**Identification of airborne bacteria from an indoor environment and initial characterisation of a multi-drug resistant *Kytococcus sedentarius* isolate**

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# **Abstract**

This study was conducted to identify the diversity of airborne bacteria in the Sheffield university lecture theatre (MBB-F2) through passive monitoring (PM) and active monitoring (AM). A total of 6 and 7 bacteria were isolated from settle plates as a PM and dehumidifier as AM respectively. Samples were taken over an hour a week on each Friday after 17.00 for eight weeks, and each sampler isolated different bacteria.

Bacteria isolated from dehumidifier were screened against a selection of antibiotics according to British Society for Antimicrobial Chemotherapy (BCAS). Disc diffusion and MIC methods indicated that *Kytococcus sedentarius* (designed MBB13) is resistant to gentamicin, ciprofloxacin and erythromycin; and the latter is normally a successful treatment for its infections. *Kytococcus sedentarius* is found on the skin, mucosae and oropharynx and also an opportunistic pathogen causing pitted keratolysis (PK), valve endocarditis and hemorrhagic pneumonia. Thus, features of this organism, together with the complete genome sequence, annotation and *in vitro* studies were used to describe this organism.

Data confirmed that *K. sedentarius* MBB13 is a strict aerobe; unable to cause PK due to its inability to degrade keratin; and under salt stress, it is able to produce glycine betaine as a main compatible solute and other solutes such as glutamate, proline, acetate, lactate and ectoine.

*Kytococcus sedentarius* MBB13 is an actinomyces and its genome revealed four WhiB proteins (1, 2, 3 and 7) that are restricted to actinomyces and four sigma factors (A, B, H and J). In *Mycobacterium tuberculosis*, WhiB proteins act as nitric oxide-responsive transcription regulators at least in part through interaction with the major sigma factor A ‘ σA’. The fundamental role of WhiB proteins in actinomyces’ developmental processes proposed that they could play a role in entry into and emergence from the non-replicative persistent state. The *M. tuberculosis* WhiB1 and WhiB2 are essential and encode DNA-binding proteins with a nitric oxide (NO) sensitive [4Fe-4S] cluster. NO is an important component of the host response to *M. tuberculosis* and a high dose of NO generated by macrophages can kill the bacilli but a low dose can promote transition to the dormant non-replicating state. Here, *K. sedentarius* MBB13 WhiB1 and WhiB2 are NO-sensitive; WhiB1 is O2-insensitive but not WhiB2; and the cluster of WhiB1 is essential for the protein folding. A quantitative real time PCR analysis following exposure to a variety of growth and antibiotics indicates differential in upregulation of *whiB* genes. *In vivo* protein-protein interaction indicates that all four WhiB proteins of *K. sedentarius* MBB13 interact with the *C*-terminal domain of σA but not with the other sigma factors.

The sensitivity of WhiB1 and WhiB2 to NO, differentiation of *whiB* genes expression due to variety of growth and antibiotics, ability of WhiB proteins to interact with SigA and the structural characterization of folding WhiB1 suggest that those proteins may have a role in regulating the pathogenicity of *K. sedentarius* MBB13.

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# **List of Abbreviations**

AmpR – Ampicillin resistance

ARGs – Antibiotic resistance genes

bp – base pairs

cAMP – cyclic adenosine monophosphate

CD spectroscopy – Circular dichroism spectroscopy

DNIC – Dinitrosyl-iron-cysteine complex

DTT – Dithiothreitol

Fe-S – Iron-sulphur cluster

HGT – Horizontal gene transfer

Hygro-BR – Hygromycin B resistance

IPTG – Isopropyl-β-D-thiogalactopyranoside

KanR – Kanamycin resistance

kDa – Kilo Dalton

MBB – Microbiology and molecular biotechnology

NO – Nitric oxide

OD – Optical density

PAGE – polyacrylamide gel electrophoresis

PCR – Polymerace chain reaction

PK – Pitted keratolysis

qRT-PCR – Quantitative polymerace chain reaction

RNAP – RNA polymerace

rpm – Revolutions per minute

SDS – Sodium dodecyl sulphate

StrR – Streptomycin resistance

TEMED – N, N, N’ ,N’- Tetramethylethylenediamine

Tris – Tris (hydroxymethyl) methylamine

UV – Ultra violet visible spectroscopy

VBNC – Viable but nonculturable

Wbl – WhiB like

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# ***Chapter one***

## 1 Introduction

### **1.1 Pollution of air**

Air pollution is a process that introduces contamination to the atmosphere, in both indoor and outdoor environments, which can be harmful to human health. Air pollution claims more than two million lives per year worldwide (Kim *et al.*, 2015). Bacteria are important components of polluted air and can be sources of airborne diseases (Abiola *et al.*, 2018). A comprehensive assay undertaken in China throughout 2015, suggested that, although, the abundance of airborne bacteria varies and changes depending on location, for example urban areas, agricultural fields, forests, and mountains, there can be between 104 to 108 cells per m3 (Zhen *et al.*, 2017).

### **1.2 Lethal diseases can be transmitted by air**

Various effects injurious to human health, including lethal diseases, were reported to be transmitted via polluted air and tend to be linked with exposure to airborne microorganisms (e.g. viruses, bacteria and fungi) such as, respiratory inflammation, acute toxic effects, allergies and cancers (Kim *et al.*, 2018). For many years, health centres were major areas for spreading antibiotic resistance through airborne pathogens (Byarugaba, 2004). However, recently many studies suggested that, indoor environments can contain antibiotic resistant bacteria (Roberts *et al.*, 2011).

### **1.3 Airborne transmission**

Airborne bacteria transmit through human respiratory fluid emitted via coughing, sneezing and talking. In industrialized countries, people spend ~85% of their time indoors which allow airborne bacteria to transmit between individuals (Hospodsky *et al.*, 2012). Microbial communities can easily shift between environments. Therefore, occupational and residential environments have been under attention to study the link between those environments with airborne communities (Kelley and Gilbert, 2013).

### **1.4 Factors that affect the spread of airborne pathogens in indoor environment**

People activities within the indoor environment are thought to be the main source for the spread and diversity of airborne microbial contamination through the following factors, coughing, walking, talking, sneezing and washing (Hayleeyesus and Manaye, 2014). Besides, the indoor environment also influences the microorganism’s growth and multiplication through several factors, including air exchange rate, air movement, temperature, humidity, building structures and location, design and ventilation systems (Meadow *et al.*, 2014). A number of epidemiological studies were reviewed by the World Health Organisation (WHO) and suggested that indoor dampness-related factors are linked to several respiratory health issues that affect humans, such as asthma, upper respiratory tract symptoms, respiratory infections, coughs, wheeziness and dyspnoea (Heseltine and Rosen, 2009).

### **1.5 Culturable airborne bacteria**

Airborne bacteria are classified into two groups; culturable and viable but nonculturable (VBNC) (Tong, 1999). It has been claimed that only 0.1% of the total microbial population of environment can be successfully cultured, which is due to the bacterial growth requirements (Hill *et al.*, 2000). Several methods have been reported to isolate airborne population such as, passive and active monitoring for culturable bacteria (Napoli *et al.*, 2012). Many effective markers have indicated that, VBNC cells are alive though confirmation of protection of genomic DNA, uptake of labeled amino acids and cellular membrane integrity (Ramamurthy *et al.*, 2014). Therefore, several methods were proposed to detect VBNC communities through their metabolic activities, which can detect the accumulation of rhodomine (a chemical compound existing within the living cells) (Darzynkiewicz *et al.*, 1982; Ramamurthy *et al.*, 2014), or Quantitative PCR (qPCR) by the detection of global expression genes (Ramamurthy *et al.*, 2014). In this current study, culturable airborne bacteria are targeted for indoor environment assessment (student lecture theatre).

### **1.6 Assessment of airborne taxa in different places**

Exposure to airborne bacteria is a key element in the spread of infectious diseases (Hospodsky *et al.*, 2012). In the last decade, indoor airborne bacteria have been studied widely employing different sampling techniques (Gόrny and Dutkiewicz, 2002). Although, humans are the main source of microbial diversity, ventilation also affects the microbial diversity of indoor rooms (Kelley and Gilbert, 2013). For instance, watery environments such as, hospital therapy pools and showerheads are rich in *Mycobacteria*, as well as many Proteobacteria and rarer groups (Kelley and Gilbert, 2013), while shower curtains and drinking water contain different *Sphingomonas* and *Methylobacterium* species (Vaz-Moreira *et al.*, 2011; Kelley and Gilbert, 2013). A comprehensive study applied in two Neonatal Intensive Care Units suggested that those units are particularly susceptible to opportunistic pathogens and found the following species, *Neisseria*, *Pseudomonas*, *Enterobacter*, *Streptococcus* and *Staphylococcus* (Hewitt *et al.*, 2013). Another study investigated the diversity of bacteria on surfaces in offices in three metropolitan cites (New York, San Francisco and Tucson, USA) and claimed that offices inhabited by males were more contaminated than those inhabited by females, and those offices were rich in genera found on human skin such as *Streptococcus*, *Corynebacterium*, *Flavimonas*, *Lactobacillus* and *Burkholderiales*, oral bacteria such as, *Prevotella*, *Pseudomonas, Neisseria*, *Actinomyces* and TM7 bacteria, human digestive tract bacteria such as, *Bacteroidetes*, *Lactobacillus*, *Enterobacteriaceae* and several pathogens such as *Neisseria*, *Shigella*, *Streptococcus* and *Staphylococcus*, and soil genera such as, *Bacillus* and rhizosphere as, *Bradyrhizobium* (Hewitt *et al.*, 2012). Kelley and Gilbert (2013) reported a number of common bacteria collected from various hospital air samples as follows, *Staphylococcus epidermidis*, *S. haemolyticus*, *Kytococcus sedentarius*, *Ralstonia pickettii,* *Enterobacter spp*., *Kocuria rhizophila*, *Methylobacterium extorquens*, *Micrococcus luteus*, *Microcystis aeruginosa*, *Prochlorococcus marinus* and *Methylocella silvestris*.

### **1.7 Antibiotic resistance of airborne bacteria**

Although, exposure to the contaminated air of work environments can lead to various infections and allergic reactions, those environments may also accelerate the spread of antibiotic resistance genes (ARGs). Detection of resistance to antibiotics in non-pathogen bacteria could give novel genetic determinants of resistance (Brągoszewska and Biedroń, 2018). Genes encoding resistance to drugs can be acquired by pathogenic bacteria from other bacteria that a live within the surrounding environment and that can be through genetic recombination mechanisms (Messi *et al.*, 2015).

For example, hospital air contains multi-drug resistant bacteria (MDR) such as, vancomycin-resistant enterococci (VRE) and *Staphylococcus aureus* (MRSA) and Enterobacteriaceae, which become a problem in hospitals. Surprisingly these MDR bacteria could also be isolated from indoor and outdoor environments (Messi *et al.*, 2015). Therefore, it was beneficial to detect antibiotic resistance genes (ARGs) directly instead of isolating the bacteria that contaminated the air and test their antibiotic resistance. Identification of ARGs can be achieved using specific PCR primers (Messi *et al.*, 2015). For example, to screen airborne bacteria resistance to macrolide, lincosamide and streptogramin, those genes that are essential to be detected are *erm*(A), *erm*(B), *erm*(C), *erm*(F) and *mef*(A), and for tetracycline, *tet*(M), *tet*(O), *tet*(S), *tet*(K) and *tet*(L) (Sapkota *et al.*, 2006; Messi *et al.*, 2015).

### **1.8 Sampling of the culturable airborne communities**

#### **1.8.1 Passive monitoring**

Passive monitoring uses settle plates or ‘petri dishes’ containing culture media that is exposed for a period time and then incubated (Napoli *et al.*, 2012). This approach is only capable of monitoring biological particles that sediment out of the air and settle over the exposure period and, as a result, this method does not detect smaller particles or droplets remaining suspended in the air (Eaton *et al.*, 2018). Passive monitoring is also unable to sample specific volumes of air, so the results, at best, can only be considered semi-quantitative (Harner *et al.*, 2006). Settle plates may become overgrown in heavily contaminated conditions (Wang *et al.*, 2008). However, settle plates are easy to use and inexpensive and require no specialised equipment (Harner *et al.*, 2006).

#### **1.8.2 Active monitoring**

Active monitoring requires the use of a microbiological air sampler to physically draw a pre-determined volume of air through, or over, a particle collection device (Napoli *et al.*, 2012). Two main types are in general use:

#### **1.8.2.1 Impingers**

Impingers use a liquid medium to collect particles from air drawn by a suction pump through a thin or narrow inlet tube into a small flask containing the collection medium; the air goes toward the collection medium surface, the flow rate being determined by the diameter of the inlet tube. Once the air contacts the surface of the liquid, it changes direction abruptly and any suspended particles impinge into the collection liquid. When the sampling is completed, the collection liquid can be cultured to determine the number of viable microbes in the sample. Since the sample volume can be calculated using the flow rate and sampling period of time, the result is considered quantitative (Willeke *et al.*, 1998; Lin *et al.*, 2000; Pan *et al.*, 2019).

#### **1.8.2.2 Impactors**

Impactor samplers use a solid or adhesive medium, for example, agar gel, rather than a liquid to collect particles (Sialve *et al.*, 2015). Air is drawn into a sampling head by a pump or fan and accelerated, generally through a perforated plate (sieve samplers), or through a narrow slit (slit samplers) (Montagna *et al.*, 2017). This produces a laminar air flow onto the collection surface, usually a normal agar plate filled with a suitable medium. Air speed is measured by the diameter of the holes in sieve samplers and the width of the slit in slit samplers. When the air hits the collection surface it changes its direction and then any suspended particles are thrown out by inertia, impacting onto the collection surface. When the desired volume of air has passed through the sampling head, the agar plate is removed and directly incubated without more treatment. Following incubation, the number of viable colonies gives a direct quantitative estimate of the colony forming units in the volume of air sampled. Impaction samplers are convenient and can handle the higher rates of flow and large sample volumes necessary to monitor the quality of air in controlled environments where microbial numbers are likely to be low; microbial cells may however, be damaged induced by the sampling process and become less viable (Li, 1999; Sialve *et al.*, 2015).

The most well-known impact sampler is the Andersen sampler, a multi-stage ‘cascade’ sieve sampler that uses perforated plates with progressively smaller holes at each stage, to allow particles to be separated based on size. Another is the Casella slit sampler, in which the slit is located above a turntable on which is placed an agar plate. Then, air is drawn through the slit and the agar plate rotates, then particles are deposited evenly over its surface (Crook, 1996).

### **1.9 Aim of the study**

As explained previously in this introduction, indoor environment contain countless bacteria that can influence health negatively. Isolating airborne bacteria can produce different bacterial species. The aim of this study is: isolating bacteria from the air of student class using two methods, passive (settle plates) and active monitoring (dehumidifier). Identification of those species was determined by amplifying genes coding small subunit ribosomal RNA (16S rRNA) amplicon (Kelley and Gilbert, 2013). The abundance of bacteria isolated from active monitoring bias this study to focus on those isolated from dehumidifier.

A multi-resistance bacterium *Kytococcus sedentarius* (strain designated MBB13) was nominated for further investigation. Genome of *K. sedentarius* MBB13 was sequenced and compared to the reference genome DSM 20547. Some of the main features of the *K. sedentarius* isolate were identified genetically and tested *in vivo* as follows: oxygen relationship, salt tolerance and protein-protein interaction of WhiB-like with the major sigma A (σ A). Finally, the transcriptional factors WhiB1 and WhiB2 were isolated and characterised.

### **1.10 Motivation behind this study**

This study reviewed microbial diversity in a student classroom located between laboratories dealing with genetically modified organisms (GMO) using passive and active monitoring culture-independent isolation methods. Why is this classroom even worth exploring?

In general, built environment is primarily a habitat for a large percentage of human beings whose health may depend on the diversity of those indoor tolerant microbes. Those built environments contain chemical compounds and physical conditions unlike the natural world where microbes evolved (Kelley and Gilbert, 2013). Built environments may have unexpected consequences for the selection of microbes which find this built environment suitable to live in and more information may help to design healthier buildings. Therefore, this study may give a deeper understanding of indoor microbial diversity that may help to inform public health policy especially for immune-compromised individuals.

Using the next generation sequencing technology offers exciting opportunities to discover important information about the selected bacterium (*K. sedentarius* MBB13) such as, it is favoured habitant, metabolism and stress responses.

To date, this is the first work that covered the microbial diversity of the University of Sheffield F2 class room and showed a molecular background about *K. sedentarius* in relation to WhiB-like proteins.

# ***Chapter Two***

## 2 Materials and methods

### **2.1 Bacterial strains and plasmids**

The bacterial strains and plasmids used in this study are listed in Tables 2.1-2.3.

Table 2.1: Bacterial strains available at the beginning of this study

|  |  |  |
| --- | --- | --- |
| **Storage no** | **Description**  **(Strain/plasmid/resistance)** | **Source** |
| JRG3196 | *Escherichia coli* DH5α/pDB55 | Invitrogen |
| JRG6787 | *E. coli* DH5α/pMyNT, (overexpression plasmid), Hygromycin B resistant (Hygro-BR) | Addgene |
| Gly6289  JRG6952 | *E. coli* DH5α / pKT25,  A two hybrid BATCH system vector, which encodes the T25 fragment of *Bordetella pertussis* adenylate cyclase upstream of the multiple cloning site (MCS), Kanamycin resistant (KnR) | Lab collection |
| Gly6290  JRG6953 | *E. coli* DH5α / pKT25-zip,  A two hybrid BATCH system control vector in which the leucine zipper of GCN4 is fused in frame to T25 (KnR) | Lab collection |
| Gly6291  JRG6954 | *E. coli* DH5α / pUT18,  A two hybrid BATCH system vector, which encodes the T18 fragment of *Bordetella pertussis* adenylate cyclase downstream of the MCS, Ampicillin resistant (AmpR) | Lab collection |
| Gly6292  JRG6955 | *E. coli* DH5α / pUT18-zip,  A two hybrid BATCH system control vector, in which the leucine zipper of GCN4 is fused in frame to T18 (AmpR) | Lab collection |
| Gly6293  JRG6956 | *E. coli* BTH101,  Host strain for the two hybrid BATCH system: F-, *cya-99*, *araD139*, *gal15*, *galK16*, *rpsL1*, *hsdR2*, *mcrA1* and *mcrB1,* Streptomycin resistant (StrR) | Lab collection |
| JRG6161 | *Mycobacterium smegmatis* MC2155 | Lab collection |
| JRG6068 | *Pseudomonas putida* KT2440 | Lab collection |

Table 2.2: Bacterial strains isolated during this study

|  |  |  |
| --- | --- | --- |
| **Storage no** | **Description**  **(Strain/plasmid/resistance)** | **Source** |
| MBB1 | *Staphylococcus hominis* | In this study |
| MBB2 | *Staphylococcus saprophyticus* | In this study |
| MBB3 | *Staphylococcus haemolyticus* | In this study |
| MBB4 | *Staphylococcus succinus* | In this study |
| MBB5 | *Pantoea ananatis* | In this study |
| MBB6 | *Micrococcus yunnanensis* | In this study |
| MBB7 | *Kocuria rhizophila* | In this study |
| MBB8 | *Pseudomonas fluorescens* | In this study |
| MBB9 | *Sphingomonas ginsenosidimutans* | In this study |
| MBB10 | *Sphingomonas yunnanensis* | In this study |
| MBB11 | *Novosphingobium barchaimii* | In this study |
| MBB12 | *Staphylococcus pasteuri* | In this study |
| MBB13 | *Kytococcus sedentarius* | In this study |

Table 2.3: Bacterial strains constructed in this study

|  |  |  |
| --- | --- | --- |
| **Storage no** | **Description**  **(Strain/Vector/resistance)** | **Source** |
| Gly6286  JRG6949 | *E. coli* (DH5α) / pMyNT-*whiB1* (Hygro-BR) | This study |
| Gly6287  JRG6950 | *E. coli* (DH5α) / pMyNT-*whiB2* (Hygro-BR) | This study |
| Gly6288  JRG6951 | *E. coli* (DH5α) / pMyNT-*whiB7* (Hygro-BR) | This study |
| Gly6308  JRG6971 | *M. smegmatis* / pMyNT-*whiB1* (Hygro-BR) | This study |
| Gly6309  JRG6972 | *M. smegmatis* / pMyNT-*whiB2* (Hygro-BR) | This study |
| Gly6910  JRG6973 | *M. smegmatis* / pMyNT-*whiB7* (Hygro-BR) | This study |
| Gly6271  pGS2598 | *E. coli* (DH5α) / pKT25-*whiB1* (KanR) | This study |
| Gly6272  pGS2599 | *E. coli* (DH5α) / pKT25-*whiB2* (KanR) | This study |
| Gly6294  JRG6957 | *E. coli* (DH5α) / pKT25-*whiB3* (KanR) | This study |
| Gly6273  pGS2600 | *E. coli* (DH5α) / pKT25-*whiB7* (KanR) | This study |
| Gly6312  JRG6975 | *E. coli* (DH5α) / pKT25-*whiA* (KanR) | This study |
| Gly6295  JRG6958 | *E. coli* (DH5α) / pUT18-*sigmaA* C-terminal domain (CTD) (AmpR) | This study |
| Gly6296  JRG6959 | *E. coli* (DH5α) / pUT18-*sigmaB* (AmpR) | This study |
| Gly6267  pGS2601 | *E. coli* (BTH101) / pUT18-*sigmaH* (AmpR and StrR) | This study |
| Gly6328  JRG6991 | *E. coli* / pUT18-*sigmaJ* (AmpR) | This study |
| Gly6313  JRG6976 | *E. coli* / pUT18-*whiA* (AmpR) | This study |
| Gly6297  JRG6960 | *E. coli* (BTH101) /  pKT25-*whiB1* and pUT18-*sigmaA* CTD (AmpR, StrR and KanR) | This study |
| Gly6298  JRG6961 | *E. coli* (BTH101) /  pKT25-*whiB1* and pUT18-*sigmaB* (AmpR, StrR and KanR) | This study |
| Gly6235 | *E. coli* (BTH101) /  pKT25-*whiB1* and pUT18-*sigmaH* (AmpR, StrR and KanR) | This study |
| Gly6329  JRG6992 | *E. coli* (BTH101) /  pKT25-*whiB1* and pUT18-*sigmaJ* (AmpR, StrR and KanR) | This study |
| Gly6314  JRG6977 | *E. coli* (BTH101) /  pKT25-*whiB1* and pUT18-*whiA*, AmpR, StrR and KanR | This study |
| Gly6299  JRG6962 | *E. coli* (BTH101) /  pKT25-*whiB2* and pUT18-*sigmaA* CTD (AmpR, StrR and KanR) | This study |
| Gly6300  JRG6963 | *E. coli* (BTH101) /  pKT25-*whiB2* and pUT18-*sigmaB* (AmpR, StrR and KanR) | This study |
| Gly6236 | *E. coli* (BTH101) /  pKT25-*whiB2* and pUT18-*sigmaH* (AmpR, StrR and KanR) | This study |
| Gly6330  JRG6993 | *E. coli* (BTH101) /  pKT25-*whiB2* and pUT18-*sigmaJ* (AmpR, StrR and KanR) | This study |
| Gly6315  JRG6978 | *E. coli* (BTH101) /  pKT25-*whiB2* and pUT18-*whiA*, AmpR, StrR and KanR | This study |
| Gly6301  JRG6964 | *E. coli* (BTH101) /  pKT25-*whiB3* and pUT18-*sigmaA* CTD (AmpR, StrR and KanR) | This study |
| Gly6302  JRG6965 | *E. coli* (BTH101) /  pKT25-*whiB3* and pUT18-*sigmaB* (AmpR, StrR and KanR) | This study |
| Gly6303  JRG6966 | *E. coli* (BTH101) /  pKT25-*whiB3* and pUT18-*sigmaH* (AmpR, StrR and KanR) | This study |
| Gly6331  JRG6994 | *E. coli* (BTH101) /  pKT25-*whiB3* and pUT18-*sigmaJ* (AmpR, StrR and KanR) | This study |
| Gly6316  JRG6979 | *E. coli* (BTH101) /  pKT25-*whiB3* and pUT18-*whiA* (AmpR, StrR and KanR) | This study |
| Gly6304  JRG6967 | *E. coli* (BTH101) /  pKT25-*whiB7* and pUT18-*sigmaA* CTD (AmpR, StrR and KanR) | This study |
| Gly6305  JRG6968 | *E. coli* (BTH101) /  pKT25-*whiB7* and pUT18-*sigmaB* (AmpR, StrR and KanR) | This study |
| Gly6237 | *E. coli* (BTH101) /  pKT25-*whiB7* and pUT18-*sigmaH* (AmpR, StrR and KanR) | This study |
| Gly6332  JRG6995 | *E. coli* (BTH101) /  pKT25-*whiB7* and pUT18-*sigmaJ* (AmpR, StrR and KanR) | This study |
| Gly6317  JRG6980 | *E. coli* (BTH101) /  pKT25-*whiB7* and pUT18-*whiA* (AmpR, StrR and KanR) | This study |
| Gly6318  JRG9681 | *E. coli* (BTH101) /  pKT25-*whiA* and pUT18-*sigmaA* CTD (AmpR, StrR and KanR) | This study |
| Gly6319  JRG6982 | *E. coli* (BTH101) /  pKT25-*whiA* and pUT18-*sigmaB* (AmpR, StrR and KanR) | This study |
| Gly6320  JRG6983 | *E. coli* (BTH101) /  pKT25-*whiA* and pUT18-*sigmaH* (AmpR, StrR and KanR) | This study |
| Gly6333  JRG6996 | *E. coli* (BTH101) /  pKT25-*whiA* and pUT18-*sigmaJ* (AmpR, StrR and KanR) | This study |
| Gly6306  JRG6969 | *E. coli* (BTH101) /  pKT25-zipand pUT18-zip (AmpR, StrR and KanR) | This study |
| Gly6307  JRG6970 | *E. coli* (BTH101) /  pKT25and pUT18 (AmpR, StrR and KanR) | This study |

### **2.2 Primers used in this study**

The oligonucleotide primers used in this study are listed in Table 2.4.

