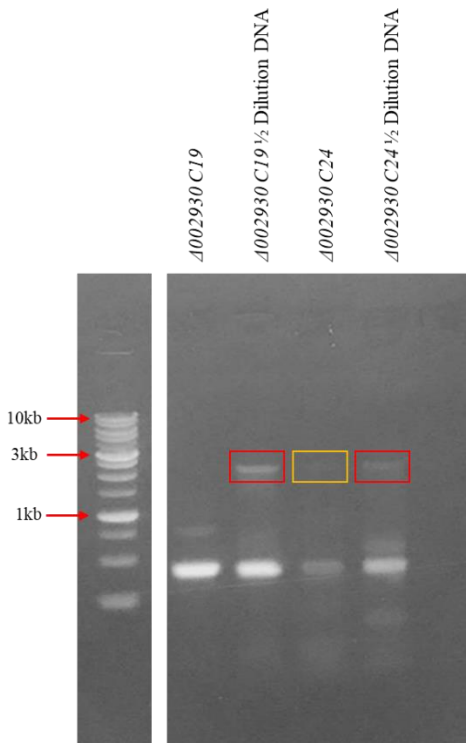

Figure 3: Agarose gel electrophoresis image of PCR amplification for confirmation of cassette presence using P1 primers in *TmSidC* colonies. Expected band size of 2.1kb was achieved for colonies highlighted in red boxes. Faint bands present in the orange boxes confer a possible correct integration.

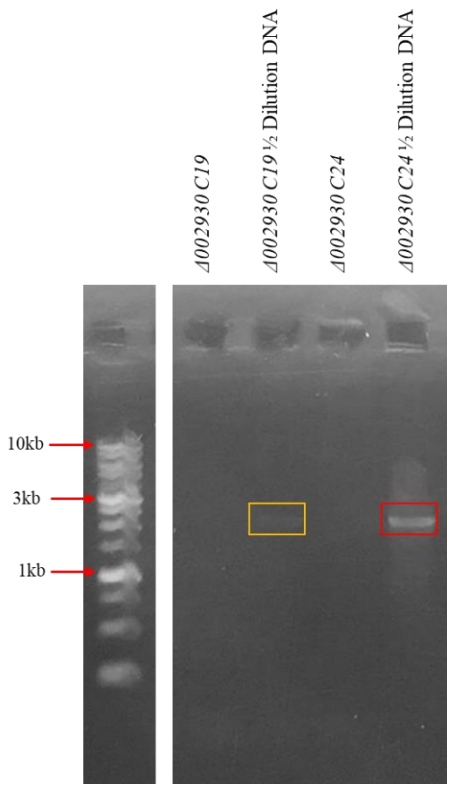

Figure 4: Agarose gel electrophoresis image of PCR amplification for confirmation of cassette presence using P6 primers in *TmSidC* colonies. Expected band size of 2.1kb was achieved for colonies highlighted in red boxes. Faint bands present in the orange boxes confer a possible correct integration.

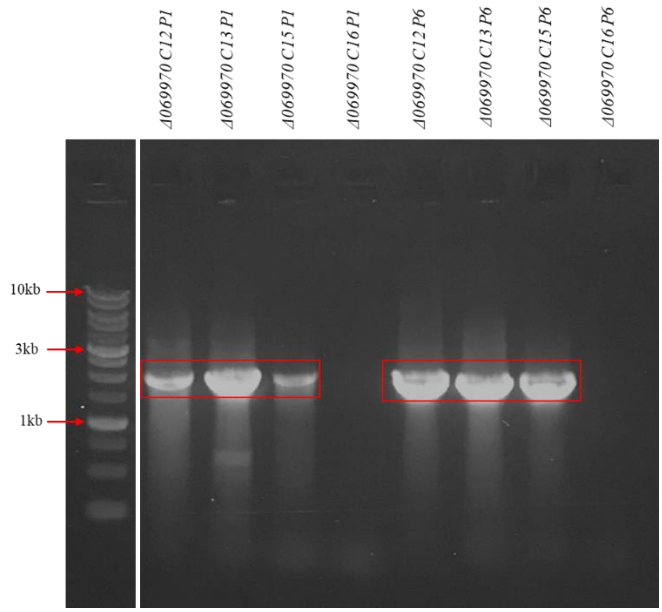

Figure 5: Agarose gel electrophoresis image of PCR amplification for confirmation of cassette presence using P1 and P6 primers in *TmSidD* colonies. Expected band sizes are achieved for colonies highlighted in red boxes (2.145kb for P1 and 2.08kb for P6 fragments).

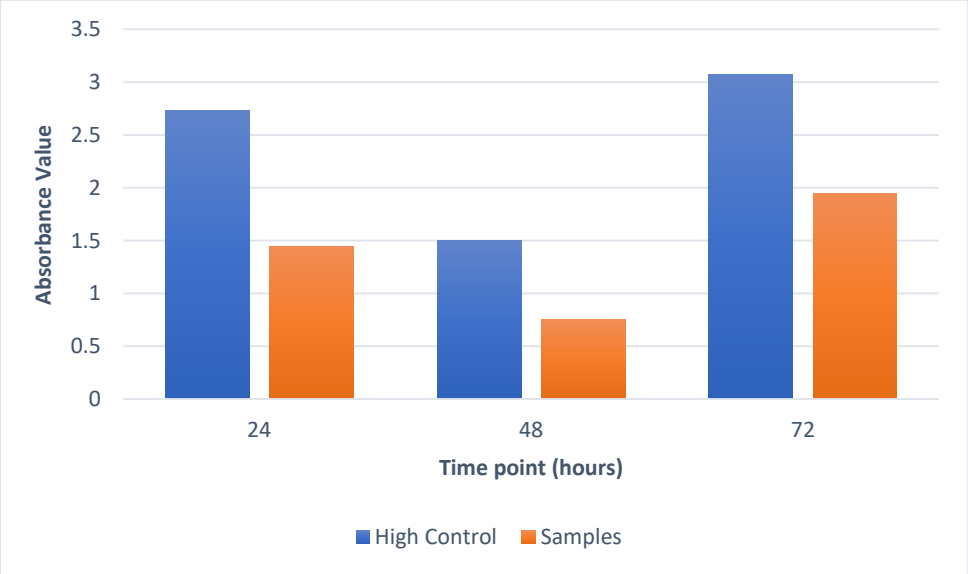

Figure 6: Graph displaying absorbance values of LDH assay at specific timepoints relating to those used within the macrophage assay.

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