Table 2.4: List of primers

|  |  |  |  |
| --- | --- | --- | --- |
| Target or purpose | Primer | Sequence (from 5’ to 3’) | Reference |
| Universal primers for the bacterial 16S rRNA gene | *27*-F | AGAGTTTGATCMTGGCTCAG | (Budi *et al.*, 1999) |
| *1492*-R | CGGTTACCTTGTTACGACTT |
| Amplification of WhiB1 coding region | *whiB1*-F | ATATATCCATGGACTGGCGAAGCAAAGCGG | This study |
| *whiB1*-R | ATATATAAGCTTTCAGCTGGCGCGGCGGGC |
| Amplification of WhiB2 coding region | *whiB2*-F | ATATATCCATGGACGAACTTCAGATCGTCG | This study |
| *whiB2*-R | ATATATAAGCTTTCAGGCGGTGAAGACCGC |
| Amplification of WhiB7 region | *whiB7*-F | ATATATCCATGGTGGCCCTGGCAGATCACC | This study |
| *whiB7*-R | ATATATAAGCTTTCAGGCCGCGATGGGGTG |
| qRT-PCR-WhiB1 | *whiB1RNA*-F | GTCTCGACGAGGACCCGGAA | This study |
| *whiB1RNA*-R | GGTCTCGATCGCCCACTGC |
| qRT-PCR-WhiB2 | *whiB2RNA*-F | TCAGATCGTCGGACATCCCG | This study |
| *whiB2RNA*-R | TAGTCGAGGCACTCGGAACG |
| qRT-PCR-WhiB3 | *whiB3RNA*-F | GACCCCGAGGAGTTCTTCCAC | This study |
| *whiB3RNA*-R | TCGCGGCACTCGAGGATGAC |
| qRT-PCR-WhiB7 | *whiB7RNA*-F | CTCATCGACCAAGCCCGTGT | This study |
| *whiB7RNA*-R | ACTTGGCGTACTCGACACCC |
| qRT-PCR-gyrA\_1 | *gyrA\_1RNA*-F | CCGCTCGTACAACAAGTGCG | This study |
| *gyrA\_1RNA*-R | TCGATCAACGGGTAGCGCAT |
| Construction of pKT25-*whiB1* | *whiB1hs*-F | ATATATTCTAGAGATGGACTGGCGAAGCAAAGCGG | This study |
| *whiB1hs*-R | ATATATGGTACCTCAGCTGGCGCGGCGGGCGC |
| Construction of pKT25-*whiB2* | *whiB2hs*-F | ATATATTCTAGAGATGCACGAACTTCAGATCGTCG | This study |
| *whiB2hs*R | ATATATGGTACCTCAGGCGGTGAAGACCGCGCGCC |
| Construction of pKT25-*whiB3* | *whiB3hs*-F | ATATATTCTAGAGATGGACAGCACGGCTCGCCAA | This study |
| *whiB3hs*-R | ATATATGGTACCTCATGCACCACGAAGCCCGCG |
| Construction of pKT25-*whiB7* | *whiB7hs*-F | ATATATTCTAGAGATGTTGGCCCTGGCAGATCACC | This study |
| *whiB7hs*-R | ATATATGGTACCTCAGGCCGCGATGGGGTGCTTGC |
| Construction of pKT25-*whiA* | *whiA25hs*-F | ATATATTCTAGAGATGACCGCATCCCTCAAGGAC | This study |
| *whiA25hs*-R | ATATATGGTACCGCTCAGTCCTGCGGGGTGTCGGT |
| Construction of pUT18-*sigmaA* CTD | *SigmaAhs-F* | ATATATAAGCTTGATGGACGCTGTCTCCTTCACC | This study |
| *SigmaAhs-R* | ATATATGGTACCCGGTCTAAATAGTCGCGAAGAAC |
| Construction of pUT18-*sigmaB* | *SigmaBhs-F* | ATATATAAGCTTGATGATTCACGACGATTTTCCG | This study |
| *SigmaBhs-R* | ATATATGGTACCCGGGCATTCGATAAACCTCCCGG |
| Construction of pUT18-*sigmaH* | *SigmaHhs-F* | ATATATAAGCTTGATGACACCCACCACCAGCGAGA | This study |
| *SigmaHhs-R* | ATATATGGTACCCGCGAGGAGACCTCCTTACCTTG |
| Construction of pUT18-*whiA* | *whiA18hs-F* | ATATATAAGCTTGATGACCGCATCCCTCAAGGAC | This study |
| *whiA18hs-R* | ATATATGGTACCGCGTCCTGCGGGGTGTCGGTGGC |  |

\*Restriction enzyme sites are underlined; CCATGG, NcoI; AAGCTT, HindIII; TCTAGA, XbaI; GGATCC, BamHI.

### **2.3 Culture media and growth condition**

#### **2.3.1 Rich media**

##### **2.3.1.1 Nutrient medium**

*Staphylococcus hominis*, *Micrococcus yunnanensis*, *Staphylococcus saprophyticus*, *Staphylococcus haemolyticus*, *Pantoea ananatis*, *Staphylococcus succinus*, *Kocuria rhizophila*, *Pseudomonas fluorescens*, *Sphingomonas ginsenosidimutans*, *Sphingomonas yunnanensis*, *Staphylococcus pasteuri*, *Novosphingobium barchaimii* and *Kytococcus sedentarius* were cultured in sterilised nutrient broth or on nutrient agar.

|  |  |  |
| --- | --- | --- |
|  | **Nutrient broth** | **Nutrient agar** |
| Distilled water | 1 L | 1 L |
| Beef extract | 1 g | 1 g |
| Yeast extract | 2 g | 2 g |
| Peptone | 5 g | 5 g |
| Sodium chloride (NaCl) | 5 g | 5 g |
| Bacteriological agar | - | 15 g |

##### **2.3.1.2 Iso-sensitest medium (ISO)**

All bacteria were cultured using sterilised ISO media for antibiotic susceptibility testing based on the British Society for Antimicrobial Chemotherapy (BSAC) 2013 procedure.

|  |  |  |
| --- | --- | --- |
|  | **ISO broth** | **ISO agar** |
| Distilled water | 1 L | 1 L |
| Hydrolysed casein | 11 g | 11 g |
| Peptones | 3 g | 3 g |
| Glucose | 2 g | 2 g |
| Sodium chloride (NaCl) | 3 g | 3g |
| Soluble starch | 1 g | 1 g |
| Disodium hydrogen phosphate | 2 g | 2 g |
| Sodium acetate | 1 g | 1 g |
| Magnesium glycerophosphate | 0.2 g | 0.2 g |
| Calcium gluconate | 0.1 g | 0.1 g |
| Cobaltous sulphate | 0.001 g | 0.001 g |
| Cupric sulphate | 0.001 g | 0.001 g |
| Zinc sulphate | 0.001 g | 0.001 g |
| Ferrous sulphate | 0.001 g | 0.001 g |
| Manganous chloride | 0.002 g | 0.002 g |
| Menadione | 0.001 g | 0.001 g |
| Cyanocobalamin | 0.001 g | 0.001 g |
| L-Cysteine hydrochloride | 0.02 g | 0.02 g |
| L-Tryptophan | 0.02 g | 0.02 g |
| Pyridoxine | 0.003 g | 0.003 g |
| Pantothenate | 0.003 g | 0.003 g |
| Nicotinamide | 0.003 g | 0.003 g |
| Biotin | 0.0003 g | 0.0003 g |
| Thiamine | 0.00004 g | 0.00004 g |
| Adenine | 0.01 g | 0.01 g |
| Guanine | 0.01 g | 0.01 g |
| Xanthine | 0.01 g | 0.01 g |
| Uracil | 0.01 g | 0.01 g |
| Bacteriological agar | - | 15 g |

##### **2.3.1.3 Luria-Bertani (LB) medium**

*Escherichia coli* was normally cultured in sterilised LB or on LB agar (Fisher 40377-41041).

|  |  |  |
| --- | --- | --- |
|  | **LB broth** | **LB agar** |
| Distilled water | 1 L | 1 L |
| Tryptone | 10 g | 10 g |
| Yeast extract | 5 g | 5 g |
| Sodium chloride (NaCl) | 10 g | 10 g |
| Bacteriological agar | - | 15 g |

##### **2.3.1.4 Middlebrook 7H9 (broth) and 7H10 (agar)**

*Mycobacterium smegmatis* was routinely grown in sterilised 7H9 or on 7H10. For 7H9, ADS solution (see below) was added after sterilization by filtration. For gene expression purposes, 7H9 was prepared without ADS.

|  |  |  |
| --- | --- | --- |
|  | **7H9** | **7H10** |
| Distilled water | 1 L | 1 L |
| Ammonium sulfate | 0.5 g | 0.5 g |
| Disodium phosphate | 2.5 g | 1.5 g |
| Monopotassium phosphate | 1 g | 1.5 g |
| Sodium citrate | 0.1 g | 0.4 g |
| Magnesium sulfate | 0.05 g | 0.025 g |
| Calcium chloride | 0.0005 g | 0.0005 g |
| Zinc sulphate | 0.001 g | 0.001 g |
| Copper sulfate | 0.001 g | 0.001 g |
| Ferric ammonium citrate | 0.04 g | 0.04 g |
| L-Glutamic acid | 0.05 g | 0.05 g |
| Pyridoxine hydrochloride | 0.001 g | 0.001 g |
| Biotin | 0.0005 g | 0.0005 g |
| Malachite green | - | 0.00025 g |
| Bacteriological agar | - | 15 g |

To prepare Middlebrook 7H9 medium with ADS.

|  |  |
| --- | --- |
| Middlebrook 7H9 medium powder | 4.7 g |
| ADS:  2% glucose  0.2% BSA  0.8% NaCl  50% Glycerol | 0.1 L |
| Water | 0.9 L |

#### **2.3.2 Growth media for antibiotic susceptibility testing**

##### **2.3.2.1 Iso/disc antibiotics**

As described in Section 2.3.1.2, ISO agar medium was prepared and left to cool to about 45°C. Bacterial suspensions were mixed with the autoclaved ISO agar to match the turbidity of 0.5 McFarland standard (0.111 OD600) (Beściak and Surmacz-Górska, 2011), then the mixture was poured into petri dishes, (30 ml/plate) and left to cool in a microbiological safety cabinet for 20 min. Antibiotic discs were then placed at the centre of the plates. The plates were incubated at 37°C for 18 h. Measurement of antibiotic inhibition zones and interpretation of the data was carried out according to the BSAC (2013).

##### **2.3.2.2 Iso/minimum inhibitory concentration (MIC)**

As described in Section 2.3.1.2, the ISO broth medium was prepared and left to cool to about 45°C, then 100 µl of ISO broth was added to each well of a 96 well plate using a multi-channel pipette. Then 100 µl of bacterial suspension (0.5 McFarland standard; 0.111 OD600) (Beściak and Surmacz-Górska, 2011), containing 2048 mg/ml of the tested antibiotic was mixed with the first well to obtain 1024 mg/ml/bacterial suspension, then 100 µl of the first well was transferred to the second well to obtain 512 mg/ml, then this serial dilution was repeated in each well until the final concentration of 1 mg/ml. Data interpretation was done according to the BSAC guideline (2013).

#### **2.3.3 Growth media for the bacterial BACTH two-hybrid system**

##### **2.3.3.1 MacConkey/maltose medium**

Preparation of MacConkey/maltose agar plates was carried out by adding 40 g of MacConkey agar (Sigma BCBS2083V) to 1 L of distilled water. The medium was autoclaved and left on bench to cool to approximately 50°C, then maltose (1% final concentration), IPTG (0.5 mM) and appropriate antibiotics were added. For maltose 20% stock solutions, were prepared and filtered for sterilization. For additional testing, 0.05 mM 2,2’-Bipyridyl (dissolved in 20% ethanol), 64 µg Erythromycin or 5% NaCl were added. For antibiotics and the iron chelator, sterilization was done by filtration (0.20 µm non-pyrogenic sterile-R Minisart Syringe Filter).

##### **2.3.3.2 LB/X-gal medium**

As described in Section 2.3.1.3, LB agar was prepared and left to cool to about 45°C, 0.5 mM IPTG, 40 µg/ml X-gal and appropriate antibiotics were added before pouring the plates. For X-gal, a stock solution (20 mg/ml) was prepared in dimethyl formamide.

#### **2.3.4 Bacterial growth supplements**

##### **2.3.4.1 Antibiotic susceptibility test**

Antibiotic discs were purchased from Oxoid and class/loadings are listed in Table 2.5. For MIC measurements, antibiotic stock solutions were purchased from various companies (Table 2.6).

Table 2.5: Antibiotic discs used in this study

|  |  |  |  |
| --- | --- | --- | --- |
| **Name** | | **Amount** | **Class** |
| Erythromycin (E) | | 5 µg | Macrolide |
| Doripenem (DOR) | 10 µg | | β-Lactam  β-Lactam  β-Lactam  β-Lactam  β-Lactam |
| Imipenem (IPM) | 10 µg | |
| Meropenem (MEM) | 10 µg | |
| Piperacillin (PRL) | 75µg | |
| Piperacillin-tazobactam (TZP) | 85 µg | |
| Tetracycline (TE) | 10 µg | | Tetracycline |
| Amikacin (AK) | 30 µg | | Aminoglycoside  Aminoglycoside |
| Gentamicin (CN) | 10 µg | |
| Vancomycin (VA) | 30 µg | | [Glycopeptide](https://en.wikipedia.org/wiki/Glycosylation) |
| Ciprofloxacin (CIP) | 1 µg | | Quinolone |
| Polymyxin B (PB) | 300 units | | Colistin |
| Rifampin (RD) | 5 µg | | Antimycobacterial |
| Chloramphenicol (C) | 10 µg | | Chloramphenicol |

Table 2.6: Antibiotic stocks used in this study

|  |  |  |
| --- | --- | --- |
| **Antibiotics** | **Lot/patch number** | **Company** |
| Erythromycin | WXBC1653V | Sigma |
| Doripenem | 25MG/32128 | Sigma |
| Imipenem | 200MG/PHR1796 | Sigma |
| Meropenem | 300MG/1392454 | Sigma |
| Piperacillin | 350MG /1541500 | Sigma |
| Piperacillin-tazobactam | 116M4759V | Sigma |
| Tetracycline | 046M4809V | Sigma |
| Amikacin | A0368000 | Sigma |
| Gentamicin | 80K1461 | Sigma |
| Vancomycin | 10ML/SBR00001 | Sigma |
| Ciprofloxacin | 116M4062V | Sigma |
| Polymyxin B | 1MU/P4932 | Sigma |
| Rifampin | 500MG/PHR1806 | Sigma |
| Chloramphenicol | SLBR8869 | Sigma |
| Ampicillin sodium salt | G25794 | Sigma |
| Streptomycin sulfate | S6501-25G/064k0564 | Sigma |
| Kanamycin sulfate | 11815024 | Thermofisher |
| Hygromycin B | Z29C032 | Alfa aesar |

##### **2.3.4.2 Keratin degradation test**

LB agar was prepared as described in Section 2.3.1.3 and then supplemented with 4 mg/ml of pieces of keratin azure ~1.5 mm length (Sigma). The LB-keratin azure agar was autoclaved and plates were poured in the Biological safety cabinet. Then a sterile disc (Sigma) was dipped into overnight cultures using sterile forceps and placed in the middle of the plate. The plates were incubated for 6 days at 37°C in darkness. This method has been adapted from (Scott and Untereiner, 2004).

##### **2.3.4.3 Sample preparation for Nuclear Magnetic Resonance (NMR)**

Overnight cultures (50 ml in 250 ml conical flasks) of *K. sedentatius* MBB13 were grown in Nutrient broth containing different NaCl concentrations at 37°C for 16 h with shaking (250 rpm). Bacteria were collected by centrifugation for 10 min at 3000 *g*. Supernatant was discarded and the pellets were resuspended in 1 ml dH2O. Then the sample tubes were placed in ice and the bacteria were lysed by sonication (3 x 20 s with 15 s cooling between each sonication step). Samples then were transferred to Eppendorf tubes and centrifuged at 13000 *g* for 7 min. The supernatants were transferred to pre-weighed Eppendorf and frozen overnight at -80°C. The tubes were recapped with lids that had been pierced with a needle and transfer to a freeze-dryer for 72 h. After that, the lids with the holes were removed from the Eppendorf tubes and the tubes were sealed. The tubes were then re-weighed and stored at room temperature until sent to the NMR suite for analysis. The samples were dissolved in 530 μl D2O and 5 μl trimethylsilyl propionate (100 mM). NMR spectra were collected using a Bruker 600 mHz.

##### **2.3.4.4 Anaerobic growth**

To grow bacteria on plates, 30 µl of overnight bacterial cultures were streaked on LB agar plates, which were then placed into an anaerobic jar (Oxoid) containing an Oxoid Anaerobic Gas Generating Kit (the kit was opened and 10 ml of water was immediately added), anaerobic catalyst (Oxoid) and anaerobic indicator (opened and placed into the jar immediately before sealing). The jar was sealed and placed at 37°C overnight. For anaerobic broth cultures, 25 ml universal tubes with silicone septa screw caps were autoclaved and then sterilised LB (10 ml) was placed in the tubes and the caps were screwed tightly. The medium and headspace were made anaerobic by bubbling with oxygen-free nitrogen for 8 min. A metal reusable hypodermic needle (autoclaved) was used to introduce the nitrogen gas and a microlance needle (0, 8 x 40 mm) (BD) was used to allow the gas to exit. Then 50 µl (0.01 OD600) from an overnight bacterial culture was injected into the anaerobic medium and the cultures were incubated at 37°C overnight.

##### **2.3.4.5 Medium supplements**

Agar and broth media were supplemented with appropriate antibiotics at the following final concentrations: Ampicillin 100 µg/ml, Streptomycin 50 µg/ml, Kanamycin 35 µg/ml and Hygromycin B 100 µg/ml. For protein expression, IPTG (1.0 mM) or acetamide (0.2%) were added to the medium of *E. coli* or *M. smegmatis* cultures, respectively, to induce recombinant protein expression.

#### **2.3.6 Bacterial growth conditions**

Bacteria were grown from a single colony or a glycerol stock. Incubation time was 18 h for all bacteria, except *M. smegmatis* which was 72 h. For protein expression, *M. smegmatis* was cultured on 7H10 or in 7H9 media at 37°C. 7H9 cultures were agitated at 250 rpm. When the OD600 reached 0.5-0.9, the cultures were induced with 0.2% acetamide and incubated for a further 12 h.

#### **2.3.7 Bacterial growth measurement**

*Escherichia coli* and *M. smegmatis* growth was estimated in broth media by reading the OD at 600 (OD600). For *M. smegmatis*, the culture was supplemented with 2.0 ml/l glycerol and 0.5 ml/L Tween 80 to prevent the cells clumping.

#### **2.3.8 Bacterial strain storage**

For long term storage, bacterial cell pellets from a 5 ml culture were suspended in 1 ml LB broth (or 7H9 for *M. smegmatis*) and 1 ml of 80% (v/v) glycerol with the appropriate antibiotics. Then 1 ml of the mixture was transferred into a 1.5 ml Eppendorf tube and stored at -70°C.

### **2.4 Preparation and transformation of competent cells**

#### **2.4.1 Chemical competent cells**

##### **2.4.1.1 Preparation**

A single colony of *E. coli* DH5α was placed in into 5 ml of LB medium and grown at 37°C overnight with shaking. One ml of this bacterial suspension was transferred into 100 ml of pre-warmed LB medium in 250 ml flask, and incubated at 37°C with shaking (250 rpm) until OD600 ~0.5 (2 h estimated time). The culture was transferred into two Falcon tubes and both tubes were centrifuged at 4°C / 6000 x *g* for 20 min. Then the supernatants were discarded and the cells carefully suspended in 50 ml of chilled RF1 (see below) and kept on ice for 15 min. The cells were centrifuged at 4°C / 6000 x *g* for 20 min. Then the supernatants were discarded and the cells carefully suspended in 8 ml of chilled RF2 (see below) and kept on ice for 20 min. Then each 400 µl of RF2/bacterial suspension was transferred into an Eppendorf tube and stored at - 80°C.

|  |  |  |  |
| --- | --- | --- | --- |
| **RF1 (pH 5.8)** |  | **RF2 (pH 6.8)** |  |
| Potassium chloride (KCl) | 100 mM | MOPS | 10 mM |
| Manganese (II) chloride tetrahydrate (MnCl2.4H2O) | 5 mM | KCl | 10 mM |
| Potassium acetate (CH3CO2K) | 30 mM | CaCl2.2H2O | 75 mM |
| Calcium chloride dihydrate (CaCl2.2H2O) | 10 mM | Glycerol 15% | 15% |
| Glycerol | 15% |  |  |

##### **2.4.1.2 Transformation**

Frozen competent cells (Section 2.4.1.1) were thawed on ice for 30 min. Then an aliquot (100 µl) of competent cells was mixed with (0.1 ng-1 µg) pre-chilled plasmid or ligation mix. The mixture was left on ice for 2 min. The mixture was exposed to 42.2°C (heat shock) for 1.5 min. Again the mixture was left on ice for 2 min, then 800 µl of LB medium was added to the mixture and incubated at 37°C for 1 h. Then the mixture was centrifuged at 6000 x *g* for 1 min. The supernatant was discarded and the pellets were resuspended and spread on LB agar containing the appropriate antibiotic(s) before incubation at 37°C for 18 h.

#### **2.4.2 Electrocompetent cells**

##### **2.4.2.1 Preparation**

Primary overnight cultures (5 ml) of either *E. coli* or *M. smegmatis* were grown in defined media (described in Section 2.3.1.3 or 2.3.1.4 respectively). A 250 ml flask containing LB or 7H9 was inoculated with 300 µl of the overnight culture of *E. coli* or *M. smegmatis*. For *E. coli*, the new culture was incubated at 37°C with shaking at 250 rpm until the OD600 reached ~0.6 (estimated time ~2 h). For *M. smegmatis*, the incubation time was 18 h until the OD600 reached ~0.9. The cultures were immediately placed on ice for 30 min for *E. coli* or ~2 h for *M. smegmatis*. The cultures were poured into 50 ml Falcon tubes and centrifuged at 3500 *x g* for 10 min at 4°C. The supernatant was discarded and pellets were suspended with 10% (v/v) cold glycerol and centrifuged at 3500 *x g* for 10 min at 4°C. This was repeated twice more. The cell pellets were suspended in 1000 µl 10% (v/v) cold glycerol then, aliquots (100 µl) of the cell suspension were transferred to pre-cooled Eppendorf tubes in dry ice and stored at - 80°C.

##### **2.4.2.2 Transformation**

Frozen competent cells (Section 2.4.2.1) were thawed on ice for 30 min. Then the competent cells were mixed with plasmid (~0.5 µg). The mixtures were transferred to cooled electroporation cuvettes and electroporated at 1.8 kV for either *E. coli* or *M. smegmatis* by the Hybaid Cell Shock unit. Then, 800 µl of LB or 7H9 was added to the mixtures which were transferred into Eppendorf tubes and incubated for 1 h (*E. coli*) or 4 h (*M. smegmatis*) at 37°C. Mixtures were centrifuged at 12000 *x g* for 1 min and the supernatants discarded. The pellets were resuspended and spread on LB (*E. coli*) or 7H10 (*M. smegmatis*) plates with appropriate antibiotics and incubated at 37°C for 18 h (for *E. coli*) or 27 h (for *M. smegmatis*).

### **2.5 Isolation of bacteria using passive (settle plates) or active (dehumidifier) sampling techniques**

Bacteria present in an occupied lecture theatre (MBB-F02) at the University of Sheffield were sampled from the third week of October until the first week of December 2015 (8 weeks), and isolation of collected bacteria was conducted each Friday. The study used two methods; dehumidifier sampling (active monitoring) and settle plates using open petri dishes containing nutrient agar (NA) medium (passive monitoring). Samples were taken each Friday after the students had left the room (normally after 5 pm when the room was unoccupied). For the dehumidifier (Meaco 12L Platinum) samples, the water tank had been washed three times and disinfected by ethanol three times and left to dry in a microbiology cabinet before fitting into the machine. The dehumidifier was located close to the exit door next to the first row of student benches. The dehumidifier was switched on for one hour to collect room air moisture (normally the dehumidifier collected 50 ml of water-vapour per hour). For the settle plates, four petri dishes were placed on tables adjacent to the location of the dehumidifier (~1-2 m away). The plates were left open while the dehumidifier was switched on to suction mode. After that, the plates were closed and the dehumidifier was switched off. Then the plates and the dehumidifier tank were taken to the laboratory. Water-vapour from the dehumidifier was transferred to a 50 ml Falcon tube and centrifuged. The supernatant was discarded and the ~50 μl of liquid that remained was streaked on a NA plate. The plates containing dehumidifier water-vapour residue and those which were left open (settle plates) were incubated at 37°C for 18 h. The following day, colonies were sub-cultured on fresh NA plates and were incubated at 37°C for 18 h. One colony of each sub-cultured bacterium was inoculated into 5 ml NB medium in a 50 ml Falcon tube and incubated at 37°C for 18 h. These bacterial cultures were used for genomic DNA extraction (Section 2.6.2).

### **2.6 Nucleic acid methods**

#### **2.6.1 DNA storage**

Genomic and plasmid DNA were stored at -20°C.

#### **2.6.2 Bacterial genomic extraction**

Genomic DNA extraction was carried out using either Sigma GenElute or Qiagen QIAamp DNA mini kits according to the manufacturer’s instructions. An additional step, to improve cell lysis of *K. sedentarius* MBB13 was treatment of the cell suspensions with 300 µg/ml Lysozyme, 200 µg/ml Achromopeptidase, 200 µg/ml, Lysostaphin, 100 µg/ml Mutanolysin and 40 µl Proteinase K (20 mg/mL) for 30 min at 37°C (Sims *et al.*, 2009). For whole genome sequencing, DNA was eluted in EB buffer with no EDTA (30–100 µl) yielding and a minimum concentration of 10 ng/µl.

##### **2.6.2.1 Bioinformatics analysis by ACT**

Genomic DNA was sent to MicrobesNG for Next-Generation Sequencing (NGS). Four trimmed files (fastq.gz) were received from MicrobesNG. Those files were used to generate three files that can be read by ACT software (Sanger) namely,

(1) Galaxy-[Concatenated\_reference\_Genbank\_file]

(2) Galaxy-[ACT\_comparison\_file].tabular

(3) Galaxy-[Concatenated\_contigs\_Genbank\_file]

These files were created through <https://hactar.shef.ac.uk/galaxy>. The process to create these files was carried out with help from Dr. Roy Chaudhuri from the Bioinformatics Services, Department of Molecular Biology and Biotechnology at the University of Sheffield and can be found in Appendix 9.4.*a*. The ACT software was used to open the *K. sedentarius* MBB13 and reference genomes together (Appendix 9.4.*b*).

#### **2.6.3 Plasmid extraction**

Plasmid DNA extraction was carried out using Qiagen QIAprep® Spin Miniprep Kit following the instructions provided by the manufacturer.

#### **2.6.4 DNA concentration measurement**

DNA concentration was measured using the NanoPhotometer P-300 Spectrophotometer after programing the machine by selecting double-strand DNA. The machine was blanked using EB buffer.

#### **2.6.5 Agarose gel electrophoresis**

DNA fragment size and concentration of genomic DNA, PCR amplification products and plasmids were estimated by agarose gel electrophoresis. Agarose (1 g) in 1x TAE buffer (100 ml) was heated until boiling in a microwave oven (~50 s). GelRed Nucleic Acid 10,000x (Biotium 18G0327) or Ethidium bromide solution (1% solution) was added to the melted agarose (1 µl/10 ml). Then the agarose was poured into the gel tray containing the appropriate comb and left on bench to cool for ~20 min. The gel was placed in the gel tank and covered with 1x TAE buffer. DNA samples were mixed with loading dye (6x) (Biolab B7024S) and before loading into the wells. HyperLadder (Bioline) 1 kb or 100 kb was used to estimate the DNA sizes. Gels were electrophoresed at 80 V, and 400 mA for 45 min (small gel) or 70 min (large gel). DNA was viewed using a Photodocumentation System (Syngene). Estimation of DNA molecular weight by comparison with standard DNA ladders (Fig 2.1).

|  |  |
| --- | --- |
|  | **10x TAE buffer** |
| Tris | 48.4 g |
| EDTA (0.5M, pH 8.0) | 20 ml |
| Glacial acetic acid | 11.42 ml |
| dH2O | 1 L |

|  |  |
| --- | --- |
| **C:\Users\mbb\Desktop\N0551_thumb.gif** | **C:\Users\mbb\Desktop\N0552_thumb.gif** |
| Fig. 2.1: Quick-Load Purple DNA ladders (New England BioLabs; image reproduced from [www.neb.com](file:///C:\Users\mbb\Desktop\My%20thesis%20June2019\submitted%20files\www.neb.com)) | |

#### **2.6.6 DNA Gel extraction**

Gels were placed on a 2UV transilluminator (UVP) to view the DNA fragments, and DNA fragments were cut out using a scalpel. DNA was extracted from the excised agarose using QIAquick Gel Extraction kit (Qiagen) following the manufacturer’s instructions.

#### **2.6.7 Primer design**

Oligonucleotide primers were designed for amplification of DNA fragments. Primers were designed to be 50-60% G-C rich, 20-30 bases in length and 55-70°C melting temperature (Tm). For cloning purposes, restriction endonuclease sites were incorporated into the primer design. Tm was estimated using ApE plasmid editor software (an open source software programme created by M. Wayne-Davis, <http://biologylabs.utah.edu/jorgensen/wayned/ape/>). The following website was use to check the presence of restriction sites within the gene of interest (NEBcutter V2.0): <http://nc2.neb.com/NEBcutter2/index.php>. For protein overexpression, primers were designed to allow insertion of the open reading frames between the Ncol and HindIII sites of pMyNT. For the bacterial two hybrid system, primers were designed to insert genes between XbaI and KpnI sites of pKT25, and between HindIII and KpnI for pUT18. Due to amplification difficulties, *whiB3*, σA *C*-terminal domain (CTD) and σB were synthesised, and σJ was synthesised and ligated into pUT18 by Eurofins. The codon usage of the synthesised gene sequences were optimized to *E. coli* K-12 using this webpage: [http://gcua.schoedl.de/sequential\_v2.html](http://gcua.schoedl.de/sequential_v2.html%20%20). All primers were purchased from Eurofins. Gene sequences after cloning are available in Appendix 9.11.

#### **2.6.8 DNA amplification**

An AmpliTaq Gold fast or 2x MyTaq PCR master mix (MM) were used for DNA amplification. The standard reaction used was as follows:

|  |  |
| --- | --- |
|  | **11.5 µl total volume** |
| Forward primer (10 pmol/µl) | 0.5 µl |
| Reverse primer (10 pmol/µl) | 0.5 µl |
| Template DNA (50-100 ng/µl) | 1.25 µl |
| MM (AmpliTaq Gold/ 2x MyTaq Mix) | 3 µl |
| dH2O | 6.25 µl |

For Polymerase Chain Reactions (PCR), 30 cycles were performed as follows:

|  |  |
| --- | --- |
|  | **Time** |
| Initial denaturation | 98°C – 30 s |
| Denaturation | 95°C – 1 min |
| Annealing | 5°C below the Tm of each primer pair |
| Extension | 72°C – 1 min/1 kbp |
| Final extension | 72°C – 5 min |
| Hold | 4°C |

#### **2.6.9 DNA digestion by restriction endonuclease**

Restriction enzymes were purchased from New England Biolabs and the reactions were made in separate tubes as follows:

|  |  |  |
| --- | --- | --- |
|  | **Insert** | **Vector** |
| x10 Smart cut buffer | 2 µl | 2 µl |
| Restriction enzymes (20,000 U/ml) | 0.5 µl | 0.5 µl |
| DNA | 500 ng | - |
| Vector | - | 1000 ng |
| dH2O | Up to 20 µl | Up to 20 µl |

Mixtures were incubated at 37°C for 1 h. To stop restriction enzyme activity, DNA purification was carried out (alternatively, for HindIII and NcoI, mixtures were incubated at 80°C for 20 min).

#### **2.6.10 DNA ligation**

Quick ligation kits were purchased from New England Biolabs and the reactions were assembled as shown below. DNA-vector ratio was determined through the following webpage <http://www.insilico.uni-duesseldorf.de/Lig_Input.html>.

|  |  |
| --- | --- |
|  | **Volume** |
| Quick ligase | 1 µl |
| Reaction buffer | 10 µl |
| DNA | 1:3 molar ratio vector to insert |
| Nuclease-free water | Up to 10 µl |

Ligation mixtures were incubated at room temperature for 20 min. Ligation mixes were used to transform competent cells ether chemically or by electroporation as described in Sections 2.4.1.2 and 2.4.2.2.

#### **2.6.11 DNA purification**

DNA purification was carried out using QIAquick PCR kit (Qiagen) following the manufacturer’s instructions.

#### **2.6.12 DNA sequence viewing and alignment**

Plasmid DNA sequencing was carried out by either GATC Biotech Company or Core Genomic facility in Medical School at the University of Sheffield. All sequence files were viewed in either FinchTV or Snapgene programmes. The 16S rRNA gene sequence data were used to make a phylogenetic tree of the strains. Sequence data were analysed using NCBI website (<https://www.ncbi.nlm.nih.gov/>) to identify the closest match in the database (Appendix 9.1).

#### **2.6.13 Site-directed mutagenesis**

QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies 200521-5) was carried out for the site-directed mutagenesis according to the instructions of the manufacturer. Primers were designed via the following webpage: (<https://www.agilent.com/store/primerDesignProgram.jsp>).

### **2.7 RNA stabilisation, extraction and expression**

#### **2.7.1 RNA stabilisation**

Mid-log phase cultures, were sampled (2-5 ml) and mixed with 2 volumes of RNA protect reagent (Qiagen). After mixing for 10 s, the samples were incubated at room temperature for 5 min. Mixtures were then centrifuged at 6000 *x g* for 10 min at 4°C. The supernatants were discarded and the cell pellets were immediately stored at -80°C.

#### **2.7.2 RNA extraction**

RNA was extracted using a RNeasy mini kit (Qiagen) according to the manufacturer’s procedure. To test for DNA contamination, DNA was amplified using the universal 16S ribosomal primers (27-F and 1492-R) and then the PCR sample was separated by gel electrophoresis and visualised.

#### **2.7.3 DNA removal**

For RNA samples that were positive in the 16S PCR test (Section 2.7.2), the contaminating DNA was removed using the Turbo DNA-free kit (Invitrogen) standard procedure.

#### **2.7.4 RNA concentration measurement**

RNA concentration was measured using the NanoPhotometer P-300 Spectrophotometer after programing the machine by selecting RNA. The instrument was blanked using RNase-free water.

#### **2.7.5 Primer design for RNA specificity**

Primers were designed using Primer-BLAST tool in NCBI webpage with the following criteria:

|  |  |
| --- | --- |
| **Criteria** | **Standard** |
| Tm | 59-65 (62 optimal) |
| Produce size value | 200 as maximum |
| Product size | 70-200 bp |
| Primer length | 18-22 nucleotides (20 optimal) |
| GC rich | 20-60% (primer pair should have similar percentage) |
| Avoid nucleoids repeats | Examples, GTTTT or GCGCGCGC |

#### **2.7.6 Quantitative Real-Time PCR (qRT-PCR)**

To detect the relative abundance of RNA, the samples were arrayed in an optical 96 well plate and amplification was detected using Brilliant III Ultra-Fast SYBR green qRT-PCR kit (Agilent Technologies) as follows:

|  |  |
| --- | --- |
|  | **Amount** |
| 2x Brilliant III SYBR green | 10 µl |
| 10 mM F primer | 2 µl |
| 10 mM R primer | 2 µl |
| 100 mM DTT | 0.2 µl |
| RT/RNase Block | 1 µl |
| RNA | 2 µl |
| 2 µM Reference dye (100 mM stock) | 0.3 µl |
| RNAase-free | 2.5 µl |

Analysis was conducted on the total RNA from three biological and technical replicates. Cycling conditions were as follows:

|  |  |
| --- | --- |
| 50°C, 10 min | 1 cycle |
| 95°C, 3 min | 1 cycles |
| 95°C, 15 s | 40 cycles |
| 60°C, 20 s |
| 95°C, 1 min | 1 cycle |
| 55°C, 30 s |  |
| 95°C, 30 s |  |

#### **2.7.7 Analysis of quantitative real-time PCR**

The expression of the genes of interest was normalized against the control gene using the ∆CT method to determine the relative gene expression level between the two conditions (Manganelli *et al.*, 1999).

### **2.8 Protein methods**

#### **2.8.1 Protein concentration measurement**

Two methods to measure protein concentration were used, Bradford assay and Beer-Lambert law. To use the Bradford assay (Bradford, 1976), the absorbance was measured at 595 nm using Biochrom WPA Lightwave II spectrophotometer after reaction of sample with the Bradford reagent (Bio-Rad) according to the manufacturer’s procedure. For Beer-Lambert law, the theoretical extinction coefficient was first obtained from the ExPASy webpage: <https://web.expasy.org/protparam/>, then the absorbance was measured at 280 nm using a NanoPhotometer P-300 Spectrophotometer. The protein concentration was calculated as follows:

Bradford:

OD959= Protein (µl) + reagent (10% Bradford reagent + 90% H20)

106 = # mg/ml → 1000 = # µM

Beer–Lambert law:

\*106 = # µM

#### **2.8.2 Denaturing gel electrophoresis (SDS-PAGE)**

SDS-PAGE (Laemmli, 1970) was carried out in a glass plate sandwich (Bio-Rad). The two gels, resolving and stacking were prepared as follows:

|  |  |  |
| --- | --- | --- |
|  | **15% Resolving gel (ml)** | **5% Stacking gel (ml)** |
| dH2O | 2.7 | 6.95 |
| 3 M Tris-HCl (pH 8.3) | 0.95 | - |
| 0.5 M Tris-HCl (pH 6.3) | - | 1.25 |
| 30% Acrylamide | 3.8 | 1.7 |
| 10% TEMED | 0.02 | 0.02 |
| 10% SDS | 0.075 | 0.1 |
| 10% APS | 0.1 | 0.1 |

Firstly, the resolving gel was poured to occupy 2¾ of the space, with the rest left for the stacking gel. Then isopropanol was layered over the resolving gel to ensure a level surface until the gel was polymerized. Isopropanol was removed and the gel surface was washed with water. Then the stacking gel was loaded to fill the rest of the space. The comb was inserted and the gel left on the bench until it was polymerized. The gel was transferred into the gel tank containing 1x SDS running buffer and then the comb was removed from the gel. Loading dye was mixed with the protein samples at a ratio of (1:5) and the mixtures were heated to 95°C for 5 min. Samples were loaded into the wells alongside an appropriate standard protein ladder (Fig. 2.2). Polypeptides were separated by electrophoresis for 80 min at 180 V. The glass plates were removed and the gel was stained with Coomassie Blue solution for 60 min. The staining solution was removed and the gel was destained overnight. The 1x SDS running buffer, 5x loading dye, Coomassie Blue stain and destain solutions were prepared as follows:

|  |  |  |  |
| --- | --- | --- | --- |
| **1x SDS running buffer** | | **5x loading dye** |  |
| SDS | 1 g | SDS | 5% |
| Tris | 3 g | Tris-HCl pH 6.8 | 0.225 M |
| Glycine | 14.4 g | Glycerol | 50% |
| dH2O up to | Up to 1 L | Bromophenol blue | 0.05% |
|  |  | 2-Mercaptoethanol | 0.25 M |

|  |  |  |
| --- | --- | --- |
|  | **Commassie Blue stain** | **Destain** |
| Methanol | 400 ml | 400 ml |
| Acetic acid | 100 ml | 100 ml |
| Commassie Brilliant Blue | 1.15 g/L | - |
| dH2O up to | 1 L | 1 L |

Estimation of protein molecular weight was achieved by the running of pre-stained markers (CSL-BBL) (Fig 2.2).

|  |
| --- |
| **C:\Users\mbb\Desktop\PM007-0500.jpg** |
| Fig. 2.2: PiNK Plus prestained Protein Ladder (Cleaver Scientific; [www.clentlifescience.co.uk](file:///C:\Users\mbb\Desktop\My%20thesis%20June2019\submitted%20files\www.clentlifescience.co.uk)) |

#### **2.8.3 Protein overproduction of WhiB1 and WhiB2 in Mycobacterium smegmatis**

The coding regions of *whiB1* and *whiB2* were amplified from *K. sedentarius* MBB13 genomic DNA using the forward and reverse primers for *whiB1* and *whiB2* (Table 2.4). The products were digested and ligated (Section 2.6.9 and 2.6.10) into pMyNT (Hygro-BR) between the Ncol and HindIII sites. The integrity of both plasmids was checked by DNA sequencing (Section 2.6.12). Plasmids containing *whiB1* (strain JRG6949) and *whiB2* (strain JRG6947) were purified (Section 2.6.11) and used to transform (Section 2.4.2.2) *M. smegmatis* MC2155 (JRG6161) (Table 2.1). Protein overproduction was carried out in 2 L flasks containing 7H9 medium with Hygromycin-B (100 µg/ml). Cultures were grown at 37°C until OD600 ~0.9 (~18 h) (Section 2.3.6) then the cultures were induced with 0.2% acetamide and incubated at 37°C for ~12 h, with shaking (250 rpm). Cells were collected by centrifugation at 17,500 *x g* for 30 min and stored at -20°C until cell lysate preparation.

#### **2.8.4 Production of cell-free extracts**

Frozen cells of the *M. smegmatis* containing the recombinant WhiB1 and WhiB2 proteins were suspended in either 20 mM NaH2PO4, pH 7.4 or 50 mM Tris, pH 8.0, containing 500 mM NaCl, and disrupted by sonication. Cells were sonicated (9 x 25 s pulses) on ice by Soniprep 150 Plus set to the maximum amplitude. The sonicated samples were centrifuged (using Avanti J-251) at 45,500 *x g* for 20 min to separate the cell debris from lysate. The supernatant was used for purification.

#### **2.8.5 Protein purification by affinity chromatography**

WhiB1 and WhiB2 (which both had *N*-terminal His6-tags) were purified using 5 ml HiTrapTM chelating columns, on the AKTA Pure machine. Approximately, 10 ml of cell lysate was injected into the loading loop and the machine was set according to the manufacturer’s procedure. The purification buffers were prepared as follows:

|  |  |  |
| --- | --- | --- |
|  | **Binding buffer A (pH 8.0)** | **Elution buffer B (pH 8.0)** |
| Tris | 50 mM | 50 mM |
| NaCl | 0.5 M | 0.5 M |
| Imidazole | - | 0.5 M |
| dH2O | Up to 1 L | Up to 1 L |

The eluted fractions were collected and their protein concentrations were measured. Then the fractions were analysed by SDS-PAGE to determine their purity. The protein containing fractions were stored at -20°C.

#### **2.8.6 Scanning UV visible spectroscopy**

To obtain the absorption spectra of proteins, the pure fractions were transfer into cuvettes. Scanning was between 200-800 nm. The machine was baselined using a buffer blank. For measurement under anaerobic conditions, the protein solutions were transferred to Hellma® quartz cuvettes (10 mm) which had a screw cap lids to maintain anaerobic conditions.

#### **2.8.7 Circular dichroism (CD) spectroscopy**

Buffer exchange (20 mM NaH2PO4, 0.1 M NaCl, pH 7.4) was achieved by concentration and dilution (Vivaspin) or by membrane dialysis (Spectra/Por), size 3,000 Da molecular weight cut-off (MWCO). The protein (~75 µM WhiB1 or ~35 µM WhiB2) was transferred to a CD cuvette and spectra were collected using a Jasco J810 instrument.

#### **2.8.8 Liquid chromatography mass spectrometry (LC-MS)**

LC-MS was carried out by Mr. Simon Thorpe (Spectrometry Centre at the University of Sheffield) using Agilent 6530 Q-ToF (MS instrument) and Agilent 1260 infinity (LC instrument). In brief, the protein sample was diluted to 3 µM final concentration in an aqueous solution containing, 1% formic acid and 0.1% acetonitrile, and then loaded Phenomenex Aeris Widepore 3.6u XB–C18 column (50 mm x 2.1 mm). Bound protein was eluted by applying a linear gradient from 0.1% formic acid, 5% to 95% acetonitrile, at 0.4 ml/min (flow rate) for 10 min. The protein eluate was continuously introduced by the positive mode electrospray ionization (ESI) of the mass spectrometer.

### **2.9 Iron-sulfur cluster analysis methods**

#### **2.9.1 Removal of the iron-sulfur cluster from holo-WhiB1**

Protein concentration was determined by Bradford assay and Beer–Lambert law (Section 2.8.1). Three measurements with different volumes of holo-protein were made and the average was taken. Then the protein was treated with 10 mM EDTA in presence of 1 mM TCEP-HCI and kept at room temperature overnight. Removal of the iron-sulfur cluster was detected by UV-visible spectroscopy.

#### **2.9.2 Reconstitution of the WhiB2 iron-sulfur cluster**

WhiB2 was purified (Section 2.8.5) and UV-visible spectra were taken to estimate the concentration of iron-sulfur clusters. For apo-WhiB2, the protein was reduced by adding 1 mM DTT before reconstitution. All reconstitution steps were carried out inside the anaerobic cabinet. Buffers and other solutions were left for at least 18 h inside the cabinet to ensure that they were anaerobic. Iron-sulfur cluster reconstitution was achieved using the NifS-based system (Kudhair *et al.*, 2017) as follows:

|  |  |
| --- | --- |
|  | **Volume** |
| Purified-Apo-WhiB2 | 1x |
| Ammonium ferrous sulphate | 10x protein concentration |
| L-cysteine | 20x protein concentration |
| DTT | 10 mM |
| NifS (Section 2.9.2.1) | 10 µg for each 200 µM of WhiB2 |

The reaction was left inside the anaerobic cabinet overnight. Next day, the reconstituted WhiB2 was dialyzed against anaerobic buffer: 25 mM NaH2PO4, 0.25 M NaCl, pH 7.4 overnight inside the cabinet to remove non-integrated reaction components (Section 2.9.3). The dialyzed reconstituted WhiB2 was transferred into Hellma® quartz cuvette (10 mm) and the screw cap lid was sealed to keep the protein anaerobic. The [4Fe-4S] cluster content was measured by UV-visible spectroscopy.

##### **2.9.2.1 Expression and purification of desulfurase enzyme (NifS)**

NifS is a cysteine desulfurase and can be isolated from *Azotobacter vinelandii*. NifS is used to provide sulphide from L-cysteine in order to reconstitute WhiB2. Dr. Dennis R. Dean (University of Virginia) provided the plasmid pDB55 which encodes NifS, the purification was done by Dr. Bassam Khudair. In brief, the overexpression of NifS was carried out as described by (Zheng *et al.*, 1993), with few modifications as follows: protein induction was achieved by 1 mM IPTG instead of 1% lactose, incubation time 8 h instead of 2 h and cultures were incubated at 25°C.

#### **2.9.3 Oxygen (O2) sensitivity of the WhiB1 and WhiB2 iron-sulfur clusters**

To examine the O2 sensitivity of the purified and reconstituted WhiB1 and WhiB2 proteins, the UV-visible spectra of the proteins were recorded (Section 2.8.6). O2-saturated buffer: 50 mM Tris, 0.5 M NaCl, pH 7.4, 5% glycerol was added to the anaerobic protein solutions such that the final concentration of O2 was ~110 µM. UV-visible spectra were recorded at regular intervals. Alternatively, O2 was introduced by bubbling directly into the unsealed cuvette using a Hamilton gastight syringe (100 µl).

#### **2.9.4 Nitric oxide sensitivity of WhiB1 and WhiB2 iron-sulfur clusters**

Spermine NONOate (Cayman) was used to investigate the sensitivity of the WhiB1 and WhiB2 iron-sulfur clusters to NO. Spermine NONOate releases NO with half-life of 39 min. Spermine NONOate was prepared by dissolving the powder in water to the final concentration of 19.06 mM. Spermine NONOate solution (30 µl) was injected using a Hamilton syringe into 1 ml of anaerobic protein (~35 µM) in a sealed Hellma® quartz cuvette (10 mm).

#### **2.9.5 In vitro denaturation of Holo-WhiB1**

To denature the holo-WhiB1 protein, guanidinum thiocyanate (BDH Chemicals, K21375064-551) was used. Guanidinum thiocyanate was prepared by dissolving the powder in water. Guanidinum thiocyanate working solution was mixed with the protein (~100 µM) to the final concentration of 1.5 M. Then the treated protein was left in the UV-visible spectrum to take several reading points to confirm the loss of the cluster.

### **2.10 *In vivo* protein-protein interaction**

#### **2.10.1 The bacterial Adenylate Cyclase-based Two-Hybrid (BACTH)**

The interaction between WhiB proteins and RNA polymerase sigma factors was investigated using the BACTH system. First, the genes encoding the WhiB proteins and the sigma factors were amplified via PCR, the primers are listed in Table 2.4. PCR products were digested (Section 2.6.9) and ligated (Section 2.6.10) into, pKT25 between the XbaI and KpnI for *whiB* genes, and pUT18 between HindIII and KpnI for the sigma factors. As a result, each WhiB protein was cloned as an in-frame fusion at the *C*-terminal end of T25 in pKT25 and each sigma factor was sub-cloned as an in-frame fusion at the *N*-terminal end of T18 in pKT18. Plasmids were propagated in *E. coli* DH5α. Then plasmids were purified using a Qiagen miniprep kit (Section 2.6.11). The plasmids that encode the T25-WhiB and Sigma-T18 protein fusionswere used to co-transform (Section 2.4.2.2) electrocompetent *E. coli* BTH101 (JRG6956) (Table 2.1). Transformed cells were plated on LB agar medium with appropriate antibiotics (Section 2.3.3.1) and incubated at 37°C for 24 to 36 h. After a successful electroporation, one colony was picked and inoculated into 5 ml LB broth containing appropriate antibiotics and incubated at 37°C for 19h. Samples (10 µl) of the cultures were dropped onto indicator media plates (MacConkey/maltose and LB/X-gal) with the appropriate antibiotics and IPTG for induction and incubated at 30°C for 36 to 40 h. It was found to be important to inoculate the indicator plate from an overnight culture that was inoculated from a plate. For iron-starvation, salt-stress and antibiotic-stress, 0.05 mM 2,2’-Bipyridyl (dissolved in 20% ethanol) and 64 µg/ml Erythromycin, were added to the indicator media respectively.

#### **2.10.2 β-Galactosidase assay**

β-Galactosidase assays weres used to measure the protein-protein interaction quantitatively. The transformed cell cultures were incubated overnight for an OD600 0.6 to 1.0. Cultures were left on ice before 1 ml was incubated in a water bath set at 28°C for 10 min. In a new Eppendorf tube the following components were assembled:

|  |  |
| --- | --- |
|  | **Volume** |
| Transformed cell culture | 0.1 ml |
| Z-buffer | 0.7 ml |
| β-Mercaptoethanol | 1.89 µl |
| 0.1% SDS | 10 µl |
| Chloroform | 20 µl |

The preparation of Z-buffer was as follows:

|  |  |
| --- | --- |
|  | **Volume** |
| Na2HPO4.7H2O (0.06 M) | 16 g |
| NaH2PO4.H2O (0.04 M) | 5.5 g |
| KCl (0.01 M) | 0.75 g |
| MgSO4.7H2O (0.001 M) | 0.246 g |
| dH2O | 1 L |
| pH | 7.0 |

The mixture was vortexed for 10 s then and incubated at 28°C for 5 min. 200 µl of ONPG (prepared by adding 4 mg/ml in Z-buffer) was added and the reaction course was monitored with a stopped watch. Once the colour of the reaction became yellow, the reaction was stopped by adding 500 µl of 1 M Na2CO3. The reaction was centrifuged for 3 min then the absorbance of the supernatant was measured at 420 nm. β-Galactosidase activity was calculated as follows:

# ***Chapter Three***

## 3 Isolation and characterisation of bacteria from an indoor environment

### **3.1 Introduction**

Closed workplaces are important environments to measure the level of pollution that individuals are exposed to, since 90% of people’s time is spent indoors (Lee and Chang, 2000). Indoor environments contain a wide variety of bacteria which can affect air quality through air conditioning systems or humidifiers (Flannigan, 1992). Estimates of diversity of indoor microbes vary depending on the method of isolation. Therefore, many studies employed two methods of isolation i.e. passive and active monitoring, to assess the bacteria suspended in indoor air and then determined any correlations between the two methods (Napoli *et al.*, 2012). As described in Chapter 1, passive monitoring uses settle plates (Petri dishes) containing solid culture media that are exposed for a fixed period of time. Active monitoring samples airborne particles by using a suction pump to draw air into a container and then analysing the microbes collected using either liquid or solid media (Gregory, 1973; Sehulster *et al.*, 2003). Here, settle plates and a dehumidifier were employed to assess the bacteria suspended in a student lecture theatre. Dehumidifiers have been widely used to reduce air moisture and bacteria and dust are captured in the collection tank (Longo and Gasparella, 2005). Hence, the water within the collection tank contains bacteria that were present in the air within the dehumidifier’s control zone. The aim of the work reported in this chapter was to assess the bacterial diversity in the air of a lecture theatre using two different methods of isolation, active and passive monitoring, and to estimate the potential health hazards by determining the antibiotic resistance levels of the isolated bacteria.

### **3.2 Isolation of airborne bacteria from MBB-F02**

Lecture theatre F02 in the Department of Molecular Biology and Biotechnology (MBB) at the University of Sheffield (MBB-F02) was chosen to examine airborne bacteria in an indoor environment after occupation by students, staff and visitors for two months during the autumn semester of 2015. MBB-F02 was chosen because it is one of the biggest and busiest rooms in MBB. Based on the university records, at least 152 lectures and 9 seminars were delivered in F02, equivalent to ~194 h of occupation over the 2 months of the experiment. Besides, MBB-F02 has a special location being ~3-10 metres from 5 microbiology laboratories. Based on the University Biosafety committee records, those laboratories are classified as class 2 Genetically Modified Organisms (GMO) laboratories, which could potentially enhance the risk of spreading antibiotic resistant bacteria. It has been suggested that visiting health centres such as hospitals can lead to visitor infection and can be potentially risky for older people, children and most notably, immunocompromised patients (Park *et al.*, 2013). In this study, two types of aerosol sampling were conducted, one using a dehumidifier (active monitoring) and the other settle plates (open petri dishes; passive monitoring) to estimate the prevalence and identity of airborne bacteria (Section 2.4). Settle plates were left open on the benches and the dehumidifier was switched on for 1 h after the occupants had left the room. The settle-plate sampler collected 15 colonies, whereas ~250 colonies were collected from the dehumidifier over the two months of the experiment. The colonies were of varied appearance in size and colour indicating that several different bacterial species had been cultured (Fig. 3.1). This result suggests that active monitoring has the potential to detect a greater variety of species probably by virtue of sampling larger volumes of air. The data matched those of Sayer *et al.*, (1972) who claimed that, the gravity settling culture (GSC) technique can give false negative results and therefore it is not reliable for quantitative evaluation of airborne bacteria, whereas the Andersen sampler (active monitoring) detected numerous bacteria. Also Sayer *et al.* (1972) demonstrated that collecting bacteria by plate using gravity failed to detect several bacteria, such as *Staphylococcus aureus*, which were detected by Andersen sampling. In addition, it is possible that the dehumidifier captures more bacteria due to its capacity to sample air/organisms present at higher attitude (~2-3 m).

|  |  |  |
| --- | --- | --- |
| *a* | C:\Users\mbb\Desktop\IMG_20190319_123229 edited (Copy) (2).jpg | C:\Users\mbb\Desktop\IMG_20190319_123224 edited (Copy) (2).jpg |
| *b* | C:\Users\mbb\Desktop\15 NA.jpg | C:\Users\mbb\Desktop\21 NA.jpg |
| Fig. 3.1: A selection of the bacterial colonies detected by growth on (a) settle plates and (b) plating the water collected by the dehumidifier. The bacterial colonies were grown by incubation on Nutrient agar medium for 24 h at 37°C under aerobic conditions. Settle plates were opened for 1 h on a student table and the dehumidifier was run for 1 h. | | |

To investigate further the bacteria detected by passive and active monitoring, the colonies were sub-cultured. Six and seven strains were successfully sub-cultured from settle plates and dehumidifier, respectively (Fig. 3.2).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| *a* | C:\Users\mbb\Desktop\Staphylococcus hominis MBB1 (Copy).jpg MBB1 | C:\Users\mbb\Desktop\Staphylococcus saprophyticus MBB3 (Copy).jpg  MBB2 | C:\Users\mbb\Desktop\Staphylococcus haemolyticus MBB4 (Copy).jpg MBB3 | C:\Users\mbb\Desktop\Staphylococcus succinus MBB6 (Copy).gif MBB4 |
|  | C:\Users\mbb\Desktop\Pantoea ananatis MBB5 (Copy).jpg MBB5 | C:\Users\mbb\Desktop\Micrococcus yunnanensis MBB2 (Copy).jpg MBB6 |  |  |
| *b* | C:\Users\mbb\Desktop\Kocuria rhizophila MBB7 (Copy).gif MBB7 | C:\Users\mbb\Desktop\Pseudomonas fluorescens MBB8 (Copy).jpg MBB8 | C:\Users\mbb\Desktop\Sphingomonas ginsenosidimutans MBB9 (Copy).jpg MBB9 | C:\Users\mbb\Desktop\Sphingomonas yunnanensis MBB10 (Copy).gif MBB10 |
|  | C:\Users\mbb\Desktop\Novosphingobium barchaimii MBB12 (Copy).jpg  MBB11 | C:\Users\mbb\Desktop\Staphylococcus Pasteuri MBB11 (Copy).gif MBB12 | C:\Users\mbb\Desktop\Kytococcus sedentarius MBB13 (Copy).jpg  MBB13 |  |
| Fig. 3.2: Streak plates of isolates grown on Nutrient agar. (*a*) Colonies picked from settle plates (*b*) Colonies picked from plates spread with resuspended particulates present in the dehumidifier water collection tank (Fig. 3.1). Plates were incubated at 37 °C overnight. | | | | |

### **3.3 Identification of the bacterial isolates by 16S ribosomal RNA sequencing**

The visual appearance (colour/size of bacterial colonies) of the settle plate and dehumidifier isolates were different (Figs. 3.1. and 3.2). The DNA sequences of 16S rRNA genes can be used to suggest the identities of bacterial isolates (Clarridge, 2004). Therefore genomic DNA was extracted (Section 2.6.2) from liquid cultures of the 13 isolates shown in Fig. 3.2, and the conserved region of their 16S rRNA genes was amplified by PCR using universal bacteria primers (27-F and 1492-R) (Table 2.4) (Budi *et al.*, 1999) (Fig. 3.3). The DNA sequences (Section 2.6.12) of the PCR products were analysed using the BLAST programme available through the National Center for Biotechnology Information (NCBI) to identify the closest matches in the database (Table 3.1; Appendix 9.1).

|  |  |
| --- | --- |
| L 1 2 3 4 5 6 | L 7 8 9 10 11 12 13 |
| C:\Users\mbb\Desktop\gel1.JPG | C:\Users\mbb\Desktop\gel3.JPG |
| Fig. 3.3. PCR amplification of 16S rRNA genes of the isolated bacteria. Lanes L are Standard 1 kb Hyperladder; lanes 1 to 6 are the unknown microorganisms samples isolated from the settle plates (MBB1-6); lanes 7 to 13 are the unknown microorganism samples isolated from the dehumidifier trap (MBB7-13). | | |

The six species isolated by passive monitoring belonged to three families Staphylococcaceae (*Staphylococcus hominis, S. saprophyticus, S. haemolyticus* and *S. succinus*), Micrococcaceae (*Micrococcus yunnanensis*) and Enterobacteriaceae (*Pantoea ananatis*). All isolates have been reported to be common inhabitants of human environments (Novakova *et al.*, 2010; Evans *et al.*, 2019). The seven species isolated from the dehumidifier trap (active monitoring) belonged to different families, namely, Micrococcaceae (*Kocuria rhizophila*), Sphingomonadaceae (*Sphingomonas ginsenosidimutans*, *S. yunnanensis* and *Novosphingobium barchaimii*), Dermacoccaceae (*Kytococcus sedentarius*), Pseudomonadaceae (*Pseudomonas fluorescens*) and Staphylococcaceae (*S. pasteuri*). Hence it appeared that the organisms isolated from the settle plates and the dehumidifier differed and those from the latter were more diverse.

Table 3.1: Summary of 16S rRNA gene sequence analyses of oligotrophic bacteria cultured from (*a*) the settle plates, and (*b*) the dehumidifier.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Identifier | Closest match | Taxa | Gram stain  (+/-) | Sequence  Identity Percentage | NBCI Sequences ID | Reference |
| *(a)*  MBB1 | *Staphylococcus hominis* | Staphylococcaceae  Staphylococcaceae  Staphylococcaceae  Staphylococcaceae | + | 100% | LN899796.1 | (Decraene *et al.*, 2008) |
| MBB2 | *Staphylococcus saprophyticus* | + | 100% | KT720173.1 | (Decraene *et al.*, 2008) |
| MBB3 | *Staphylococcus haemolyticus* | + | 99% | KT720154.1 | (Decraene *et al.*, 2008) |
| MBB4 | *Staphylococcus succinus* | + | 100% | KJ888125.1 | (Gandolfi *et al.*, 2011) |
| MBB5 | *Pantoea ananatis* | Enterobacteriaceae | - | 99% | AP012032.1 | (Luhung *et al.*, 2018) |
| MBB6 | *Micrococcus yunnanensis* | Micrococcaceae | + | 96% | KT719656.1 | (Fang *et al.*, 2014) |
| *(b)* |  |  |  |  |  |  |
| MBB7 | *Kocuria rhizophila* | Actinobacteria | + | 99% | KC429605.1 | (Takarada *et al.*, 2008; Savini *et al.*, 2010) |
| MBB8 | *Pseudomonas fluorescens* | Pseudomonadaceae | - | 100% | KT223384.1 | (Trivedi *et al.*, 2015) |
| MBB9 | *Sphingomonas ginsenosidimutans* | Sphingomonadaceae | - | 100% | HF930756.1 | (Narciso-da-Rocha *et al.*, 2014) |
| MBB10 | *Sphingomonas yunnanensis* | Sphingomonadaceae | - | 100% | JF459957.1 | (Zhang *et al.*, 2005) |
| MBB11 | *Novosphingobium barchaimii* | Sphingomonadaceae | - | 99% | LN613112.1 | (Niharika *et al.*, 2013) |
| MBB12 | *Staphylococcus pasteuri* | Staphylococcaceae | + | 99% | KR856343.1 | (Savini *et al.*, 2009) |
| MBB13 | *Kytococcus sedentarius* | Actinobacteria | + | 98% | KC583230.1 | (Sims *et al.*, 2009) |

The most abundant bacterial taxa observed from passive monitoring matched those of a previous publication focused on the indoor microbiome (Table 3.1.*a*, Appendix. 9.1) (Decraene *et al.*, 2008; Gandolfi *et al.*, 2011; Fang *et al.*, 2014; Abiola *et al.*, 2018; Luhung *et al.*, 2018). The Staphylococcaceae are likely to be associated with human skin (Luongo *et al.*, 2017). Bacteria isolated by active monitoring (Table 3.1.*b*, Appendix. 9.1) were more likely to be water habitants such as, Sphingomonadaceae (Glaeser and Kämpfer, 2014) and Actinobacteria (Ghai *et al.*, 2014). Therefore, this study confirms the need to have an awareness of the biases that can be introduced by the method of collection and isolation in understanding bacterial communities found in indoor air.

#### **3.2.4 Screening of dehumidifier isolates for antibiotic resistance by disc diffusion tests**

Determination of bacterial susceptibility to antibiotics is crucial to manage bacterial infections (Bonev *et al.*, 2008). Therefore, it is important to screen bacteria against antibiotics and determine their effect on bacteria in the environment (Alexy *et al.*, 2004). Disc diffusion assays can be used to determine sensitivity by comparison to standards set by the British Society for Antimicrobial Chemotherapy guideline (BSAC). The isolates were tested against the antibiotics that were suggested by BSAC based on bacterial taxa (BSAC, 2013) (Section 2.3.2.1) (Table 3.2). Because the BSAC does not have standard breakpoints for *Sphingomonadaceae*, *Kocuria* spp. and *Kytococcus* spp., the non-fermentative criteria were used for *Sphingomonadaceae* and *Staphylococci* spp. criteria were used for *Kocuria* spp. and *Kytococcus* spp (Sader and Jones, 2005; Mnif *et al.*, 2006; Savini *et al.*, 2010).

The results of the disc diffusion tests on the dehumidifier isolates are shown in Table 3.2. Bacteria were screened against seven classes of antibiotics: (1) Macrolides: erythromycin; (2) β-Lactams: doripenem, imipenem, meropenem, piperacillin and piperacillin-tazobactam; (3) Tetracyclines: tetracycline; (4) Aminoglycosides: amikacin, gentamicin; (5) Glycopeptides: vancomycin; (6) Quinolones: ciprofloxacin; and (7) Colistins; polymyxin B. All the bacteria for which sensitivity criteria were available were susceptible to doripenem, imipenem and piperacillin-tazobactam, for *K. sedentarius* sensitivity to these antibiotics has not been defined (Table 3.2). *Sphingomonas ginsenosidimutans* and *Sphingomonas yunnanesis* showed an intermediate response to amikacin and resistance to gentamicin, *P. fluorescens* was resistant to meropenem and *K. rhizophlia* was resistant to ciprofloxacin. Only *K. sedentarius* was resistant to two antibiotics, ciprofloxacin and erythromycin.

In the *Sphingomonadaceae*, the resistance of *S. ginsenosidimutans* to gentamicin (aminoglycoside) was reported previously (Narciso-da-Rocha *et al.*, 2014). For *N. barchaimii*, sensitivity to all tested antibiotics including ciprofloxacin has also been reported by (Niharika *et al.*, 2013). However, to date, *Sphingomonas yunnanesis* antibiotic resistance has not been tested. Nevertheless, Vaz-Moreira et al. (2011) reported that no resistance was observed in the genera of *Sphingomonas* and *Novosphingobium*. In addition, it is generally accepted that *Sphingomonadaceae* are non-pathogenic, however, bacteria belonging to this family are abundant in hospital water systems, and some are phototrophic and chlorine-resistant (Kim *et al.*, 2007; Vaz-Moreira *et al.*, 2011). Besides that, members of Sphingomonas harbour plasmids carrying resistance genes that offering the potential for spreading resistance traits to pathogens through horizontal gene transfer (HGT) (Narciso-da-Rocha *et al.*, 2014). For example, the plasmid pNL1 that derived from *S. aromaticivorans* F199, can be transferred to a wide range of *Sphingomonas* strains. Plasmid pNL1 contains the replicase genes *repAaAb* that able to degrade xenobiotic compounds which suggests that the members of *Sphingomonas* genus are able to adapt quickly and efficiently to degrade new compounds in the environment (Basta *et al.*, 2005).

The *P. fluorescens* isolate was sensitive to all the antibiotics tested here with the exception of the β-lactam meropenem (Table 3.2). To date, no antibiotic screening tests have been reported for *P. fluorescens*. This lack of information may be due to its low pathogenicity (Leeman *et al.*, 1995).

A single resistance against ciprofloxacin was noticed for *Kocuria rhizophila*. However, Savini et al (2010) reported that *K. rhizophila* was susceptible ciprofloxacin. *K. rhizophila* has been widely used in industry as a quality control indicator in antibiotic sensitivity testing (Savini *et al.*, 2010).

Despite, *S. pasteuri* being reported to exhibit resistance to macrolides, tetracyclines, chloramphenicol, streptomycin, fosfomycin and methicillin (Savini *et al.*, 2009), no resistance was observed in this study (Table 3.2). *Staphylococcus pasteuri* causes nosocomial infections and is a blood derivatives contaminant, which suggests a role in causing human disease (Savini *et al.*, 2009).

Examination of the antibiotic sensitivity of *K. sedentarius* MBB13 showed resistance to two antibiotics ciprofloxacin (Quinolones) and erythromycin (Macrolides). The resistance of *K. sedentarius* to antibiotics has been reported previously (Marples and Richardson, 1980; Chaudhary and Finkle, 2010; Savini *et al.*, 2011). *Kytococcus sedentarius* is an opportunistic human pathogen implicated in several diseases such as Pitted keratolysis (PK) and bacteraemia (Sims *et al.*, 2009). Unexpectedly, erythromycin has been widely used to treat PK infection (Pranteda *et al.*, 2014).

In summary, seven bacteria have been isolated from air passed through a dehumidifier in MBB-F02 and tested against at least seven antibiotics. Four bacteria showed a single resistance namely *S. ginsenosidimutans*,S. *yunnanesis*, *K. rhizophila* and *P. fluorescens*, two bacteria were susceptible to all the tested antibiotics, namely *N. barchaimii* and *S. pasteuri*, and only one bacterium showed resistance to two antibiotics, *K. sedentarius*. Increasing recognition of resistance to antibiotics of *K. sedentarius* and because erythromycin is used to treat *K. sedentarius* infections, the minimum inhibitory concentration (MIC) profile of *K. sedentarius* was investigated using (BSAC, 2013) guideline for the assays.

Table 3.2: Antibiotic resistance pattern of the dehumidifier isolates.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Organism (strain ID)/ antibacterial agent | Interpretation of zone diameters (mm) | | | | Organism (strain ID)/ antibacterial agent | | | Interpretation of zone diameters | |
| mm categorization | | | | mm categorization | |
| *S.ginsenosidimutans* / (HF930756.1) (a) |  |  | | | *P.* *fluorescens* /  (KT223384.1) (b) | | |  |  |
| Amikacin  Ciprofloxacin  Doripenem  Gentamicin  Meropenem  Imipenem  Piperacillin  Piperacillin-tazobactam | 18.7±2.3  48.7±2.3  48.7±2.3  15.3±1.2  32.7±1.2  53.3±3.5  54.7±4.2  50.7±1.2 | I  S  S  R  S  S  S  S | | | Amikacin  Ciprofloxacin  Doripenem  Gentamicin  Meropenem  Imipenem  Piperacillin  Piperacillin-tazobactam | 36±0  42±2  26.7±1.2  34.7±1.2  6±0  25.3±2.3  30±0  34.7±1.2 | | | S  S  S  S  R  S  S  S |
| *S.yunnanesis* / (JF459957.1) (a) |  |  | | | *K.rhizophlia* /  (KC429605.1)(c) | | |  |  |
| Amikacin  Ciprofloxacin  Doripenem  Gentamicin  Meropenem  Imipenem  Piperacillin  Piperacillin-tazobactam | 16±4  36±1.2  50±0  16±4  31.3±4.6  55.3±1.2  58±3.5  58±2 | I  S  S  R  S  S  S  S | | | Amikacin  Ciprofloxacin  Doripenem  Gentamicin  Imipenem  Piperacillin-tazobactam  Polyxaycin-B | | 31.3±4.2  13.7±1.5  40+0  30±0  40+0  33.3±5.8  21.3±2.3 | | S  R  S  S  S  S  ND |
| *N. barchaimii* / (LN613112.1) (a) |  |  | | | *S.pasteuri* /  (KR856343.1) (c) | | |  |  |
| Amikacin  Ciprofloxacin  Doripenem  Gentamicin  Meropenem  Imipenem  Piperacillin  Piperacillin-tazobactam | 28±0  28±1.2  42.7±2.3  31.3±0  30±0  50±0  26.7±1.2  30±0 | | S  S  S  S  S  S  S  S | | Amikacin  Ciprofloxacin  Gentamicin  Chloramphenicol  Erythromycin  Tetracycline  Vanciomycin | | | 29.3±1.2  24.3±1.2  31.3±1.2  25.3±1.2  30±0  32.7±1.2  20±0 | S  S  S  S  S  S  S |
| *K.sedentarius* /  (KC583230.1) (c)  Amikacin  Ciprofloxacin  Doripenem  Gentamicin  Imipenem  Piperacillin-tazobactam  Vancomycin  Rifampin  Erythromycin  Tetracycline  Chloramphenicol | 29.3±1.7  9±0  24.3±1.5  31.7±2.1  29±1.7  10.3±1.5  32.3±3.8  39.7±5.5  9.3±0.6  27±3  20.7±1.2 | | | S  R  ND  S  ND  ND  ND  S  R  S  S |  | | |  |  |

(a) Breakpoint of non-fermentative criteria was applied for this organism. (b) Breakpoint criteria of *Pseudomonas* spp. (c) Interpretive criteria for staphylococci was applied for this organism. \* Symbols in categorization indicate sensitivity testing level as S, Susceptibility; R, resistance; I, moderate sensitivity; ND, not determined.

#### **3.2.5 Determination of minimum inhibitory concentrations (MIC) of antibiotics against Kytococcus sedentarius MBB13**

*Kytococcus sedentarius* MBB13 was the only bacterium exhibiting resistance to two antibiotics namely, ciprofloxacin and erythromycin, as judged by the disc diffusion method (Table 3.2; Appendix 9.2).

Increased resistance to antibiotics by *K. sedentarius* has been previously reported (Old and McNeill, 1979; Levenga *et al.*, 2004; Savini *et al.*, 2011; Folayan *et al.*, 2018). Besides, *K. sedentarius* is an opportunistic pathogen causing, pitted keratolysis, hemorrhagic pneumonia and valve endocarditis (Sims *et al.*, 2009).

To determine and confirm the antibiotic resistance profile of *K. sedentarius* MBB13, the MIC method was carried out using the 96-well microtiter plate assay (Section 2.3.2.2). In these tests, *K. sedentarius* MBB13 showed resistance to ciprofloxacin, erythromycin and also gentamicin (Table 3.3; Appendix. 9.3). Singh and Naik (2005) suggested that gentamicin sulphate cream is an effective treatment for Pitted keratolysis caused by *K. sedentarius*. These resistance patterns suggest that *K. sedentarius* MBB13 is a multi-resistant strain.

Table 3.3: Measurement of MICs for a range of antibiotics against *K. sedentarius* MBB13.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Antibacterial agent | MIC and interpretation | | Reference range | | |
| (mg/L) / 24 h | Categorization | R> | I | S≤ |
| Amikacin | 4 | S | 16 | 16 | 8 |
| Ciprofloxacin | 32 | R | 1 | - | 1 |
| Doripenem | 0.25 | ND | - | - | - |
| Gentamicin | 8 | R | 1 | - | 1 |
| Imipenem | 16 | ND | - | - | - |
| Piperacillin sodium salt | 256 | ND | - | - | - |
| Vancomycin | 0.25 | S | 4 | - | 4 |
| Rifampin | 0.004 | S | 0.5 | 0.12-0.5 | 0.06 |
| Erythromycin | 128 | R | 2 | 2 | 1 |
| Tetracycline | 2 | I | 2 | 2 | 1 |
| Chloramphenicol | 8 | S | 8 | - | 8 |

Interpretive criteria for staphylococci was applied for *Kytococcus sedentarius* MBB13. \* Symbols in categorization indicate sensitivity testing level as S, Susceptibility; R, resistance; I, moderate sensitivity; ND, not determined by BSAC. British Society for Antimicrobial Chemotherapy (BSAC) was used as reference guidance.

### **3.4 Discussion**

Indoor environments contain opportunistic pathogens which due to either their pathogenicity or antibiotic resistance are a potential hazard to human health and particularly to the immunocompromised (Abiola *et al.*, 2018). Different groups of bacteria were isolated from a student lecture theatre using different methods namely, passive monitoring and passage of air through a dehumidifier. As suggested in the literature, the range of microbes detected in indoor environments varies depending on the method of isolation (Napoli *et al.*, 2012). This suggested that to evaluate the presence of organisms, different methods of isolation maybe required. The methods used here only detect culturable organisms; the viable-but-nonculturable (VBNC) organisms remain undetected. Therefore, to detect the VBNC, several methods have been suggested such as, microfluidics which detect their metabolic activity and toxic proteins (Jiang *et al.*, 2014), or metagenomics that collects genes sequenced from the environment (Ghai *et al.*, 2014), or by quantitative PCR to detect their DNA (Rowan *et al.*, 2015).

The detection and isolation of a wider range of organisms from the dehumidifier water-vapour suggested that screening these organisms against a selection of common antibiotics is essential. This step is to assess the level of tolerance to communal antibiotics since pathogenic multi-resistant organisms have become a serious global public health problem (Zhu *et al.*, 2011). The seven isolated species were screened against at least seven antibiotics selected according to BCAS (2013) (Table 2.1). Two of the isolates *S. ginsenosidimutans* and *S. yunnanensis* belonged to Sphingomonadaceae showed a single resistance to amikacin and moderate resistance to gentamicin. A number of Sphingomonadaceae members including, *S. ginsenosidimutans* were isolated from hospital tap-water and had susceptibility for all antibiotics used including amikacin and gentamicin (Narciso-da-Rocha *et al.*, 2014). This result may increase the risks associated with this family since antibiotic resistance was detected here. *Kytococcus sedentarius* MBB13 showed resistance to ciprofloxacin and erythromycin. However, it has been reported that most *K.sedentarius* are susceptible to erythromycin and ciprofloxacin (Old and McNeill, 1979; Ertam *et al.*, 2005; Chaudhary and Finkle, 2010). This result may give an indication that *K. sedentarius* MBB13 is a resistant strain. Erythromycin has been widely used to treat *K. sedentarius* infections (pitted keratolysis, peritonitis and bacteraemia) (Kuskonmaz *et al.*, 2006; Chaudhary and Finkle, 2010; Pranteda *et al.*, 2014). Therefore *K. sedentarius* MBB13 was selected for further analysis. To confirm the resistance phenomenon of *K. sedentarius* MBB13, MIC assays were employed. Beside the resistance to erythromycin and ciprofloxacin, *K. sedentarius* MBB13 was also resistant to gentamicin in those tests. However, Old and McNeill (1979) reported that *K. sedentarius* was susceptible to gentamicin. Guidelines for therapy of *Kytococcus* spp. illnesses are lacking, due to the absence of interpretive guidelines for evaluating growth inhibition in the presence of antibiotics. Thus, *Staphylococci* spp. breakpoints have been used in the literature to understand behaviour under drug exposure (Mnif *et al.*, 2006; Chan *et al.*, 2012). However, this may cause diagnostic confusion in treating this genus by antibiotics.

To conclude, employing two methods (settle plates and dehumidifier) of isolating airborne bacteria produced two different groups of bacteria. Antibiotic sensitivity screening showed that one of the bacteria captured by the dehumidifier, *K. sedentarius* MBB13, was resistant to erythromycin, gentamicin and ciprofloxacin. Despite that fact that *K. sedentarius* is an opportunistic pathogen and potentially multidrug-resistant bacterium, little is known about the molecular aspects involved in the mechanisms of either in resistance to drugs or being a strictly aerobic, marine habitant (Sims *et al.*, 2009). Therefore, the *K. sedentarius* MBB13 genome was examined in the next stage of this project.

# ***Chapter Four***

## 4 Analysis of the *K. sedentarius* MBB13 genome

### **4.1 Introduction**

Several studies including the results presented in (Chapter 3) have reported that *K. sedentarius* exhibits resistance to some antibiotics (Old and McNeill, 1979; Marples and Richardson, 1980; Levenga *et al.*, 2004; Savini *et al.*, 2011; Folayan *et al.*, 2018). *Kytococcus sedentarius* has been isolated from several environments, including marine (Sims *et al.*, 2009), human blood and lung tissues (Marples and Richardson, 1980; Levenga *et al.*, 2004), indoor air (Folayan *et al.*, 2018) and Silicone Gastrostomy Devices (Dautle *et al.*, 2003), as well as human skin (Folayan *et al.*, 2018). Some strains of *K. sedentarius* are reported to produce the antibiotics, monensin A and B (e.g. strain TR-2) (Pospisil *et al.*, 1998).

*Kytococcus sedentarius* is a strictly aerobic, coccoid, gram positive actinobacterium considered to be part of commensal microflora (Sims *et al.*, 2009). However, *K. sedentarius* may also become pathogenic under certain conditions. For example, due to production of extracellular enzymes (serine proteases P1 and P2) that degrade human callus, *K. sedentarius* is implicated in pitted keratolysis (Longshaw *et al.*, 2002). It is associated with fatal haemorrhagic pneumonia for acute myeloid leukaemia patients (Levenga *et al.*, 2004), cerebral cyst infection and ventriculoatrial shunt infection for those with congenital hydrocephalus (Greene *et al.*, 1980). Moreover, a closely related strain, *Kytococcus schroeteri* is associated with postoperative spondylodiscitis and pneumonia, and in many cases was resistant to antibiotics (Shah *et al.*, 2019). Eleven human infections have been recorded due to members of *Kytococcus* (Folayan *et al.*, 2018). For example, a 55 year old neutropenic man diagnosed with myeloid leukaemia suffered from fatal haemorrhagic pneumonia associated with *K. sedentarius* (Levenga *et al.*, 2004). In another case a 7 year old boy with congenital hydrocephalus contracted a cerebral cyst and ventriculoatrial shunt infections associated with *K. sedentarius* (Greene *et al.*, 1980).

The aim was to sequence the *K. sedentarius* strain: MBB13, compare it to the DSM 20547 strain and determine the genomic features underpinning some of the typical *K. sedentarius* characteristics (respiration, antibiotic production, keratin degradation and salt tolerance). The genome of the reference strain *K. sedentarius* (DSM 20547 = 541T = ATCC 14392 = JCM 11482 = CCM 314) has been sequenced (Sims *et al.*, 2009).

### **4.2 Genome assembly**

Comparative genomics is an important process in understanding the content of bacterial genomes and to analyse phenotypic differences between strains and species, and then correlate these to differences in genome sequences (Carver *et al.*, 2005). Therefore, the Artemis Comparison Tool (ACT) (Section 2.6.2.1) was used to explore the *K. sedentarius* MBB13 genome sequence by pair-wise comparison (Edwards and Holt, 2013).

It is over 20 years since the sequencing of the first bacterial genome (Land *et al.*, 2015). DNA sequencing is classified to four generations. In brief, the first generation (Sanger sequencing or chain termination method) was developed by Frederick Sanger (1977) and determines the nucleotide sequence using DNA polymerase and labelled dideoxy nucleotides. This method can be used for sequencing short DNA fragments (Parida and Mohapatra, 2016). The second generation (next generation sequencing ‘NGS’ or massive parallel sequencing) fragments the genome into small pieces and then assembles the entire genome matching up overlapping sequences (Pérez‐Enciso and Ferretti, 2010). The third generation (long-read or single molecule sequencing) is used to sequence a single molecule, therefore breaking long strands of DNA into small segments and assembly of the DNA fragments is unnecessary (Land *et al.*, 2015; Bleidorn, 2016). The fourth generation is another single-molecule technique but nanopore-based sequencing where ultra-long, label-free, high throughput reads are possible with low amount of material (Yanxiao Feng *et al.*, 2015).

The genome of *K. sedentarius* MBB13 has been sequenced using NGS (illumina method) by MicrobesNG, University of Birmingham ([microbesng.uk](file:///C:\Users\Jeffrey\Downloads\microbesng.uk)). The genome was generated as contig files (four compressed files), which required assembly to overlap the sequence reads into one file containing the whole genome sequence (Fig. 4.1). The process takes place through the Galaxy website (<https://hactar.shef.ac.uk/galaxy/>). Contig files of *K. sedentarius* strain MBB13 were assembled using strain DSM 20547 (reference sequence) for annotation. Galaxy generates three different FASTA files which are then used by the ACT software for genome visualisation.

|  |
| --- |
|  |
| Fig. 4.1: A schematic of the genome sequence assembly of Kytococcus sedentarius MBB13. (*a*) The four contigs files are ready for assembly. (*b*) The contigs files are overlapped using the Galaxy website. (*c*) The whole-genome is generated as one readable file. |

### **4.3 Comparing the genome of *K. sedentarius* MBB13 to that of strain DSM 20547**

Pairwise reciprocal best-hit FASTA analysis was used to compare *K. sedentarius* strain MBB13 and the reference genome strain DSM 20547 (available from [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov))*.* The result summarised the relationship based on shared amino acid identity and revealed a common core set of genes (Fig. 4.2). The genome sequence of *K. sedentarius* MBB13 is 2695.4 kb in length with 97% identical to the reference genome (DSM 20547). In prokaryotes, the percentage of GC-content is 25-75%, and actinobacteria are considered as high GC (Chen and Zhang, 2003; Ventura *et al.*, 2007). *Kytococcus sedentarius* MBB13 had 71.55% of GC similar to DSM 20547 (71.63%) (Fig. 4.2 and Table 4.1). The origin of replication was located upstream of *dnaA*, and at this point the GC skew value shifts (Fig 4.3).

|  |  |
| --- | --- |
| *K. sedentarius* DSM 20547 (2,785,024 bp) | |
| C:\Users\mbb\Desktop\Genome_data\new genome2\Galaxy122-[Comparison_PNG_image] clean.png | Identical (97%) |
| *K. sedentarius* MBB13 (2,695,411 bp) | |
| Fig. 4.2: Comparison depicting linear diagram of K. sedentarius MBB13 and DSM 20547, generated by the Galaxy assembly website (<https://hactar.shef.ac.uk/galaxy>). Arrows indicate the position of contigs, red blocks indicate conserved regions and blue blocks indicate conserved regions in reverse orientation. Identity percentage measurement was generated from [blast.ncbi.nlm.nih.gov](http://www.blast.ncbi.nlm.nih.gov). | |

|  |
| --- |
| C:\Users\mbb\Desktop\Genome map\1.png |
| Fig. 4.3: Circular genome visualisation of K. sedentarius MBB13 and DSM 20547. The burgundy circle indicates the reference genome, and the green circle indicates the MBB13 genome. The %GC content is shown in an adjacent feature track (black circle). The GC skew is shown the last circle from inside (green and violet). This CGView map is generated via the online website of <http://stothard.afns.ualberta.ca/cgview_server/>. |

Table 4.1: Overall sequence composition of both sequence of *K. sedentarius* MBB13 and DSM 20547.

|  |  |  |
| --- | --- | --- |
|  | MBB13 | DSM 20547 |
| Number of bases | 2,695,411 | 2,785,024 |
| GC percentage | 71.55 | 71.63 |
| Total genes | 2499 | 2554 |
| Assigned genes | 1789 (74.63%) | 1963 (76.85%) |
| Hypothetical proteins | 710 (36.08%) | 591 (23.14%) |

The genome sequence of *K. sedentarius* MBB13 contains 2499 predicted genes. Putative functions were assigned to 74.63% of genes, while the remaining 36.08% were annotated as hypothetical proteins (Table 4.1).

### **4.4 *Kytococcus sedentarius* MBB13: relationship with oxygen**

*Kytococcus sedentarius* is described as an obligate aerobe with strictly respiratory metabolism, with some strains being capable of reducing nitrate to nitrite (Stackebrandt, *et al.*, 1995). Accordingly, *K. sedentarius* MBB13 did not grow on Luria-Bertani agar plates incubated under anaerobic conditions (Fig. 4.*a*). To test growth in liquid culture, a 5 ml aerobic cultureof *K. sedentarius* MBB13 was grown in Luria-Bertani broth at 37°C overnight and was used to inoculate anaerobic Luria-Bertani broth to a final OD600 of 0.1. After overnight incubation at 37°C to exhaust any remaining oxygen, 100 µl of this culture was transferred to either aerobic or anaerobic Luria-Bertani broth and these cultures were incubated under the corresponding conditions at 37°C overnight, at which point OD600 measurements were made (Fig. 4.*b*). The OD600 values for the aerobic cultures were four-fold greater than the anaerobic cultures, suggesting that some anaerobic growth (final OD600 0.5 compared to the initial OD600 of 0.01) had occurred.

|  |  |
| --- | --- |
| (*a*)  C:\Users\mbb\Desktop\anaerobic cond of KS\111111.JPG | (*b*) |
| Fig. 4.4: *Kytococcus sedentarius* MBB13 may require oxygen for growth. Cultures were incubated at 37°C for 18 h. (*a*) Luria-Bertani agar: left, anaerobic, right: aerobic. (*b*) Final optical densities (OD600) achieved under anaerobic and aerobic conditions. Data shown are the mean values, error bars indicate standard errors (n=3). | |

To further test any requirement for oxygen to support the growth of *K. sedentarius* MBB13, the thioglycollate broth technique was applied. Sodium thioglycollate is a reducing agent that removes molecular oxygen from the environment and maintains a low oxygen tension (Patricia, 2014). When sodium thioglycollate is present in a column of growth medium in a tube, facultative anaerobes will grow throughout the medium; strict aerobes only grow at the surface of the medium in contact with air; and strict anaerobes only grow towards the bottom of the medium (Fig 4.5). *Kytococcus sedentarius* MBB13 grew only at the top of the thioglycollate broth (Fig. 4.6). The facultative anaerobe *E. coli* exhibited growth throughout the medium. This result indicates that *K. sedentarius* MBB13 grows only aerobically.

|  |  |
| --- | --- |
| (*a*) (*b*) (*c*) | Fig. 4.5: The expected bacterial growth in thioglycollate broth for; (*a*) strict aerobe, (*b*) facultative anaerobe and (*c*) strict anaerobe. |
| C:\Users\mbb\Desktop\ss.jpg |

|  |  |
| --- | --- |
| (*a*) (*b*) | Fig. 4.6: Growth Characteristics of *K. sedentarius* MBB13 (a) and (b) E. coli in thioglycollate broth. The bacterial growth was incubated at 37 °C for 48 h. Experiment was confirmed by three repeats. |
| C:\Users\mbb\Desktop\`11111.JPG |

Interrogation of the *K. sedentarius* MBB13 genome using Biocyc (Karp *et al.*, 2017) predicted that the bacterium had the capacity to carry out 41 reactions that require molecular oxygen, including aerobic respiration, heme biosyntheisis and synthesis of unsaturated fatty acids (Table 4.2). This list suggested that further investigation of the potential metabolic modes (e.g. aerobic respiration, anaerobic respiration, fermentation) available to *K. sedentarius* MBB13 might reveal its dependence on oxygen for growth.

Table 4.2: Metabolic reactions requiring molecular oxygen that are predicted to occur in *K. sedentarius* MBB13.

|  |  |
| --- | --- |
| **Reactions that consume oxygen** | **Pathways** |
| 2 an ubiquinol[membrane] + oxygen[in] + 5 H+[in] → 2 a ubiquinone[membrane] + 2 H2O[in] + 5 H+[out] | proline or succinate to cytochrome oxidase electron transfer |
| ferroheme *o* + a reduced unknown electron carrier + oxygen → ferroheme *i* + an oxidized unknown electron carrier + H2O | heme *a* biosynthesis |
| ferroheme *i* + a reduced unknown electron carrier + oxygen → hydroxyferroheme *i* + an oxidized unknown electron carrier + H2O | heme *a* biosynthesis |
| coproporphyrinogen III + oxygen + 2 H+ → protoporphyrinogen IX + 2 CO2 + 2 H2O | heme *b* biosynthesis I (aerobic) |
| protoporphyrinogen IX + 3 oxygen → protoporphyrin IX + 3 hydrogen peroxide | heme *b* biosynthesis I (aerobic) |
| L-arginine + oxygen → 4-guanidinobutyramide + CO2 + H2O | L-arginine degradation X (arginine monooxygenase pathway) |
| L-phenylalanine + (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin + oxygen → L-tyrosine + (6R)-4a-hydroxy-tetrahydrobiopterin | L-phenylalanine degradation I (aerobic) |
| L-tryptophan + oxygen → N-formylkynurenine | L-tryptophan degradation I (via anthranilate) |
| L-aspartate + oxygen → 2-iminosuccinate + hydrogen peroxide + H+ | NAD biosynthesis I (from aspartate) |
| n-octane + 2 a reduced rubredoxin + oxygen + 2 H+ → 1-octanol + 2 an oxidized rubredoxin + H2O | octane oxidation |
| 2 an ubiquinol[membrane] + oxygen[in] + 4 H+[in] → 2 a ubiquinone[membrane] + 4 H+[out] + 2 H2O[in] | succinate to cytochrome *bd* oxidase electron transfer |
| 4-imidazolone-5-propanoate + a reduced unknown electron carrier + oxygen + H2O → 4-oxoglutaramate + ammonium + formate + an oxidized unknown electron carrier |  |
| ectoine + 2-oxoglutarate + oxygen → 5-hydroxyectoine + succinate + CO2 |  |
| a long-chain aldehyde + FMNH2 + oxygen → a long-chain fatty acid + *hν* + FMN + H2O + 2 H+ |  |
| a (5Z)-alkan-5-enoyl-CoA + oxygen → a (2E,5Z)-alka-2,5-dienoyl-CoA + hydrogen peroxide |  |
| octanoyl-CoA + oxygen → *trans*-oct-2-enoyl-CoA + hydrogen peroxide |  |
| a 2,3,4-saturated fatty acyl CoA + oxygen → a *trans*-2-enoyl-CoA + hydrogen peroxide |  |
| a decanoyl-[Acp] + oxygen → a (2E)-dec-2-enoyl-[Acp] + hydrogen peroxide |  |
| 6-*cis*-tridecenoyl-CoA + oxygen → 2-*trans*,6-*cis*-tridecadienoyl-CoA + hydrogen peroxide |  |
| 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-butanoyl-CoA + oxygen → 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-(E-buta-2-enoyl)-CoA + hydrogen peroxide |  |
| 4-*cis*-undecenoyl-CoA + oxygen → 2-trans, 4-*cis*-undecadienoyl-CoA + hydrogen peroxide |  |
| 6-*trans*-tridecenoyl-CoA + oxygen → 2-*trans*-6-*trans*-tridecadienoyl-CoA + hydrogen peroxide |  |
| propanoyl-CoA + oxygen → acryloyl-CoA + hydrogen peroxide |  |
| 4-*trans*-undecenoyl-CoA + oxygen → 2-*trans*, 4-*trans*-undecadienoyl-CoA + hydrogen peroxide |  |
| 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-octanoyl-CoA + oxygen → 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-(E-octa-2-enoyl)-CoA + hydrogen peroxide |  |
| 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-hexanoyl-CoA + oxygen → 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-(E-hexa-2-enoyl)-CoA + hydrogen peroxide |  |
| 5-*cis*, 7-*trans*-tetradecadienoyl-CoA + oxygen → 2-trans, 5-cis, 7-*trans*-tetradecatrienoyl-CoA + hydrogen peroxide |  |
| (5Z)-tetradecenoyl-CoA + oxygen → (2E,5Z)-tetradecenoyl-CoA + hydrogen peroxide |  |
| 4-hydroxybutanoyl-CoA + oxygen → 4-hydroxycrotonyl-CoA + hydrogen peroxide |  |
| (6Z,9Z,12Z,15Z,18Z,21Z)-tetracosahexaenoyl-CoA + oxygen → (2E,6Z,9Z,12Z,15Z,18Z,21Z)-tetracosaheptaenoyl-CoA + hydrogen peroxide |  |
| stearoyl-CoA + 2 a ferrocytochrome *b*5 + oxygen + 2 H+ → oleoyl-CoA + 2 a ferricytochrome *b*5 + 2 H2O |  |
| palmitoyl-CoA + 2 a ferrocytochrome *b*5 + oxygen + 2 H+ → palmitoleoyl-CoA + 2 a ferricytochrome *b*5 + 2 H2O |  |
| RH + a reduced [NADPH-hemoprotein reductase] + oxygen → ROH + an oxidized [NADPH-hemoprotein reductase] + H2O |  |
| pyridoxine 5'-phosphate + oxygen → hydrogen peroxide + pyridoxal 5'-phosphate |  |
| pyridoxamine 5'-phosphate + oxygen + H2O → ammonium + hydrogen peroxide + pyridoxal 5'-phosphate |  |
| phenylacetyl-CoA + NADPH + oxygen + H+ → 2-(1,2-epoxy-1,2-dihydrophenyl)acetyl-CoA + NADP+ + H2O |  |
| 4-hydroxyphenylpyruvate + oxygen → CO2 + homogentisate |  |
| 2-oxo-3-phenylpropanoate + oxygen → (2-hydroxyphenyl)acetate + CO2 |  |
| homogentisate + oxygen → 4-maleyl-acetoacetate + H+ |  |
| 4 a reduced *c*-type cytochrome[out] + oxygen[in] + 8 H+[in] → 4 an oxidized *c*-type cytochrome[out] + 4 H+[out] + 2 H2O[in] |  |
| 4 a reduced *c*-type cytochrome[out] + oxygen[in] + 8 H+[in] → 4 an oxidized *c*-type cytochrome[out] + 4 H+[out] + 2 H2O[in] |  |
| (6Z,9Z,12Z,15Z,18Z)-tetracosapentaenoyl-CoA + oxygen = (2E,6Z,9Z,12Z,15Z,18Z)-tetracosahexaenoyl-CoA + hydrogen peroxide |  |
|  |  |

Further analysis suggested that *K. sedentarius* MBB13 central carbon metabolism was based on glucose entering the pentose phosphate pathway to produce glyceraldehyde-3-phosphate; the glycolytic pathway was incomplete due to the absence of a gene coding for phosphofructokinase (Fig. 4.7.a). The overall pentose phosphate pathway reaction is shown in Eqn. 4.1.

3 glucose + 3 ATP + 6 NADP+ + 3 H2O → 2 glucose-6-phosphate + glyceraldehyde-3-phosphate + 3 CO2 + 6NADPH + 6 H+ [Eqn. 4.1]

Enzymes (glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase) for metabolism of glyceraldehyde-3-phosphate to pyruvate are predicted to be present.

glyceraldehyde-3-phosphate + phosphate + NAD+ + 2 ADP → pyruvate + NADH + H+ + 2ATP + H2O [Eqn. 4.2]

In many bacteria pyruvate stands at the crossroads of aerobic and anaerobic metabolism (Yasid *et al.*, 2016). Lacking pyruvate formate-lyase and pyruvate oxidase, it appears that *K. sedentarius* MBB13 was restricted to use of the pyruvate dehydrogenase complex to further metabolise pyruvate [Eqn. 4.3].

pyruvate + NAD+ + CoASH → acetyl-CoA + NADH + H+ + CO2 [Eqn. 4.3]

*Kytococcus sedentarius* MBB13 appears to have a complete Krebs cycle for oxidation of acetyl-CoA [Eqn. 4.4] and provision of biosynthetic intermediates. The presence of genes coding for phosphotransacetylase and acetate kinase suggests that acetyl-CoA can be converted to acetate with concomitant substrate level phosphorylation [Eqn. 4.5] (Fig. 4.5.b). The presence of two putative acetyl-CoA synthetase genes (*acsA\_1* and *acsA\_2*), suggest the capability to re-assimilate any excreted acetate [Eqn. 4.6].

acetyl-CoA + 3 NAD+ + FAD + phosphate + ADP → 2 CO2 + CoASH + 3 NADH + 3 H+ + FADH2 + ATP [Eqn. 4.4]

acetyl-CoA + phosphate + ADP → acetate + CoASH + ATP [Eqn. 4.5]

acetate + ATP + CoA → acetyl-CoA + AMP + PPi [Eqn. 4.6]

The reducing equivalents (NAD(P)H and FADH2) generated by the pentose phosphate pathway and Krebs cycle can be used to support biosynthesis of macromolecules or to feed an aerobic electron transport chain (Fig. 4.7.c). The *K. sedentarius* MBB13 strain possesses a non-proton translocating NADH dehydrogenase, enzymes for the biosynthesis of fully unsaturated menaquinones (MK8-12) and two alternative oxidases, cytochrome *bc1-aa3* oxidase and cytochrome *bd* oxidase; the latter being associated with aerobic respiration at low oxygen tensions (Wu *et al.*, 1996).

The Krebs cycle enzyme, succinate dehydrogenase donates electrons at the level of FADH2 to the menaquinone pool and a proline dehydrogenase (PutB/ProC) could also serve as a primary dehydrogenase in the presence of proline. Enzymes associated with anaerobic fermentative metabolism, such as pyruvate formate-lyase, hydrogenase, were not detected in the genome *K. sedentarius* MBB13. Some strains of *K. sedentarius* are able to reduce nitrate to nitrite (Sims *et al.*, 2009), a reaction which could allow nitrate to serve as an alternative terminal electron acceptor, however, the MBB13 strain did not possess genes resembling known nitrate reductases, suggesting that anaerobic respiratory growth was not possible.

Aerobic respiration is relatively energetically efficient but inevitably generates reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals, that can damage many cell components (Imlay, 2008).

The *K. sedentarius* MBB13 genome contains genes coding for sensor-regulator proteins SoxR (superoxide-responsive) and OxyR (hydrogen peroxide-responsive). The *oxyR* gene is located immediately downstream of the *ahpCF* genes, which code for an alkylhydroperoxide reductase, an enzyme involved in resistance to peroxide stress (Bsat *et al.*, 1996). By analogy with other bacteria it is likely that OxyR will activate expression of *ahpCF* in response to peroxide stress. OxyR is also likely to activate expression of catalases (*katA* and *katE*) and predicted glutathione peroxidases (*gpx1\_1* and *gpx1\_2*), since these enzymes are likely to be important in adapting to peroxide stress. SoxR is probably an activator of *sodN*, coding for a Ni-superoxide dismutase. These components of the *K. sedentarius* genome provide protection against endogenous reactive oxygen species damage associated with aerobic respiratory growth (Fig. 4.7d).

The main fatty acids of the *K. sedentarius* type strain 541 are reported to be methyl-branched *iso*-C17:1, *anteiso*-C17:0, saturated C15:0 and C17:0 (Sims *et al*., 2009). It is possible, but unlikely, that one or more of the potential oxygen-requiring lipid reactions listed in Table 4.2 are essential. However, synthesis of deoxynucleotides is essential for DNA replication. Ribonucleotide reductases (RNR) are used to catalyse the conversion of NDPs to dNDPs (Minnihan *et al.*, 2013). One class of RNR has a tyrosyl radical at its active site and generation of this radical requires molecular oxygen (Minnihan *et al.*, 2013). Another class of RNR use a vitamin B12, oxygen-independent mechanism to synthesise dNDPs (Lee *et al.*, 2012). Bacteria that lack an oxygen-independent RNR cannot grow in the absence of oxygen (Foskolou and Hammond, 2017). However, this cannot account for the inability of *K. sedentarius* MBB13 to grow anaerobically because it possesses both RNR systems (Class I, O2-dependent enzyme coded by *nrdAB* and Class II, O2-independent enzyme encoded by *nrdJ*).

From this analysis of the *K. sedentarius* MBB13 genome sequence it was concluded that the requirement of oxygen is most likely due to the absence of fermentation and anaerobic respiratory pathways, resulting in dependence on the presence of oxygen as a terminal electron acceptor.

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Fig. 4.7: Schematic representations of (a) The pentose phosphate pathway, (b) Krebs cycle, (c) alternative aerobic respiratory chains, (d) responses to detoxify reactive oxygen species. The schemes are based on analysis of genes present in the *K. sedentarius* MBB13 genome.

### **4.5 *Kytococcus sedentarius* MBB13: Salt tolerance**

*Kytococcus sedentarius* was first isolated in 1944 from a slide immersed in sea water near San Diego, USA. Since then it is has been frequently isolated from human skin and, in the context of the current study, was also identified in a culture-independent investigation of the microbial content of commercial airline cabin air (Sims *et al.*, 2009; Osman *et al.*, 2008). Its presence in the marine environment and on human skin indicates that *K. sedentarius* is salt tolerant; the type strain 541T can grow in media containing up to 10% (~1.7 M) NaCl (Sims *et al*., 2009). The optical densities of *K. sedentarius* MBB13 cultures grown for 24 h in nutrient broth supplemented with additional NaCl (nutrient broth contains 0.1 M NaCl) showed that growth negatively correlated with the increase in salinity (Fig. 4.8). Significant growth was observed when the total NaCl concentration was 0.6 M, a concentration similar to that of sea water. However, the best growth of *K. sedentarius* MBB13 was found at low salinity indicating that the strain is halotolerant (Ara *et al.*, 2013).

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| Fig. 4.8: Growth of *Kytococcus sedentarius* MBB13 in nutrient broth supplemented with different concentrations of NaCl at pH 7.5 ±0.2 after 24 h incubation at 37°C on an orbital shaker (150 rpm). OD was measured at 600 nm against a medium blank. Data points are the means of three replicates plus or minus standard deviation. |

Bacteria maintain an appropriate osmotic gradient across the cytoplasmic membrane by changing the osmotic potential of the cytosol to control water influx and efflux through aquaporins. Under conditions of high salinity, accumulation of osmoprotective compatible solutes, such as glycine betaine, proline, proline betaine, trehalose, glutamate, ectoine, carnitine, taurine and dimethylsulfonio-propionate, draws water into the cell to prevent desiccation (Wood, 2015). These compounds are excreted through mechanosensitive channels when the external osmolarity decreases (Wood, 2015). The response to high osmolarity often begins with uptake of potassium ions (K+), followed by replacement of K+ ions by one or more compatible solutes (McLaggan *et al.*, 1994). Analysis of the *K. sedentarius* MBB13 genome indicated the presence of several genes potentially involved in the response to osmotic stress (Fig. 4.9).

In *B. subtilis*, K+ ion uptake to lessen water loss during initial exposure to high salinity is mediated by two related potassium importers KtrAB and KtrCD, consisting of a homodimeric transmembrane protein (KtrB) and an associated octameric cytosolic ring protein (KtrA) (Holtmann *et al.*, 2003). Although *K. sedentarius* MBB13 possesses a *ktrA* gene, a *ktrB* candidate was not detected, suggesting that the KtrA protein has a different partner and probably does not function in gating K+ import in this bacterium (Hoffmann and Bremer, 2016).

The *E. coli* KdpFABC complex (K+-dependent ATPase) is another K+ transporter, encoded by the *kdpFABC* operon and is an alternative route to scavenge K+. KdpD is a membrane bound sensor kinase and KdpE is a cytosolic response regulator and comprise a typical prokaryotic two component signal transduction system. Upon stimulus, KdpD undergoes autophosphorylation and the phosphoryl group is transferred to the regulator KdpE. KdpE binds with high affinity at the *kdpFABC* promoter when in its phosphorylated and dimerized form, and triggers *kdpFABC* transcription (Schniederberend *et al.*, 2010). In *K. sedentarius* MBB13, KdpFABC complex and its regulators are not detected (Fig. 4.9).

Ectoine is another major compatible solute in many halophiles such as, *Chromohalobacter salexigens*, *Methylomicrobium alcaliphilum* and *Bacillus pasteurii*. Ectoine can be taken up from the environment when available. Ectoine uptake is mediated by the Ect ABC transporter, which is encoded by the *ectA*, *ectB*, and *ectC* genes. In *M. alcaliphilum*, a MarR-like transcriptional regulator (EctR1) represses the expression of the *ectABC-ask* operon by binding at the putative -10 sequence (Mustakhimov *et al.*, 2010). The *K. sedentarius* MBB13 genome possesses a MarR-like regulator and *ectABC* operon but does not contain the gene *ask* (Fig. 4.9).

Compatible solutes can be obtained from the environment or synthesised in the bacterial cytosol. Glycine betaine and choline uptake is mediated by the ABC-type transporter OpuC, which is encoded by the *opuBA-opuCD-opuCB-opuCC-lcdH* operon in the *K. sedentarius* MBB13 genome. In addition, *opuD* and *betL*, coding for Betaine/Carnitine/Choline Transporter (BCCT) family transporters, and *proP*, coding for a broad specificity Major Facilitator Superfamily (MFS) proline transporter, complete the catalogue of compatible solute uptake systems (Fig. 4.9). In *Listeria monocytogenes*, *betL* was rapidly expressed in response to an osmotic up-shift (Sleator *et al.*, 2003) and ProP acts as an osmosensor and osmoregulator, permitting it to respond rapidly to changes in osmolarity (Culham *et al.*, 2003).

Choline is a precursor of glycine betaine, conversion being catalysed by two enzymes (choline dehydrogenase, EC 1.1.91.1 and an aldehyde dehydrogenase, EC 1.2.1.8). In *E. coli*, these enzymes are encoded by the *betAB* operon (Wood, 2006). Although a *betB* gene was identified in the *K. sedentarius* MBB13 genome, *betA* was not. Gram-positive bacteria use an alcohol dehydrogenase (GbsB, EC 1.1.1.1) (Dandekar *et al.*, 1999; Wood *et al.*, 2001), and plants use a choline monoxygenase (EC 1.4.14.7) to effect the conversion of choline to betaine aldehyde (Hibino *et al.*, 2002), but no similar genes were detected in the *K. sedentarius* MBB13 genome.

The *K. sedentarius* MBB13 *putB-proC* operon codes for enzymes allowing catabolism of proline to glutamate (see above). *Kytococcus sedentarius* MBB13 also possesses the *otsAB* genes, coding for trehalose biosynthesis enzymes (Fig. 4.9). Following osmotic down-shifts the accumulated compatible solutes need to be released to minimise water influx and *K. sedentarius* MBB13 has two mechanosensitive channels (*mscL* and *ykuT/mscS*) to fulfil this function.

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| Fig. 4.9: Systems involved in osmotic adaptation by *K. sedentarius* MBB13. Analysis of the genome sequence identified transporters, channels and biosynthetic pathways that could contribute to tolerating high salinity. Membrane proteins involved in release or uptake of and cytoplasmic enzymes catalyzing the compatible solutes, glycine betaine, ectoine, glutamate and trehalose are shown. The catabolism (PutB-PutC) of L-proline is shown. MscS and MscL are mechanosensitive channels which are a transient gating systems to release compatible solutes in response to an osmotic down-shock, preventing cell lysis; they release non-specific organic compounds and water-attracting ions to reduce the cytoplasm osmotic potential and curb water influx. Glycine betaine synthesis from the precursor choline taken up by the osmotically inducible OpuC transporter [*opuCA-opuCB-opuCC-opuCD*] in response to high salinity and the BetB for glycine betaine synthesis. And EctABC for ectoine biosynthesis are shown. The operon *otsAB* allows synthesis of trehalose. These systems were identified based on their homologs in *Bacillus* spp. (Kuhlmann and Bremer, 2002; Schroeter *et al.*, 2013). |

To determine which of these potential osmoprotective systems were deployed by *K. sedentarius* MBB13 when cultures were grown in the presence of different NaCl concentrations, proton NMR analysis of cell extracts was carried out (Section 2.3.4.3) (Fig. 4.10). The 1H NMR spectra showed that glycine betaine increased significantly as the NaCl concentration increased (Kim *et al.*, 2017). Proline appeared to increase once the concentration of NaCl was raised above 1.0 M (Kim *et al.*, 2017). A species with chemical shift of ~4.1 ppm indicated that ectoine appeared only when the NaCl concentration was greater than 2.0 M. Ecotine peaks were also present at 2.2. ppm and between 1.0-1.5 (Nagata *et al.*, 1996; Khmelenina *et al.*, 1999; Ongagna-Yhombi and Boyd, 2013). Acetate at ~2.0 ppm (Krucker *et al.*, 2004) and lactate at ~1.3 ppm (Wang *et al.*, 2017) appeared from 0.5 M NaCl. Chemical shifts at ~3.7 ppm are likely be glutamate and appeared between 1.0-1.5 M NaCl (Kim *et al.*, 2017).

The analysis of the *K. sedentarius* MBB13 genome and the experiments presented here suggest that glycine betaine is the major compatible solute, but proline, glutamate, and ectoine also contribute to salt tolerance.

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| C:\Users\mbb\Desktop\NMR WITH Ectoine.jpg |
| Fig. 4.10: 1H NMR spectra of cell extracts derived from *K. sedentarius* MBB13 at 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 NaCl (M) in nutrient broth (NB) medium (containing 0.1 M NaCl). The spectra shown are equivalent to ~10 OD600 units of bacteria without additional NaCl; ~8 OD600 units of bacteria for cultures supplemented with 0.5, 1.0 or 1.5 M NaCl; ~6 OD600 units of bacteria for cultures supplemented with 2.0 M NaCl; and 0.2 OD600 units of bacteria for cultures supplemented with 3.0 M NaCl. The 1H spectra identified glycine betaine as the most abundant compatible solute, and acetate, lactate, glutamate, ectoine and proline also accumulated. Solutes indicated by dashes. |

### **4.6 *Kytococcus sedentarius* MBB13: keratinase production**

*Kytococcus sedentarius* has been reported to cause pitted keratolysis (Nordstrom *et al.*, 1987; Ertam *et al.*, 2005; Singh and Naik, 2005). Some strains produce proteases P1 and P2 that are able to degrade keratin (Longshaw *et al.*, 2002). The ability of *K. sedentarius* MBB13 to degrade keratin azure and release blue dye in the medium was tested (Section 2.3.4.2) (Fig 4.11). It appeared that *K. sedentarius* MBB13 was unable to degrade keratin. Keratinases in bacteria have been studied intensively and showed that, the active site was located in *N*-terminal of keratinases genes (Gupta and Ramnani, 2006; Brandelli *et al.*, 2010), but no similar active sites were detected in the *K. sedentarius* MBB13 genome.

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| (*a*)  *C:\Users\mbb\Desktop\keratin azure\cut\smaller size\9 days SK (Copy).jpg* | (*b*)  *C:\Users\mbb\Desktop\keratin azure\cut\smaller size\9 days P. PUTIDA (Copy).jpg* |
| (*C*)  *C:\Users\mbb\Desktop\keratin azure\cut\smaller size\9 days E.COLI (Copy).jpg* |  |
| Fig. 4.11: Keratin azure test plates following 9 days incubation. The blue dye released surrounded the bacterial growth for positive reactions. (*a*) *Kytococcus sedentarius* MBB13, (b) *Pseudomonas putida* KT2440 (positive control) (*c*) *Escherichia coli* DH5α (negative control). Discs were dipped into overnight culture of the tested strains and placed by sterile forceps in the middle of the LB-keratin azure agar. | |

### **4.7 *Kytococcus sedentarius* MBB13: monensin A and B production**

Complex reduced polyketides are natural products that are produced by modular enzyme biosynthesis systems termed polyketide synthases (PKSs). At least 20 antibiotics are derived from PKSs such as, monensin, erythromycin and lovastatin (Yuzawa *et al.*, 2018).

Monensin (A and B) are polyether ionophore antibiotics and produced by *Streptomyces cinnamonensis* ST021, and mainly used to treat coccidiosis in poultry (Tang *et al.*, 2017). They are also of interest because they promote cell apoptosis and inhibit proliferation of human cancer, including myeloma, neoplasia, glioma, lymphoma and breast cancer (Tang *et al.*, 2017). Over 20 genes are required for monensin biosynthesis, and the *mon* cluster has been characterised from *S. cinnamonensis*. The monensin biosynthesis genes were classified into 4 groups, type I polyketide synthases (PKSs), type II thioesterases, post-PKS tailoring enzymes and putative pathway specific regulators (Table 4.3). The presence of activators such as MonH, MonRI and MonRII are essential for high production of monesin (Tang *et al.*, 2017).

Table 4.3: Cluster genes involve in the production of monesin A and B in *S. cinnamonensis* ST021.

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| Gene type | Gene name |
| type I polyketide synthases (PKSs) | MonAI to MonAVIII |
| type II thioesterases | MonCII, MonAIX, and MonAX |
| post-PKS tailoring enzymes | MonBI, MonBII, MonCI, MonCII, and MonD |
| putative pathway specific regulators | MonH, MonRI, and MonRII |

\* Data adapted from (Tang *et al.*, 2017)

It has been reported that *K. sedentarius* TR-2 is a producer of monensin A and B. Matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy detected the presence of a mixture of monensin A and B , however no further genetic details were shown (Pospisil *et al.*, 1998). Investigation of the genomes of *K. sedentarius* strains MBB13 and DSM 20547 (Section 2.5.2.1), showed that, the *mon* cluster genes were not present. Therefore, both the *K. sedentarius* MBB13 and DSM 20547 are not monensin producers.

### **4.8 *Kytococcus sedentarius* MBB13: *wbl* genes and sigma factors**

Actinobacteria are the largest taxonomic unit among the 18 major lineages within the Bacteria domain (Ventura *et al.*, 2007). One characteristic of the Actinobacteria is the possession of Wbl (WhiB-like) proteins (Bush, 2018). The first Wbl to be identified was WhiB from *Streptomyces* *coelicolor*, which was shown to co-control the cessation of aerial hyphal growth (Bush, 2018). It is now known that *S. coelicolor* has 14 Wbl proteins and *M. tuberculosis* has 7, with most Actinobacteria possessing more than one Wbl protein. Wbl proteins are small (~80–140 residues) and possess a [4Fe–4S] cluster coordinated by four invariant cysteine residues, along with a conserved five-residue G[V/I]WGG motif, predicted to mediate protein-protein interactions (Bush, 2018). The iron-sulfur clusters are thought to act as redox sensors in controlling diverse biological processes, such as morphogenesis, metabolism, virulence and antibiotic resistance (Miller and Auerbuch, 2015).

The Wbl proteins of *M. tuberculosis* have been extensively studied. WhiB1 is an essential nitric oxide-responsive transcription regulator (Smith *et al*., 2010; Kudhair *et al*., 2017). WhiB2 is also essential and depletion of WhiB2 leads to filamentous growth and aberrant septation and thus likely has a role in coordinating cell division (Gomez and Bishai, 2000). WhiB3 has an oxygen- and nitric oxide-sensitive [4Fe-4S] cluster and its expression is up-regulated at low pH and inside macrophages (Geiman *et al*., 2006; Rhodes *et al*., 2007; Singh *et al*., 2007). It is thought that WhiB3 is involved in mediating redox homeostasis when *M. tuberculosis* is in a host phagosome and thus is implicated in virulence and persistence (Singh *et al*., 2009). Like WhiB3, the iron-sulfur cluster of WhiB4 is sensitive to oxygen and nitric oxide. WhiB4 likely regulates the response to oxidative stress because deletion of *whiB4* resulted in over-induction of anti-oxidant activities and enhanced resistance to oxidative stress (Chawla *et al*., 2012). The *M. tuberculosis whiB5* mutant was attenuated in a mouse model of infection, was unable to resume growth following a chronic infection and was more sensitive to nitric oxide than the parental strain (Casonato *et al*., 2012). WhiB6, is implicated in pathogenesis through its regulation of the virulence critical Type VII secretion system ESX-1 and aspects of aerobic and anaerobic metabolism (Chen *et al*., 2016). WhiB7 controls multi-drug resistance by regulating the expression of several antibiotic resistance genes (Burian *et al*., 2013). Thus, WhiB7 is required for diversity of antibiotics resistance with different targets and structures such as lincosamides, pleuromutilins, phenicols, macrolides, tetracyclines and some aminoglycosides. It has been suggested that the antibiotic resistance genes, *tap*, *erm* and *eis* are also a part of the WhiB7 regulon. WhiB7 is thought to bind DNA through an AT-hook motif in the *C*-terminal region (Bush, 2018).

The overall picture that emerges from the study of *M. tuberculosis* Wbl proteins is that they primarily function as transcription regulators. With the exception of WhiB7, which possesses an AT-hook motif (see above), the other Wbl proteins lack a recognisable DNA-binding motif and interaction with DNA is likely to be mediated by their positively charged C-terminal regions (Smith *et al*. 2012). This raises questions about how Wbl proteins could target specific promoters to regulate gene expression. At least part of the answer is thought to be that Wbl proteins interact with other proteins, including RNA polymerase sigma factors. Thus, WhiB1, WhiB3 and WhiB7 interact directly with σA and WhiB2 and WhiB6 interacted with σA in a bacterial two-hybrid assay (Feng *et al*., 2016). Thus, it was of interest to catalogue the sigma factors of *K. sedentarius* MBB13. Analysis of the *K. sedentarius* MBB13 genome revealed the presence four sigma factors (σA, σB, σR and σJ) (Table 4.4; Fig 4.12.*b*). In *M. tuberculosis*, the housekeeping sigma factor, σA, binds to core RNA polymerase during exponential growth phase and is the major sigma factor present in the cell. Under stress conditions alternative sigma factors replace σA to permit expression of different suites of genes allowing the bacterium to adapt to the new environmental conditions. Thus, σB is involved in adaptation to nutrient starvation and stationary phase (Sachdeva *et al*., 2010); σH (σR) plays a role in the response to oxidative stress and heat shock (Raman *et al*., 2001); and σJ is implicated in survival in host macrophages (Sachdeva *et al*., 2010).

Table 4.4: Best match of Wbl protein and Sigma factors families of *K. sedentarius* MBB13 to those in *Mycobacterium tuberculosis*.

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| Superfamily | Gene | Proposed function | Identity (%) |
| *Wbl* | *whiB1* | • Involved in growth, dormancy and reactivation | 73 |
| *whiB2* | • Involved in cell division | 87 |
| *whiB3* | • Involved in redox balance and virulence | 61 |
| *whiB7* | • Involved in redox balance, antibiotic resistance and virulence | 61 |
| *Sigma factors* | *SigmaA*  *(HrdB)* | • RNA polymerase sigma-70 factor | 89 |
|  | *SigmaB* | • Sigma-B/F/G subfamily | 35 |
|  | *SigmaR(*or *H)* | • ECF RNA polymerase sigma factor | 69 |
|  | *SigmaJ* | • RNA polymerase sigma-70 family | 39 |

\*Proposed functions are adapted from blast.org. \*If no gene name next to the bacterium, blast did not detect any.

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| *(a)* Fe-s cluster domain(N-terminal) DNA binding (C-terminal)  KS whiB1 ------------------MDWRSKAACLDEDPELFFPIGNT-GPA-IEQIEKAKKVCARCEVTETCLQWAIETGQDAGVWGGLSEDERRALKRRNA--RARRAS---  KS whiB2 ----VHELQIVGHPEQAVPSWQERALCAQTDPEAFFPEKGG-S------TREAKRVCTGCDVRSECLDYALENDERFGIWGGLSERERRKLKRRAV--FTA------  KS whiB3 ----MDSTARQPGPVADLWDWQFEGLCRTTDPEEFFHPEGERGSARRLRDERAKRVCQRCPVILECREHALAAKEPYGVWGGLSEDEREQELARRS---RRGLRGA-  KS whiB7 ----MLALADHQTLIDQARVAGRPLPCLENDPDTWFADTPT-G------VEYAKSLCHACPVRTLCLEGALERREPWGVWGGELIEAGRVLPRKRPRGRPRKHPIAA    Mtb whiB1 ------------------MDWRHKAVCRDEDPELFFPVGNS-GPA-LAQIADAKLVCNRCPVTTECLSWALNTGQDSGVWGGMSEDERRALKRRNA--RTKARTGV-  Mtb whiB2 LVPEAPAPFEEPLPPEATDQWQDRALCAQTDPEAFFPEKGG-S------TREAKKICMGCEVRHECLEYALAHDERFGIWGGLSERERRRLKRGII-----------  Mtb whiB3 ----MPQPEQLPGPNADIWNWQLQGLCRGMDSSMFFHPDGERGRARTQREQRAKEMCRRCPVIEACRSHALEVGEPYGVWGGLSESERDLLLKGTM-GRTRGIRRTA  Mtb whiB7 -------VSVLTVPRQTPRQRLPVLPCHVGDPDLWFADTPA-G------LEVAKTLCVSCPIRRQCLAAALQRAEPWGVWGGEIFDQGSIVSHKRPRGRPRKDAVA-  \* \* . :\* . \*\* :\* \* : \* \*: : \*:\*\*\*  Variable region functional region |
| *(b)* ND1  Mtb SigA ------------------------------------------------------------------------------------------------------  KS SigA VTPPTSEKSAETPTVSEPVTAVLGKQLLDKPGLIEFVERAVDNGKVATDKVQQAIEGASLTPTQAQRLLKNLRSQGVEVHFDQETAARLAQEQRGVKPRASR  ND1    Mtb SigA VAAT-----KASTATDEPVKRTATKSPAASASGAK-TGAKRTAAKSASGSPP-AKR—-ATKPAARSVKPASAPQDTTTSTIPKRKTRAAAKSAAAKAPSARG  KS SigA SRTTRSTTAKTATAKSTTAKSTATKSTTAKSTAAKSTTAKSTAAKSTAAKSTTAKSTTAKSTAAKSTTAASGTSAKGT-ASTTRKATTAAKGTAAKSTTAKG  :\* \*::\*\*.. .\* \*\*\*\*\* :\*.::.\*\* \* \*\* \*\*\*\*\*::.. \*\* \*.. \*\*:\*.. \*\*. . . \* : .\*\*: :\*\*\*.:\*\*\*: :\*:\*  ND1  Mtb SigA HATKPRAPKDAQHEAATDPEDALDSVEELDAEPDLDVEPGEDLDLDAADLNLDDLEDDVAPDADDDLDSGDDEDHEDLEAEAAVAPGQTADDDEEIAEPTEK  KS SigA RTAAKGTTKSAAE---TNARNA------AADAPELDEIAAEDEAEERAA-----------------LA-GK—-D------TVELVAGETSQAAPQKAESTEE  ::: : \*.\* . \*: .:\* \*:\*\* .\*\* : \* \* \*. \* . :. \*:\*:: : \*\* \*\*:  ND1  Mtb SigA DKASGDFVWDEDESEALRQARKDAELTASADSVRAYLKQIGKVALLNAEEEVELAKRIEAGLYATQLMTELSERGEKLPAAQRRDMMWICRDGDRAKNHLLE  KS SigA ESESRGFVLRADDEDDA-PAQQVVTAGATADAVKDYLKQIGKVALLNAEQEVDLAKRIEAGLFAEQRL----NSGDKIDAKLKRELWWVASDGKNAKNHLLE  :. \* .\*\* \*:.: \*:: . \*:\*\*:\*: \*\*\*\*\*\*\*\*\*\*\*\*\*\*:\*\*:\*\*\*\*\*\*\*\*\*:\* \* : : \*:\*: \* :\*:: \*:. \*\*..\*\*\*\*\*\*\*  ND1 ND2  Mtb SigA ANLRLVVSLAKRYTGRGMAFLDLIQEGNLGLIRAVEKFDYTKGYKFSTYATWWIRQAITRAMADQARTIRIPVHMVEVINKLGRIQRELLQDLGREPTPEEL  KS SigA ANLRLVVSLAKRYTGRGMLFLDLIQEGNLGLIRAVEKFDYTKGYKFSTYATWWIRQAITRAMADQARTIRIPVHMVEVINKLARVQRQMLQDLGREPTPEEL  \*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\* \*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*.\*:\*\*::\*\*\*\*\*\*\*\*\*\*\*\*\*  ND2 LD CD    Mtb SigA AKEMDITPEKVLEIQQYAREPISLDQTIGDEGDSQLGDFIEDSEAVVAVDAVSFTLLQDQLQSVLDTLSEREAGVVRLRFGLTDGQPRTLDEIGQVYGVTRE  KS SigA AKELDMTPEKVVEVQKYGREPISLHTPLGEDGDSEFGDLIEDSEAVVPSDAVSFTLLQEQLHSVLDTLSEREAGVVSMRFGLADGQPKTLDEIGRVYGVTRE  \*\*\*:\*:\*\*\*\*\*:\*:\*:\*.\*\*\*\*\*\*. :\*::\*\*\*::\*\*:\*\*\*\*\*\*\*\* \*\*\*\*\*\*\*\*\*:\*\*:\*\*\*\*\*\*\*\*\*\*\*\*\*\* :\*\*\*\*:\*\*\*\*:\*\*\*\*\*\*:\*\*\*\*\*\*\*  CD  Mtb SigA RIRQIESKTMSKLRHPSRSQVLRDYLD  KS SigA RIRQIESKTMSKLRHPSRSQVLRDYLD  \*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\* |
| Fig. 4.12: Alignment of Wbl and major sigma factor A proteins between *K. sedentarius* MBB13 (KS) and *M. tuberculosis* H37Rv (Mtb) amino acid sequences. (*a*) The conserved cysteines residues and unique G(I/V)WGG domain (tryptophan containing) of Wbl proteins are highlighted in green and yellow respectively, and AT-hook of Wbl7 is in blue. (*b*) The Sigma factor N-terminal 1 and 2, linker and C-terminal domains are indicated as ND1, ND2, LD and SD respectively. *M. tuberculosis* H37Rv was obtained from [ncbi.nlm.nih.gov](file:///C:\Users\Jeffrey\Downloads\ncbi.nlm.nih.gov) (RefSeq NC\_000962.3). |

### **4.9 *Kytococcus sedentarius* MBB13: Antibiotic resistance**

#### **4.9.1 Ciprofloxacin resistance**

Antibiotic sensitivity screening indicated that *K. sedentarius* MBB13 was resistant to ciprofloxacin, a quinolone family antibiotic that targets DNA gyrase. Several mechanisms for ciprofloxacin resistance have been reported, including expression of multi-drug efflux pumps, mutations in drug uptake systems and missense mutations in GyrA, GyrB, ParC and ParE (Hooper, 1999). The *K. sedentarius* MBB13 DNA gyrase is encoded by the *gyrB\_1-gyrA\_1* operon (Hooper, 1999). In several bacteria, ciprofloxacin resistance is linked to GyrA mutations of Ser (S112 in *K. sedentarius* MBB13 GyrA) and/or Asp (D116 in *K. sedentarius* MBB13) in the highly conserved quinolone resistance motif HPHGDSAVYD motif (Hooper, 1999). Replacement of Ser by Leu or Ile results in resistance (Hooper, 1999), but this motif is conserved in the *K. sedentarius* MBB13 GyrA. Mutations within amino acid motifs associated with ciprofloxacin resistance have also been recognized in GyrB. For example, D495R or D495H mutations result in ciprofloxacin resistance in *M. tuberculosis* (Hooper, 1999). However, the GyrB motifs that are altered in ciprofloxacin resistant strains are intact in *K. sedentarius* GyrB. Therefore, it was concluded that the observed ciprofloxacin resistance is not due to a resistant mutant DNA gyrase.

DNA gyrase can be regarded as a secondary quinolone target and resistance is often associated with mutations in *parC* and/or *parE*, which code for Topoisomerase IV, which is homologous to DNA gyrase and is required for decatenating newly replicated chromosomes. The *parC* and *parE* genes were annotated as *gyrA\_2* and *gyrB\_2* in the *K. sedentarius* MBB13 genome are located on opposite strands of the chromosome and are separated by three genes (*atoE*, *yokF* and *yhdG\_2*). ParC-mediated quinolone resistance is associated with mutations analogous to those described above for GyrA. *Kytococcus sedentarius* MBB13 ParC has a Thr residue in place of the highly conserved Ser residue of the HPHGDSAVYD motif (see above), whether this could contribute to ciprofloxacin tolerance is as yet unknown, because a systematic analysis has not been reported. ParE mutations associated with quinolone resistance are associated with mutations at the positions equivalent to 492 and 530 in *K. sedentarius* MBB13 GyrB\_2 (ParE), involving replacement of an Asp residue by Asn and an Asn residue by Asp (Hooper, 1999). However, at both these positions in *K. sedentarius* MBB13 ParE the ‘sensitive’ amino acids are present. Thus, it was concluded that ciprofloxacin resistance was not associated with mutations in ParC-ParE.

Quinolone resistance in *M. smegmatis* has been linked to over-expression of the major facilitator superfamily (MFS) efflux pump LfrA (Liu *et al.*, 1996), but a similar protein was not encoded by the *K. sedentarius* MBB13 genome. The MFS efflux pumps NorA, Bmr and Blt contribute to quinolone resistance in the Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis* (Hooper, 1999). The *K. sedentarius* MBB13 genome encodes a protein (annotated as Bmr3) with 31% identity, 53% similarity over 490 amino acids to the *B. subtilis* Bmr3 protein and 37% identity, 55% similarity over 458 amino acids to the *S. cyanogenus* LanJ protein, which effluxes the antibiotic landomycin, preventing killing of the producer strain. Thus, it is possible that Bmr3 contributes to ciprofloxacin resistance by pumping the drug out of the cytoplasm. This could be tested in the future by constructing a *K. sedentarius* MBB13 deletion mutant and retesting sensitivity.

In conclusion, analysis of the genome sequence suggests that the observed ciprofloxacin resistance is probably mediated through the action of efflux pumps, possibly involving over-expression of *bmr3*.

#### **4.9.2 Erythromycin resistance**

Resistance to erythromycin is conferred by methylation of a specific adenine base in the 23S rRNA by enzymes encoded by *erm* genes, or by plasmid borne efflux pumps (*msr* genes), or by mutations in ribosomal proteins L4 (RplD) or L22 (RplV). The genetic information available for *K. sedentarius* MBB13 only includes the chromosome, so no information on any potential plasmid borne genes is available. RplV linked erythromycin resistance is associated with a 9 base pair deletion that removes a 3 amino acid sequence - M82, K83 and R84 (*E. coli* numbering; (Chittum and Champney, 1994); these amino acids are present in *K. sedentarius* MBB13 RplV. RplD-mediated resistance again arises from several mutations, e.g. K63E, G69A, T70P and R168S (*E. coli* numbering), but none of these mutations are present in the *K. sedentarius* MBB13 RplD. Erythromycin resistance in many bacteria can also be conferred by base substitutions in the 23S rRNA (G2057A, A2058G, A2059C/G; *E. coli rrlA* numbering); however none of these mutations are present in the *K. sedentarius* MBB13 genome (Vester and Douthwaite, 2001). Furthermore, the *K. sedentarius* MBB13 genome contains no close matches to the *S. aureus emrC* gene, which encodes a methyltransferase that methylates A2058 of the 23S rRNA resulting in resistance to macrolide antibiotics (Vester and Douthwaite, 2001). The best match was *rsmA* (*ksgA*) at 20% identical, 43% similar over 244 amino acids. KsgA di-methylates 16S rRNA during maturation of the small RNA subunit and thus is unlikely to methylate 23S rRNA to provide resistance to erythromycin. Thus, this analysis of the *K. sedentarius* MBB13 genome sequence has not identified the mechanism underpinning the observed erythromycin resistance, but has ruled out some possibilities and suggests that perhaps over-expression of efflux pumps is likely to be involved.

### **4.10 Discussion**

Although *K. sedentarius* is an opportunistic pathogen, very little is currently known about the molecular basis of factors contributing to its properties. In this chapter, the genetic knowledge of the isolate *K. sedentarius* MBB13 was extended by performing comparative analysis of the genome. First of all, the genome revealed a high similarity percentage to the reference with 97% with some gaps in the image of the genome alignment (Fig 4.2), the gaps reflect genes missing or acquired which could occur through exposure to environmental factor changes as a HGT process (Soucy *et al.*, 2015).

*In vivo* tests and genome investigation of *K. sedentarius* MBB13 have also been studied in this chapter. The results of this study indicate that fermentation and anaerobic respiratory pathways are absent which suggests the reason it is a strict aerobe even with the presence of RNR. Besides that, proposing the cytochrome *c* pathway that consist of a cytochrome *bc1-aa3* oxidase and cytochrome *bd* oxidase, based on genomics indicate its dependence on oxygen. Despite the fact that some strains of *K. sedentarius* reduce nitrate to nitrite (Sims *et al.*, 2009) but *K. sedentarius* MBB13 may not reduce nitrate due to absence of nitrate reductases resembling genes.

NMR results indicate that glycine betaine is the main compatible solute compound, other solutes were also observed such as ectoine, proline, glutamate, acetate and lactate (Fig 4.7). Up to date, this is the first demonstration of genes involved in compatible solutes that compared the genome data of *K. sedentarius*.

It has been claimed that *K. sedentarius* strain M17C that was isolated from an infected human foot is able to degrade keratin due to the presence of keratinase enzymes (Longshaw *et al.*, 2002). However, *K. sedentarius* strain MBB13 genome does not possess the genes involved in keratin degradation and did not degrade keratin azure (Fig 4.9). This finding suggests that not all *K. sedentarius* strains are keratin degraders including MBB13. Additionally, genome of strain M17C was unavailable online.

*Kytococcus sedentarius* is considered as a new producer of monensin antibiotic A and B. In *S. cinnamonensis*, the *mon* cluster was identified and gene inactivation suggested that two genes are important for the monesin production (*monH* and *monRI*). In *K. sedentarius* both strains MBB13 and DSM 20547 but not TR-2, the cluster was not identified. Up to date, TR-2 genome is still unavailable to investigate the production of monesin either by the bioinformatics tools or *in vivo* experiments.

To conclude this chapter, *K. sedentarius* MBB13 is strictly aerobic most likely due to due to the absence of fermentation and anaerobic respiratory pathways. The strain of *K. sedentarius* MBB13 is unable to degrade keratin. *Kytococcus sedentarius* MBB13 grows well under conditions with up to 0.6 M NaCl, most likely due to the presence of the following compatible solutes, glycine betaine, proline, glutamate and ectoine. Genome of *K. sedentarius* MBB13 does not have MonH, MonRI and MonRII, therefore is most likely unable to produce monesin. *Kytococcus sedentarius* MBB13 possesses four Wbl proteins with high similarity to those in *M. tuberculosis*. Wbl proteins are transcription factors some of which bind to the major sigma factor (σA) (Lipeng Feng *et al.*, 2016). Therefore, the characterization of the *K. sedentarius* MBB13 Wbl proteins was undertaken as described in the next chapter.

# ***Chapter Five***

## 5 Isolation and biochemical characterization of *K. sedentarius* MBB13 WhiB1 and WhiB2 proteins

### **5.1 Introduction**

The White-B-like (Wbl) family of proteins was discovered in *Streptomyces coelicolor* (Hopwood *et al.*, 1970), and were genetically examined by Chater (1972) because mutants exhibited impaired production of aerial spores leading to white instead of grey colonies. Wbl proteins are found only within the actinomycetes and likely act as transcription regulators (Bush, 2018; Section 4.8). Wbl proteins have been isolated from a number of actinomycetes (Bush, 2018), and the Wbl proteins from *Mycobacterium tuberculosis* were isolated as [4Fe-4S]-, [2Fe-2S]- and apo- forms. After protein reconstitution, only the [4Fe-4S]- forms were found (Alam *et al.*, 2009; Burian *et al.*, 2013; Kudhair *et al.*, 2017). *Kytococcus sedentarius* MBB13 has four genes coding for Wbl proteins (*whiB1, whiB2, whB3* and *whiB7*) (Section 4.8). The biochemical and biophysical properties of Wbl proteins have been studied after isolation of recombinant proteins expressed in *Escherichia coli*. More recently *M. tuberculosis* WhiB1 was overproduced in *Mycobacterium smegmatis* under the control of the inducible acetamidase promoter (Daugelat *et al.*, 2003). This approach facilitated the isolation of a stable holo-WhiB1 protein, allowing the first structure of a Wbl family member to be solved (Kudhair *et al*., 2017). In this chapter overproduction of *K. sedentarius* MBB13 WhiB1 and WhiB2 proteins in *M. smegmatis* MC2155 is reported along with some of the properties of the purified proteins.

The *whiB1* gene of *M. tuberculosis* is essential and is thought to act as a specific NO-sensing DNA-binding protein, the effect of NO on *M. tuberculosis* is concentration dependent; high levels can kill but low levels promote entry into the latent state (Kudhair *et al*., 2017). Therefore reprogramming gene expression by WhiB1 could contribute to developmental adaptations when host generated NO is encountered (Smith *et al.*, 2010). In *Corynebacterium glutamicum*, deletion of *whcE* (homolog of *whiB1*) increases sensitivity to oxidative stress and heat (Bush, 2018). In *Streptomyces*, *wblE* (homolog of *whiB1*) is essential but more investigation is still required (Bush, 2018).

The *whiB2* of *M. tuberculosis* is also essential for growth (Gomez and Bishai, 2000). The *Mycobacteriophage* TM4 has a gene, *whiBTM4*, coding for a paralog of WhiB2 that when expressed in *M. smegmatis* results in the same phenotype as a *whiB2* conditional mutant (Rybnicker *et al*., 2010). The *Streptomyces* WhiB2 (*whiB*) controls aerial growth and sporulation through a mechanism involving an unrelated DNA-binding protein WhiA (Bush, 2018). Also it has been reported that WhiB2 responds to several stresses that affect the cell envelope, such as antibiotics and SDS, suggesting that WhiB2 could be important in the early stages of infection (Konar *et al.*, 2012).

The *K. sedentarius* MBB13 WhiB1 and 2 proteins retain the *N*-terminal cysteine cluster and the G(I/V)WGG domain in the *C*-terminal (Fig 5.1). The *K. sedentarius* WhiB1 protein is 73% identical to *M. tuberculosis* WhiB1, 65% identical to *S. coelicolor* WblE, 83% identical to *C. glutamicum* WhcE and 74% identical to *M. smegmatis* WhiB1. The *K. sedentarius* WhiB2 protein is 76% identical to *M. tuberculosis* WhiB2, 85% identical to *S. coelicolor* WhiB, 85% identical to *C. glutamicum* WhcD, 77% identical to *M. smegmatis* WhiB2 and 53% identical to *Mycobacteriophage* TM4 WhiBTM4. But both WhiB1 and WhiB2 of *K. sedentarius* MBB13 have not been previously characterized.

The aim of this work was to better understand *K. sedentarius* MBB13 WhiB1 and WhiB2 by determining the properties of the proteins and establish whether they operate as O2- and/or NO-sensors.

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| (*a*)  SC WblE MDNWREHAACRTEDPDLFFPIGTTGPAALQTEQAKAVCRTCPVQEQCLRWALDTGQTLGV 60  KS WhiB1 -MDWRSKAACLDEDPELFFPIGNTGPAIEQIEKAKKVCARCEVTETCLQWAIETGQDAGV 59  CG WhcE -MDWRHEAICREEDPELFFPVGNSGPALAQIASAKMVCNRCPVTSQCLAWALETGQDAGV 59  Mtb WhiB1 -MDWRHKAVCRDEDPELFFPVGNSGPALAQIADAKLVCNRCPVTTECLSWALNTGQDSGV 59  MS WhiB1 -MDWRHKAVCRDEDPELFFPVGNSGPALAQIADAKLVCNRCPVTTECLSWALESGQDAGV 59  :\*\* .\* \* \*\*\*:\*\*\*\*:\*.:\*\*\* \* .\*\* \*\* \* \* \*\* \*\*:::\*\* \*\*  SC WblE WGGTSELERRALKRREAARRRSG---- 83  KS WhiB1 WGGLSEDERRALKRRNARARRAS---- 82  CG WhcE WGGMSEDERRALKRRKNRGRGRARIAV 86  Mtb WhiB1 WGGMSEDERRALKRRNARTKARTGV-- 84  MS WhiB1 WGGMSEDERRALKRRNARTKARTGV-- 84  \*\*\* \*\* \*\*\*\*\*\*\*\*: : |
| (*b*)  MP WhiBTM4 -------------------------------------------------------MHMHM 5  KS WhiB2 ---------------------------------------------VHELQIVGHPEQAVP 15  SC WhiB -------------------------------------------MTELVQQLLVDDADEEL 17  CG WhcD MEDSA--------------GDVSA--KLKAGQT------RTAL----EMTLDDLFGAVEQ 34  Mtb WhiB2 -----------------------------------------MVPEAPAPFEEPLPPEATD 19  MS WhiB2 MSYESGDFDRVVRFDNRLLGSVSHAPHIDTGSTPTGAAGRPQLSLVPDSF-DVAPEAEED 59      MP WhiBTM4 GGDPSAICAQTDPELWFPDKGQSTRDAKRMCMRCPLLDECRALALRDPHLVGVWGGLSAQ 65  KS WhiB2 SWQERALCAQTDPEAFFPEKGGSTREAKRVCTGCDVRSECLDYALENDERFGIWGGLSER 75  SC WhiB GWQERALCAQTDPESFFPEKGGSTREAKKVCLACEVRSECLEYALANDERFGIWGGLSER 77  CG WhcD EWQEQALCAQTDPEAFFPEKGGSTREAKRICQGCPVRDECLEFALEHDERFGIWGGLSER 94  Mtb WhiB2 QWQDRALCAQTDPEAFFPEKGGSTREAKKICMGCEVRHECLEYALAHDERFGIWGGLSER 79  MS WhiB2 QWQERALCAQTDPEAFFPEKGGSTREAKRICQGCEVRDACLEYALAHDERFGIWGGLSER 119  : \*:\*\*\*\*\*\*\* :\*\*:\*\* \*\*\*:\*\*::\* \* : \* \*\* . . .\*:\*\*\*\*\* :  MP WhiBTM4 ERRRIRKGASA-- 76  KS WhiB2 ERRKLKRRAVFTA 88  SC WhiB ERRRLKKAAV--- 87  CG WhcD ERRRLKREIS--- 104  Mtb WhiB2 ERRRLKRGII--- 89  MS WhiB2 ERRRLKRGII--- 129  \*\*\*:::: |
| Fig. 5.1: Sequence alignments of *K. sedentarius* MBB13 (KS) (a) WhiB1 and (b) WhiB2 to their homologs from *Mycobacterium tuberculosis* H37Rv (Mtb), *Mycobacteriophage* TM4 (MP), *Streptomyces coelicolor* A3(2) (SC), *Corynebacterium glutamicum* MB001 (CG) and *Mycobacterium smegmatis* MC2 155 (MS). Protein sequences were obtained from [ncbi.nlm.nih.gov/protein/](https://www.ncbi.nlm.nih.gov/protein/), ID numbers and percentage identity of genes can be found in Appendix 9.7. Identical residues are marked (\*), strongly similar residues are marked (:) and weak similar residues are marked (.). Alignments were thought MultAlin tools (Corpet, 1988). |

### **5.2 Overproduction of *K. sedentarius* MBB13 WhiB1 and WhiB2 in *M. smegmatis* MC2 155**

*Mycobacterium smegmatis* and *K. sedentarius* are both actinobacteria (Sims *et al.*, 2009; Bush, 2018). Kudhair *et al.* (2017) showed that, WhiB1 isolated after expression in *M. smegmatis* is more O2 stable than recombinant WhiB1 purified from *E. coli*. Therefore, *M. smegmatis* MC2 155 was used for overexpression of the *K. sedentarius* MBB13 *whiB1* and *whiB2*.

Overproduction of WhiB1 and WhiB2was carried out after transforming *M. smegmatis* MC2 155 (Section 2.4.2.2) (Table 2.2). The encoded proteins have a *N*-terminal His6-tag followed by a tobacco etch virus (TEV) protease tag and are expressed under the control of the acetamidase promoter (Fig 5.2). Cultures of transformed *M. smegmatis* MC2 155 cells were grown to OD600 ~1.0 then induced with 0.2% acetamide. Incubation was continued 12 h for protein overproduction (Section 2.8.3). The cell-free extracts contained high levels of WhiB1 and WhiB2 in the soluble fractions (Fig 5.3). Purification of WhiB1 and WhiB2 was carried out by affinity chromatography (Section 2.8.5). Cell lysates of *M. smegmatis* were prepared by suspending the cell pellets in binding buffer (20 mM NaH2PO4, pH 7.4 or 500 mM NaCl, 50 mM Tris, pH 8.0), and then sonicating and clarifying the extracts by centrifugation (Section 2.8.4). Elution of both Wbl proteins from HiTrap chelating columns was achieved by application of linear imidazole gradient. WhiB1 containing fractions were dark brown, unlike WhiB2 which were colourless. The purity of both proteins were confirmed by SDS-PAGE (Fig 5.4) for WhiB1 and (Fig 5.5) for WhiB2.

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| (*a*) |
| or  *whiB1* gene  249 bp  *whiB2* gene  267 bp  NcoI-HF  HindIII-HF  HindIII-HF  NcoI-HF  HindIII  *whiB2* gene  267 bp |
| **C:\Users\mbb\Desktop\Capture.JPG** |
| (*b*)  MKHHHHHHPSAGENLYFQGA-WhiB1 or WhiB2  His6-tag TEV cleavage site |
| Fig. 5.2: Schematic diagram illustrating the construction of pMyNT-based expression plasmids for *K.* *sedentarius* MBB13 *whiB1* and *whiB2*. (a) The *whiB1* and *2* of *K. sedentarius* MBB13 were amplified by PCR after editing the primers by adding restriction enzymes (NcoI and HindIII) as indicated. (*b*) The plasmids encode either His6-TEVCS-WhiB1 or His6-TEVCS-WhiB2. |

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|  | L 1 2 3 4 |  | L 5 6 7 8 9 10 |
| ~72 kDa  ~24 kDa  ~8 kDa | **C:\Users\mbb\Desktop\IMG_20171012_103110 w1.jpg** | 72 kDa  ~24 kDa  ~8 kDa | **C:\Users\mbb\Desktop\2333.jpg** |
| Fig. 5.3: Overproduction of *K. sedentarius* MBB13 WhiB1 and WhiB2 proteins from *M. smegmatis* MC2155. The images show Coomassie blue-stained SDS-PAGE gels. Lanes L, protein ladder (sizes in kDa are indicated); lanes 1 and 2, extracts from uninduced cultures expressing WhiB1, soluble and insoluble fractions, respectively; lanes 3 and 4, extracts from induced cultures expressing WhiB1, soluble and insoluble fractions, respectively; lanes 5 and 6, irrelevant proteins; lanes 7 and 8, extracts from uninduced cultures expressing WhiB2, soluble and insoluble fractions, respectively; lanes 9 and 10, extracts from induced cultures expressing WhiB1, soluble and insoluble fractions, respectively. The locations of the overproduced soluble proteins are indicated by the red ellipses. | | | |

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| (*a*) | |
| (*b*) | 1 2 3 4 5 6 7 8 |
| 72~ kDa  24~ kDa  8~ kDa |  |
| Fig. 5.4: Isolation of *K. sedentarius* MBB13 WhiB1 from *M. smegmatis* MC2 155. (*a*) HiTrap chelating column of elution profile of cell lysate containing WhiB1, the blue trace shows the protein absorbance and the red trace shows the concentration of imidazole from 0-0.5 M. (*b*) Coomassie stained SDS-PAGE gel of the elution profile fractions. Lane 1, protein ladder (sizes are indicated); lanes 2-3, non-specific bound proteins usually containing high histidine contents; lanes 4-8, fractions containing recombinant WhiB1. | |

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| (*a*) | |
| (*b*) | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 |
| 72~ kDa  24~ kDa  8~ kDa | **C:\Users\mbb\Desktop\w2.jpg** |
| Fig. 5.5: Isolation of *K. sedentarius* MBB13 WhiB2 from *M. smegmatis* MC2 155. (*a*) HiTrap chelating column of elution profile of cell lysate containing WhiB2, the blue trace shows the protein absorbance and the red trace shows the concentration of imidazole from 0-0.5 M. (*b*) Coomassie blue-stained SDS-PAGE gel of the elution profile fractions. Lane 1, protein ladder (sizes indicated); lanes 2-7, non-specific bound proteins; lanes 8-15, fractions containing recombinant WhiB2. | |

### **5.3 Biochemical and biophysical characterisation of recombinant WhiB1**

#### **5.3.1 UV-visible spectroscopy**

The WhiB1 protein fractions that eluted from the HiTrap chelating column were brown and colourless (Fig 5.4.*b*). The strong absorbance at 420 nm in the UV-visible spectrum of protein fraction (lane 6 in Fig 5.4.*b*) indicated the presence of a [4Fe-4S] cluster in the purified WhiB1 protein (Fig 5.6, purple line). In contrast, the spectrum of protein fraction (lane 7 in Fig 5.4.*b*) lacked absorbance at 420 nm, suggesting that the WhiB1 in this fraction was in the apo-form (Fig 5.6, green line). A [2Fe-2S] cluster is normally associated with peaks at 350 nm and 550-650 nm (Rybniker *et al.*, 2010), which are absent in WhiB1. Kudhair *et al.* (2017) reported that overproduction of WhiB1 in *M. smegmatis* yields only the [4Fe-4S] form, unlike overproduction in *E. coli* which resulted in three forms [4Fe-4S]-, [2Fe-2S]- and apo- during aerobic purification (Smith *et al.*, 2012).

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| Fig. 5.6: UV-visible spectroscopic analysis of different fractions of WhiB1 eluted from HiTrap chelating chromatography. Purple trace shows the spectrum of fraction 6 (60 µM WhiB1; Fig 5.4.*b*). The absorbance at 420 nm indicates the presence of a [4Fe-4S] cluster. The green trace shows the spectrum of fraction 7 (37 µM WhiB1; Fig 5.4.*b*). The absence of detectible features in the visible region of the spectrum suggests that this fraction contained apo-WhiB1. |

#### **5.3.2 Stability of [4Fe-4S] WhiB1 cluster in the presence of oxygen**

The UV-visible spectrum of the isolated WhiB1 showed only one peak at 420 nm (Fig 5.6). This peak normally indicates the presence of [4Fe-4S] cluster. Iron-sulfur clusters are often sensitive to oxidation in air (Smith *et al.*, 2012). To test the stability of the WhiB1 [4Fe-4S] in the presence of air, O2-saturated buffer was added to anaerobic sample of holo-protein such that the final concentration of O2 was 110 µM (Section 2.9.3). The UV-visible spectra were recorded before and after O2 exposure (Fig 5.7). After 12 h of incubation under these conditions, no changes in 420 nm region of the spectrum were seen, suggesting that WhiB1 has an O2-stable [4Fe-4S] cluster, consistent with data reported by Kudhair *et al.* (2017).

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| Fig. 5.7: Oxygen insensitivity of [4Fe-4S] WhiB1. The spectrum of blue and red traces were recorded at 0 and 12 h respectively after exposure to air-saturated buffer (50 mM Tris, 0.5 M NaCl, pH 7.4, 5% glycerol) with a final concentration of 110 µM of O2. Reaction was incubated at 25°C. The final concentration of the protein after exposure to air-saturated buffer was ~19 µM. |

#### **5.3.3 WhiB1 reacts with Spermine NONOate, a source of nitric oxide (NO)**

NO functions as a cytotoxin that is generated in macrophages during the first immune response against microbe invasion. Due to its chemistry, it readily undergoes redox and ligand binding reactions (Crack and Le Brun, 2019). Several transcription factors that contain Iron-sulfur clusters are known to respond to NO as well as O2 (Smith *et al.*, 2010; Kudhair *et al.*, 2017). Reactions were carried out using the NO donor (Spermine NONOate) as described in Section 2.9.4. WhiB1 (58 µM) was exposed to 571.8 µM of Spermine NONOate for 6 h under anaerobic conditions. The UV-visible spectrum showed a feature centred at 420 nm (the [4Fe-4S] absorbance) before treatment. After addition of the spermine NONOate, an increase in absorbance at 350-360 nm and decrease at 420 nm were recorded with isosbestic point at 400 nm (Fig 5.8). This result suggested that a dimeric dinitrosyl-iron-cysteine complex had been formed (Fig 5.9) (Kudhair *et al.*, 2017; Crack and Le Brun, 2019).

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| Fig. 5.8: UV-visible spectra of holo-WhiB1 before and after exposure to Spermine NONOate. Spectra of WhiB1 (58 µM cluster) before (blue trace) and after (red trace) 6 h incubation in the presence of 571.8 µM Spermine NONOate. |

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| Monomeric dinitrosyl-iron-cysteine complex | Dimeric dinitrosyl-iron-cysteine complex |
| Fig. 5.9: Diagram showing dinitrosyl-iron-cysteine (DNIC) complexes. | |

#### **5.3.4 Circular dichroism (CD) spectroscopy of WhiB1**

As previously described, WhiB1 can be isolated in [4Fe-4S]- or apo- forms, therefore CD spectra were obtained to better understand the effect of the cluster on WhiB1 secondary structure. The samples were dialysed in 20 mM NaH2PO4, 0.1 M NaCl, pH 7.4 in order to decrease the salt in the buffer (high concentrations of salt can disturb the spectroscopy reading Appendix 9.10). The desalting was carried out using the vivaspin 5000 MWCO column. Both protein states (holo- and apo-) were analysed to compare their secondary structures (Fig 5.10). The difference was detected clearly using far UV-CD through a negative molar ellipticity between ~204-220 nm of apo- and holo- which indicate presence mostly random coil and α-helix respectively (Nelson and Cox, 2013). These results indicate that the WhiB1 protein structure depends on the presence of the [4Fe-4S].

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| Fig. 5.10: CD analysis of secondary structure of holo- and apo-WhiB1. The spectra of both samples (proteins concentration ~35 µM) were recorded in 20 mM NaH2PO4, 0.1 M NaCl, pH 7.4 at room temperature. Holo-protein (blue trace) shows a typical α-helix spectrum, while red trace shows a much stronger feature at 204 nm, suggesting the presence of increased content of random coil. |

#### **5.3.5 The iron-sulfur cluster is essential for folding of WhiB1**

As judged by the CD analysis, the presence of the cluster in WhiB1 changed structure of the protein significantly (Fig 6.10). Apo- and holo-WhiB1 were also analysed using 1D proton NMR spectroscopy. To prepare apo-WhiB1, holo- protein (~20 µM) was treated with 1.5 M guanidinium thiocyanate and incubated at room temperature for 12 h (Section 2.9.5). Then holo-WhiB1 was refolded (Fig 5.11). Unlike *M. tuberculosis* WhiB4 that showed resistance to guanidine hydrochloride (Alam *et al.*, 2007). For the 1D 1H proton NMR spectra preparation, both samples were dialysed against 25 mM NaH2PO4, 0.25 M NaCl, pH 7.0 and the spectra were obtained by Dr. Andrea Hounslow (NMR facility, the University of Sheffield).

1D 1H proton NMR is a method to qualitatively measure whether a protein is folded or not. If the protein is folded, the signals are narrow, sharp, well dispersed and cover a large range of chemical shifts, however, poor signals, with broader peaks indicate the protein is unfolded. This is due to the different protein proton environment, in a unfolded protein the protons occupy many different environments leading to broad signals, however, when the protein folds, the protons are more constrained and the chemical shifts reflect the environments occupied by the protons, leading to sharper, well-defined signals (Kwan *et al.*, 2011).

As expected, the holo-WhiB1 showed sharp peaks covering a broad range of chemical shifts (Fig 5.12.*a*) especially in the area between 7-10 ppm. Apo-WhiB1 (Fig5.12.*b*) showed poor signals, with broader peaks in the same region, 7-10 ppm. This result indicated that, the Iron-sulfur cluster is important for protein folding. Cysteine (Cys) residues are important for iron-sulfur cluster acquisition (Smith *et al.*, 2012), thus, NMR was used to detect the Cys residues. Same sample of holo-WhiB1 used in Fig 6.11.*a*, was recorded in the presence of 100% D2O at 800 MHz (Fig 5.11.*c*). Area between 10-17 ppm was maximised and showed peaks likely to be Cys residues associated with the iron-sulfur cluster (Kudhair *et al.*, 2017).

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| Fig. 5.11: Time-course of WhiB1 [4Fe-4S] loss during denaturation with 1.5 M guanidinium thiocyanate monitored by UV-spectroscopy. Holo-WhiB1 (~20 µM) was treated with 1.5 M guanidinium thiocyanate and incubated at room temperature for 12 h. Spectra were obtained at the indicated times. |

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| (*a*) |  | Fig. 5.11: 1D 1H-NMR of WhiB1. Spectra were obtained using proteins prepared in 25 mM NaH2PO4 (pH 7.0) containing 0.25 M NaCl. (*a)* apo- and *(b*) holo-WhiB1 (*c*) Holo-WhiB1 spectrum in 100% D2O 25 mM NaH2PO4, 0.25 M NaCl and (pH 7.0). The chemical shifts of the potential iron-sulfur ligands (cysteine residues) are arrowed. |
| (*b*) |  |
| (*c*) | C:\Users\mbb\Desktop\1111111.JPG |

### **5.4 Biochemical and biophysical characterisation of recombinant WhiB2**

#### **5.4.1 UV-visible spectrum of WhiB2**

Fractions eluted from the HiTrap chelating column when extracts containing recombinant WhiB1 were coloured (see above). In contrast, fractions containing WhiB2 were colourless, indicating that purified WhiB2 lacked an iron-sulfur cluster (apo-WhiB2) (Fig 5.4.*b*). This was confirmed by UV-visible spectroscopy, which showed the absence of features ([4Fe-4S], 420 nm; [2Fe-2S], 405, 500, 550 and 600 nm) (Khoroshilova *et al.*, 1997). This suggests that the WhiB2 iron-sulfur cluster is O2-sensitive.

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| Fig. 5.12: UV-visible spectrum of WhiB2 eluted from HiTrap chelating chromatography. The trace shows a peak at 280 nm arising from the aromatic amino acids present in WhiB2 (34 µM). Features associated with [4Fe–4S] cluster (420 nm) or [2Fe–2S] cluster (405, 500, 550 and 600 nm). |

#### **5.4.2 Holo-WhiB2 can be reconstituted in vitro**

The ability of WhiB2 to acquire an iron-sulfur cluster was tested by carrying out an *in vitro* reconstitution reaction under anaerobic conditions (Section 2.9.2). UV-visible spectra were recorded at several points during the reaction (Fig 5.13.*a*). During anaerobic reconstitution, a brown colour developed over time and an increase in a broad absorbance at ~420 nm, typical for [4Fe-4S] containing proteins was observed. The spectrum showed other features between ~500-700 nm which probably indicate the formation of [2Fe-2S]. It was been suggested previously that fully reconstituted by Wbl proteins contain [4Fe-4S] clusters but [2Fe-2S] forms can be observed (Alam *et al.*, 2009). To confirm the formation of [2Fe-2S] clusters during the reconstitution of WhiB2, absorption changes at 420 and 600 nm were plotted against time. As shown in (Fig 5.13.*b* and *c*), the cumulative increase in absorbance at 420 ([4Fe-4S]) and 600 nm ([2Fe-2S]) increased with similar kinetics. Therefore, it might be suggested that the [2Fe-2S] form of WhiB2 is an intermediate in the assembly of the [4Fe-4S] cluster and the reconstitution reactions were incomplete.

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| (*a*) |  |
| (*b*) |  |
| (*c*) |  |
| Fig. 5.13: UV-visible spectra during reconstitution of the iron-sulfur cluster of WhiB2. (*a*) Spectra of WhiB2 iron-sulfur cluster formation over 15 h. Reaction was carried out anaerobically inside the anaerobic cabinet using closed screw capped quartz cuvette containing WhiB2, 34 µM; FeNH4(SO4)2, 0.4 mM; NifS, 2 µg; DTT, 10 mM and L-cysteine, 0.2 mM. (*b* and *c*) The absorbance change at 420 and 600 nm, respectively, over time during the reconstitution reaction. \* Readings were normalised. | |

#### **5.4.3 The WhiB2 iron-sulfur cluster is** **O2-sensitive**

The UV-visible spectrum of the reconstituted WhiB2 showed peaks at 420 and ~600 nm indicating the presence of [4Fe-4S]- and [2Fe-2S]-forms (Khoroshilova *et al.*, 1997). To test the stability of these iron-sulfur clusters when exposed to air, the screw cap of the cuvette containing anaerobic reconstituted (protein concentration ~25 µM) was opened. UV-visible spectra were recorded at several time points (Fig 5.14). The cluster associated peaks decreased by 50% in the first 10 h. The protein then precipitated after losing the cluster suggesting that some intermolecular disulphide bonds formed between the apo-WhiB2 molecules (Rybniker *et al.*, 2010). Although, the *S. coelicolor* WhiD (homolog of *M. tuberculosis* WhiB3) [4Fe-4S] cluster reacts with O2 to generate a [2Fe-2S] form (Jakimowicz *et al.*, 2005), as observed for *M. tuberculosis* WhiB1 (Smith *et al*., 2010) there was no evidence for a [2Fe-2S] intermediate in the O2-mediated disassembly of *K. sedentarius* WhiB2.

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| Fig. 5.14: UV-visible spectra of cluster loss from reconstited-WhiB2. Protein concentration after reconstitution ~25 µM. Screw capped quartz cuvette containing WhiB2 was left open to expose the protein to air. UV-visible spectra of the reconstituted [4Fe-4S]-WhiB2 showing cluster breakdown after exposure to air. Arrow indicates the direction of change. |

#### **5.4.4 WhiB2 reacts with Spermine NONOate**

As mentioned before, the iron-sulfur clusters of Wbl proteins react with NO (Smith *et al.*, 2010; Kudhair *et al.*, 2017). *In vitro* reconstitution was carried out for apo-WhiB2 (~25 µM) (Section 2.9.4), using closed screw-capped quartz cuvette inside the anaerobic cabinet. Spermine NONOate (571.8 µM) was injected into the protein solution using a Hamilton syringe with microlance needle (0.8 x 40 mm) and the treated protein was left for 2 h anaerobically. Before addition of Spermine NONOate, the UV-visible spectrum showed a feature centred at 420 nm (the [4Fe-4S] absorbance) and features at longer wavelengths suggesting the presence of a [2Fe-2S] form. After addition of the Spermine NONOate, an increase in absorbance at ~360 nm and decrease at 420 nm were recorded with isosbestic point at 400 nm (Fig 5.15). As observed in WhiB1, the result suggested the formation of dimeric dinitrosyl-iron-cysteine complex had occurred (Fig 5.9) (Kudhair *et al.*, 2017; Crack and Le Brun, 2019).

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| Fig. 5.15: UV-visible spectra of WhiB2 before and after treatment with Spermine NONOate. WhiB2 (~25 µM cluster) before (blue trace) and after (red trace) 2 h incubation in the presence of 571.8 µM Spermine NONOate. The arrows indicate the direction of the absorbance changes. The treated trace was normalised to the untreated readings. |

#### **5.4.5 Liquid chromatography mass spectrometry (LC-MS) study of WhiB2**

Mass spectrometry has been used to show that several iron-sulfur proteins are capable of retaining cluster sulfide when their iron-sulfur clusters are disassembled in the presence of oxygen (Ibrahim *et al.*, 2015). LC-MS was used to investigate whether WhiB1 and WhiB2 also exhibited this behaviour. The LC-MS spectra were collected by Mr. Simon Thorpe, (Spectrometry centre, the University of Sheffield) and the samples were prepared as described in Section 2.8.8. The major peak in WhiB2 spectrum was a species with a molecular mass of 12344 Da, ~4 Da less than the predicted molecular weight (12347.79 Da), equivalent to apo-WhiB2 with two intra-molecular disulphide bonds. A second peak at 12375 Da was 31 Da greater than the mass of apo-WhiB2 indicating the retention of one cluster sulfide (S = 32 Da) (Fig 5.16.*a*). A third species 17 Da larger than oxidsed apo-WhiB2 remains unassigned. For WhiB1, the major species had a molecular weight of 11526 Da, equivalent to reduced apo-WhiB1 (11525.96 Da) (Fig 5.16.*b*). Two minor species 16 and 54 Da larger than apo-WhiB1 were not equivalent to retention of either cluster sulfide or iron atoms.

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| (*a*) |
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| (*b*) |
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| Fig. 5.16: LC-MS analysis of WhiB2 and WhiB1. Protein samples (55 and 70 µM cluster) for WhiB2 and WhiB1 respectively, in 50 mM Tris, 0.5 M NaCl, 8.0 pH were applied the presence of 0.1 % formic acid to LC instrument for 10 min before mass analysis. (*a*) The peak at 12344 Da shows the oxidised monomeric apo-WhiB2, and the peak marked S° represents the addition of one sulfur atom retained by apo-WhiB2. (*b*) The peak at 11526 Da represents the monomer of apo-WhiB1 in its reduced form. |

### **5.5 Discussion**

In this chapter, *in vitro* biochemical and biophysical characteristics of *K. sedentarius* WhiB1 and WhiB2 iron-sulfur cluster have been studied. Isolation and purification of *K. sedentarius* WhiB1 and WhiB2 were achieved for the first time. Overexpression of both proteins was done using *M. smegmatis*.

Overproduction of WhiB1 protein in *M. smegmatis* produced two different form of the protein, one with a [4Fe-4S]- and the other being an apo-form as judged by UV-visible spectra (Fig 5.6). Overproduction of WhiB1 in *E. coli* which adopted three forms [4Fe-4S]-, [2Fe-2S]- and apo- (Smith *et al.*, 2010).

Overproduction of WhiB2 on the other hand was different as the protein was isolated only as the apo- form (Fig 5.12). These data are consistent with a previous study of *M. tuberculosis* WhiB2, which has an O2–sensitive iron-sulfur cluster (Konar *et al.*, 2012). *In vitro* reconstitution of the WhiB2 iron-cluster under anaerobic conditions indicated that [4Fe-4S] and [2Fe-2S] clusters were formed (Fig 5.14). Generally, the seven Wbl proteins of *M. tuberculosis* were found to contain traces of a [2Fe-2S] cluster after aerobic isolation but when reconstituted anaerobically, only the [4Fe-4S] cluster forms were found (Alam *et al.*, 2009). For *M. tuberculosis* reconstituted-WhiB1 it was reported that, occurrence of [2Fe-2S] form was due to the incomplete acquisition of iron and the [2Fe-2S] form was not observed by UV-visible spectroscopy when [4Fe-4S] WhiB1 was exposed to O2 (Smith *et al.*, 2010). Similarly, *S. coelicolor* the [4Fe-4S] cluster of WhiD (homolog ofWhiB3) that is degraded after exposure to O2 without passing through a [2Fe-2S] reaction intermediate (Crack *et al.*, 2009).

*Kytococcus sedentarius* WhiB1 has a [4Fe-4S] cluster that is stable even after prolonged incubation in air. Analysis of both proteins WhiB1 and WhiB2 by LC-MS (Fig 5.16) indicate that, WhiB1 did not retain sulfur adducts upon the degradation of cluster. Unlike, WhiB2 which retained up to an extra of one sulfur adducts attached to the apo- form upon the dissociation of the cluster. Those data suggest that WhiB1 under aerobic conditions with absence of stabilizing tag or a partner protein loses its [4Fe-4S] forming an intermediate cluster form which is different from WhiB2, which then suggest a different roles in each *K. sedentarius* WhiB protein.

Proton 1D NMR spectrum of WhiB1 showed that the [4Fe-4S] cluster is essential for the protein’s structure (Fig 5.11). Also, the CD spectra of holo-WhiB1 demonstrates that the cluster changed the property of the protein in comparison to the apo- form (Fig 5.10). Similar observations suggested that, holo-WhiD of *S. Streptomyces* is similar to holo-WhiB1 (Crack *et al.*, 2009). And the structure of the reduced and oxidized of apo- form of *M. tuberculosis* WhiB1, WhiB2, WhiB3, WhiB4 and WhiB7 showed a large proportion of disordered structure (Alam *et al.*, 2007; Alam *et al.*, 2009).

A number of iron-sulfur cluster proteins were reported to be NO-responsive resulting in changes to the structure of the protein that promote DNA-binding (Smith *et al.*, 2010; Kudhair *et al.*, 2017). In many [4Fe-4S] cluster proteins the reaction with NO is very rapid and changes the structure of the protein to the octa-nitrosylated cluster (Fig 5.17) (Crack *et al.*, 2012). This study confirm that WhiB1 and WhiB2 react with Spermine NONOate as an NO donor and therefore behave similarly to those to other Wbl proteins (Fig 5.8 and 5.15) (Konar *et al.*, 2012; Kudhair *et al.*, 2017).

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| Fig. 5.17: Effect of NO on [4Fe-4S] Wbl protein to form tetranuclear iron octanitrosyl. It is most likely this formation is two RRE species. Diagram was adapted with modification from (Singh *et al.*, 2007; Crack *et al.*, 2012). |

# ***Chapter Six***

## 6 Characterization of *K. sedentarius* MBB13 WhiB proteins and determination of their relationship with sigma factors

### **6.1 Introduction**

In *M. tuberculosis*, there are 13 Sigma (σ) factors designed as SigA (σA) to SigM (σM) (Manganelli *et al.*, 2004). σA is a member of sig70 (σ70) that essential in both *M. smegmatis* and *M. tuberculosis* (Gomez *et al.*, 1998; Manganelli *et al.*, 2004). Other sigma factors are non-essential and produced only in response to stress conditions (Wösten, 1998). σ70 family shares four regions (1 to 4) and each region is divided, and the 4.2, 2.3, 2.4 and 2.5 regions are required to recognize the elements (-35, -10 and extended -10) of the targeted promotes (Wösten, 1998). In Mycobacteria, Wbl proteins were reported to interact with σA, such as WhiB3:σA in *M. tuberculosis* and *M. bovis*. Moreover, *M. tuberculosis* WhiB7 binds specifically with region 4 (the *C*-terminal domain ‘CTD’) via R515 (Burian *et al.*, 2013). In *M. bovis*, a mutation on R515 of σA-CTD (region 4) cause attenuation on WhiB3:σA (Steyn *et al.*, 2002). More specifically, all Wbl proteins from *M. tuberculosis* except WhiB5 interact with σA-CTD, WhiB3, 4, 6 and 7 interact via the residue R515 but not WhiB1 and 2 which indicate a different binding site is involved (Feng *et al.*, 2016). It has been also reported that other Sigma factors interact with Wbl proteins. For example, *M. tuberculosis* WhiB2: σB (Zheng *et al.*, 2012) and *S. coelicolor* WblC (homolog of *M. tuberculosis* WhiB7: σR (homolog of *K. sedentarius* MBB13σH) (Yoo *et al.*, 2016). The genome of *K. sedentarius* MBB13 has only four sigma factor encoding genes (A, B, H and J) and four Wbl genes (1, 2, 3 and 7) and therefore provides an opportunity to carry out a comprehensive analysis of the interactions between Wbl proteins and sigma factors in this bacterium.

### **6.2 Characterization of Wbl:Sigma factors (σ) complexes**

#### **6.2.1 Wbl proteins interact with region 4.0 of SigA (σA)**

Interaction between Wbl proteins and sigma factors (σ) A, B, H and J of *K. sedentarius* MBB13 was carried out using the Bacterial Adenylate Cyclase Two-Hybrid System (BATCH). BACTH was used as an *E. coli*-based *in vivo* assay for screening protein-protein interaction based on the reconstitution of complementary fragments (T25 and T18) of adenylate cyclase (CyaA) catalytic domains from *Bordetella pertussis* (Figure 5.17) (Karimova *et al.*, 1998). Interaction between the two fragments causes cyclic AMP (cAMP) synthesis which then binds to the catabolite activator protein (CAP also known as cAMP receptor protein ‘CRP’) resulting in DNA-binding at CAP dependent promoters. This change in gene expression can be detected (maltose and lactose utilization) using indicator media (Fig 6.1).

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| Fig. 6.1: Diagram of the two hybrid BACTH system. When the catalytic domain fragments (T25 and T18) of *Bordetella pertussis* adenylate cyclase are expressed as a full length protein (1-399 amino acid fused together) in *Escherichia coli,* itgenerates cAMP, but if the two fragments are expressed separately, no cAMP is produced. When interaction occurs between two genetically fused interacting proteins (X and Y), the adenylate cyclase catalytic domain fragments come close enough to restore cAMP synthesis. Cyclic AMP binds CAP (CRP) to permit binding at specific promoters in *E. coli* *∆cya* such as the *lac* operon, which leads to a transcription of the reporter gene (*lacZ*). Diagram was adapted from (Karimova *et al.*, 1998). |

A comprehensive interaction analysis for K. *sedentarius* MBB13 WhiB proteins and sigma factors was carried out by transforming *E. coli* BTH101 (∆*cya*) with plasmids encoding either T25-WhiB1, 2, 3 or 7, and one of T18-sigma A, B, H or J (Fig 6.2.*a*) (Section 2.6.7). Transformed cells were grown on indicator media, MacConkey/maltose (Section 2.3.3.1) and Luria-Bertani broth containing X-gal (Section 2.3.3.2) and IPTG for induction. The positive control was pKT25-zip and pUT18-zip strain. The negative control contained the empty vectors encoding the T25 and T18 fragments. Interaction plates were incubated for ~30 h at 30°C. The positive and negative colonies on MacConkey medium were red and white, and blue and white for Luria-Bertani broth-X-gal medium, respectively. The results suggested that all Wbl proteins interacted with σA–CTD, but not with with σB, σH and σJ. (Fig. 6.2.*b*.*c*).

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| --- | --- | --- | --- |
| (*a*) | | | |
| (*b*) | σA–CTDσB σH σJ | (*c*) | σA–CTD σB σH σJ |
| WhiB1  WhiB2  WhiB3  WhiB7 | C:\Users\mbb\Desktop\LB3.jpg | WhiB1  WhiB2  WhiB3  WhiB7 | C:\Users\mbb\Desktop\mac2.jpg |
| Fig. 6.2: In-vivo interaction of WhiB proteins and sigma factors (σ) using the BATCH system. (*a*) A diagram showing the construction of plasmids that encode T25-Wbl proteins by ligating *whiB* genes into pKT25 between Xbal and KpnI sites; and plasmids encoding T18-sigma by ligating sigma factor genes into pUT18 plasmid between HindIII and KpnI sites. Both plasmids were used to co-transform *E. coli* BTH101 (∆*cya*). Interactions were detected on (*b*) Luria-Bertani broth medium in the presence of X-gal, (*c*) MacConkey medium in the presence of maltose. Both plates were supplemented with ampicillin, kanamycin and IPTG. Controls were pKT25-zip and pUT18-zip plasmids as a positive control (+) and empty pKT25 and pUT18 plasmids as a negative control (-) (bottom row). | | | |
|  | | | |

Quantitative measurement of the interactions was obtained by estimating β-galactosidase activates. The results indicated that WhiB1 and WhiB2 interact with σA–CTD significantly (Fig 6.3). The WhiB1:σA–CTD was ~40-fold greater than the negative control followed by WhiB2:σA–CTD at ~10-fold, and both WhiB3:σA–CTD and WhiB7:σA–CTD did not show a significant increase.

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| Fig. 6.3: Bacterial two-hybrid system analysis of interaction between WhiB 1, 2, 3 and 7 and σA-CTD, σB, σH and σJ of *K. sedentarius* MBB13. Wbl and sigma proteins were fused to T25 and T18 domains respectively of *B. pertussis* adenylate cyclase in *E. coli* BTH101. Interaction between fusion partners enhanced the β-galactosidase production. Data points are the means of four replicates plus or minus standard deviation, significance (t-test) is indicated. |

#### **6.1.2 Effects of iron-starvation-, Erythromycin- and NaCl-stresses on interactions between Wbl proteins and σA-CTD**

The effects of iron-starvation, salt-stress and Erythromycin on the ability of Wbl proteins and σA-CTD to form complexes was tested using BACTH (Section 2.10.1 and 2.10.2). As judged by visual appearance on Luria-Bertani broth-X-gal, erythromycin and NaCl decreased the interactions WhiB2, 3 and 7 but not WhiB1 proteins with σA–CTD (Fig 6.4.*a*). However, β-galactosidase activity measurements showed that WhiB1:σA–CTD interaction decreased ~40% in the presence of bipyridyl, ~60 % for 5% erythromycin and ~90% for NaCl, in comparison to the normal conditions (Fig 6.4.*b*.*c*.*d*.*e*). Moreover, reducing the availability of iron impaired WhiB2: σA interactions more than WhiB3:σA in comparison to the negative control. These results indicate that, Wbl iron-sulfur clusters react differently to iron-starvation, suggesting that accessibility to the clusters differs between *K. sedentarius* MBB13 Wbl proteins, which is consistent with results reported by Alam *et al.* (2009). It has been stated that the response of a host against bacterial infections is to restrict the availability of iron (Sritharan, 2016). This result indicates that iron-starvation might disassemble the WhiB:σA interactions. The effects of erythromycin and salt stress on the formation of WhiB:σA complexes were tested aerobically. Significant reductions in β-galactosidase activity was observed in response both stresses, compared to the positive control (Fig 6.4). However, the output from the leucine zipper control was also affected by the different growth conditions.

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| --- | --- | --- | --- | --- | --- | --- | --- |
| (*a*) | Nor | Bip | Ery | | NaCl | (*b*) | |
|  | |
|  | σA | | | | |
| WhiB1  WhiB2  WhiB3  WhiB7 | C:\Users\mbb\Desktop\normall2.jpg | C:\Users\mbb\Desktop\bib.jpg | C:\Users\mbb\Desktop\ery.jpg | | C:\Users\mbb\Desktop\naclttt.jpg |
| Controls | C:\Users\mbb\Desktop\control.jpg | |  | |  |  | |
| (*c*) |  | | (*d*) | | | | (*e*) |
|  | | | |  | | |  |
| Fig. 6.4: BACTH analysis of the effects of stress factors on interaction of *K. sedentarius* MBB13 Wbl proteins and σA-CTD. (*a*) Luria-Bertani agar medium in the presence of X-gal and supplemented with ampicillin, kanamycin, IPTG and the different stresses as follows, Nor; normal condition, Bip; 0.05 mM 2,2’ bBipyrdyl, Ery; 64 µg/ml erythromycin and Salt: 5% NaCl, and grown at 30°C for 40 h. Controls showed same result and the tested conditions. (*b*, *c*, *d* and *e*) β-Galactosidase activities between the fused partners in the presence of different stress as indicated and the tested cultures were grown at LB liquid media at 37°C for 18 h. Control cultures were empty vector (negative control) and the GCN4 leucine zipper (Zip-Zip) (positive control). Both plates and liquid cultures were supplemented with ampicillin, kanamycin and streptomycin. The charts show the mean and standard deviation for four independent cultures; significance (t-test) are indicated. | | | | | | | |

Therefore, the data were reanalysed by normalising all the data to the activities of the positive control under the different growth conditions (Fig 6.5). This suggested that the WhiB1:σA interaction was significantly lower under iron-starvation and salt-stress conditions; that WhiB2:σA interaction was lower under all the stress conditions tested; but WhiB3:σA and WhiB7:σA interactions were unaffected.

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| Fig. 6.5: Ratio of ß-galactosidase activities of Wbl: σA-CTD of *K. sedentarius* MBB13 to the GCN4 leucine zipper (Zip-Zip) ‘the positive control’ under stress conditions as indicated by the BACTH system. Liquid cultures were grown at 37°C for 18 h, in LB with appropriate antibiotics and IPTG. The chart shows the mean and standard deviation for four independent cultures; significance (t-test) are indicated. |

### **6.2 Differential expression of *whiB* genes in response to growth phase and antibacterial agents**

As mentioned previously, Wbl proteins are transcription factors that are exclusively found within the actinobacteria. Geiman *et al.* (2006) reported that, *M. tuberculosis* *wbl* genes (strain CDC1551) are differentially expressed during the different phases of growth and in the presence of different antibiotics. Therefore, expression of *K. sedentarius* MBB13 *wbl* genes was determined using a quantitative real-time PCR assay (qRT-PCR).

#### **6.2.1 Primer design and validation**

The primers for the tageted genes were designed to produce amplicons of ~100 bp using the criteira in Section 2.7.5, and their amplification efficiencies were tested with genomic DNA as a template (Fig 6.6). This showed the amplification of DNA fragments of the predicted sizes. Total RNA was extacted from triplicate cultures of *K. sedentarius* MBB13 at different phases of the growth cycle in the presence of different antibiotics. RNA prepartions were processed using the DNA-free kit to remove any contaminating genomic DNA (Section 2.7.3), and the absence of contamining DNA was confirmed by the inability to amplify 16S rRNA gene using universal bacterial primers (Section 2.6.8; Table 2.3; Fig 6.7 ). Contamination by DNA would result in errors in any quantitative real-time PCR experiment (Laurell *et al.*, 2012). Then *gyrA* (reference gene), *whiB1* and *whiB2* primers were used with different dilutions of genomic DNA to ensure that the amplification efficiencies were similar (Table 6.1, 6.2 and Fig 6.8).

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| Base Pairs  1,000  500/517  200  100 | | **C:\Users\mbb\Desktop\666666.jpg** | | Fig. 6.6. Validation of specificity the Real-time PCR primers analysed by 10% gel electrophoresis. Primer pairs were used with *K. sedentarius* MBB13 genomic DNA as template. Lane 1; 100 bp ladder, Lane 2; *gyrA*, Lane 3-6; *whiB1*, *2*, *3* and *7* respectively. | |
|  | (*a*) | |  | | (*b*) |
| Kilobases  3.0  1.5 | C:\Users\mbb\Desktop\dnrremovalfromrnasamples\untreated.jpg | | Kilobases  3.0  1.5 | | C:\Users\mbb\Desktop\dnrremovalfromrnasamples\treated.jpg |
| Fig. 6.7: Genomic DNA in extracted total RNA. PCR products were separated on agarose gel (2%) and visualized using GelRed DNA stain (Section 2.5.5). Amplification of genomic DNA from total RNA extracted from the targeted genes (*a*) before and (*b*) after treating with DNA-free kit (Section 2.6.3). Lane L, 1 kb DNA marker; lane 1, *gyrA*; lane 2, *whiB1*; lane 3, *whiB2*; lane 4, *whiB3* and lane 5, *whiB7*. | | | | | |

Table 6.1: Validation of amplification efficiency for the primers used in qRT-PCR. A preliminary experiment was initiated to validate the prime pairs. The purified genomic DNA of *K. sedentarius* MBB13 was serially diluted and used as the template in duplicate qRT-PCR reaction.

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| --- | --- | --- | --- | --- |
| Genomic DNA (ng) | Log10 genomic DNA | *gyrA* average CT value | *whiB1* average CT value | *whiB2* average CT value |
| 100 | 1.273001272 | 19.52167 | 21.655 | 22.19833 |
| 50 | 0.670941281 | 20.27667 | 22.26833 | 23.165 |
| 25 | 0.068881289 | 21.01 | 22.8 | 23.61667 |
| 12.5 | -0.533178702 | 22.13833 | 23.58333 | 24.09167 |

Table 6.2: Gradient and intercept values of the used primers in qRT-PCR.

|  |  |  |
| --- | --- | --- |
| Gene name | Gradient | Intercept |
| *gyrA* | -1.4201 | 21.633 |
| *whiB1* | -1.4201 | 21.633 |
| *whiB2* | -1.1261 | 24.297 |

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|  |
| Fig. 6.8: Graph of efficiency validation of amplification for primers used in qRT-PCR. Average CT value plotted against log10 genomic DNA concentration for calculation of the intercept and gradient of lines. Lines points represent *whiB1*, *whiB2* and *gyrA* as indicated. |

#### **6.2.2 Kytococcus sedentarius MBB13 wbl gene expression during the bacterial growth cycle**

*Kytococcus sedentarius* MBB13 cultures were grown in Luria-Bertani broth medium at 37°C with shaking at 250 rpm. Samples (5 ml) were taken from triplicate cultures at early exponential phase (EEP; OD600 of 0.75), and then at late exponential phase (LEP; OD600 of 0.9) and finally in staionary phase (SP; OD600 of 2.25) (see growth cruve of *K. sedentarius* MBB13 in Appendix 9.6). Total RNA was prepared (Section 2.7).

qRT-PCR analysis suggested that *whiB1, whiB3* and *whiB7* expression in *K. sedentarius* MBB13 in Luria-Bertani broth was higher in LEP in compared to SP. For *whiB2* no significant change was obseved (Fig 6.9). Thefore, the the expression of *K. sedentarius* MBB13 *wbl* genes after exposure to antibiotics was assessed for LEP cultures.

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| Fig. 6.9: qRT-PCR analysis of relative expression levels of *whiB1*, *2*, *3* and *7* during different phases of growth. Total RNA was isolated from *K. sedentarius* MBB13 cultures grown to early exponential phase (OD600 0.75), late exponential (OD600 0.9) and stationary (OD600 2.25). Relative expression level was calculated using the *gyrA* transcript for normalization. Averages of four repeats was taken to represent the error bars; significance (t-test) are indicated. | |

#### **6.2.3 Kytococcus sedentarius MBB13 wbl gene expression in response to antibiotics**

*Kytococcus sedentarius* MBB13 cultures were grown in Luria-Bertani broth medium at 37°C with shaking at 250 rpm until the OD600 reached 0.9 (LEP), then triplicate cultures were treated with 2,048 µg/ml of either erythromycin, ciprofloxacin, gentamicin or tetracycline. After this, the cultures were incubated under the same conditions for 30 min. Then ~5 ml of the cultures before and after treating with antibiotics were taken for the total RNA purification (Section 2.7).

qRT-PCR analysis revealed a significant increase in *whiB7* (4-fold) in response to ciprofloxacin, *whiB3* (1.5-fold) and (1-fold) *whiB7* in response to gentamicin and *whiB1* (1-fold) in response to tetracycline. No signifcant increase after exposure to erythromycin was obsereved (Fig 6.10).

It is possible to conclude that, proteins synthesis inhibitor agents, gentamicin induces *whiB3* and *whiB7* and tetracycline induces *whiB1* but not erythromycin (Neu, 1992), and the inhibiting cell division agent (ciprofloxacin) induces *whiB7*(Loveless *et al.*, 2010; de Lima Procópio *et al.*, 2012).

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| Fig. 6.10: qRT-PCR analysis of relative expression levels of *whiB1*, *2*, *3* and *7* under various antibiotic stress. Total RNA was isolated from *K. sedentarius* MBB13 grown until late exponential phase. Relative expression level was calculated using the *gyrA* transcript for normalization. Averages of four repeats were taken; significance (t-test) are indicated. | |

#### **6.2.4 Kytococcus sedentarius MBB13: does WhiA have a role in cell division through WhiB2**

As shown previously, σA-CTD interacts with Wbl proteins of *K. sedentarius* MBB13. Other species of actinomyces, e.g. *Streptomyces venezuelae*, a homolog of *K. sedentarius* MBB13 WhiB2 works with an unrelated transcription regulator, WhiA, to control a joint regulon. Mutation of *whiA* causes a failure to halt aerial growth (Bush *et al.*, 2013). *Kytococcus sedentarius* MBB13 has a *whiA* gene. Thus, to investigate the possible interaction between Wbl, sigma factors and WhiA in *K. sedentarius* MBB13, the BACTH system was used. WhiA was fused inframe into pUT18 and pKT25 respectively to investigate its interactions with Wbl proteins and sigma factors. The results indicate that there was no significant interaction on the indicator plates or ß-galactosidase activities (Fig 6.11).

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|  | | (*a*) | *(b)* |
| WhiB1, 2 ,3 and 7  σA, σB, σH and σJ | | **C:\Users\mbb\Desktop\BACH system\Oct 2018 results نتائج الثيسس هنا\plats pics of 2HS , unde rnormal,salt5% and ery microgl\WhiA\WHIA DONE.jpg** | C:\Users\mbb\Desktop\BACH system\Oct 2018 results نتائج الثيسس هنا\plats pics of 2HS , unde rnormal,salt5% and ery microgl\WhiA\WHIA DONE2.jpg |
| (*c*) |  | | |
| Fig. 6.11: Protein-protein interaction between WhiB1, 2, 3 and 7 and σA (CTD), σB, σH and σJ against WhiA of *K. sedentarius* MBB13 using BACTH system. WhiA was fused in frame into both pUT18 and pKT25 in order to interact with WhiB-pKT25 and σ-pUT18 respectively. Detection of interaction was by (*a*) Luria-Bertani broth medium plate in the presence of X-gal and IPTG as an inducer grown at 30°C for 40 h. (*b*) β-Galactosidase activities between the fused partners, cultures were grown at 37°C for 18 h. Negative control was the empty vector, and the GCN4 leucine zipper (Zip-Zip) was the positive control. Both plates and liquid cultures were supplemented with ampicillin, kanamycin and streptomycin. The chart shows the mean and standard deviation for four independent cultures; significance (t-test) are indicated. | | | |

### **6.3 Discussion**

*In vivo* protein-protein interaction of *K. sedentarius* MBB13 Wbl:σ were studied. σA-CTD interacts with all WhiB proteins of *K. sedentarius* MBB13. The interaction between Wbl proteins and σA-CTD could reprogram transcription through altering binding at promoter DNA and/or the function of the sigma factor (Dove *et al.*, 2003); (Paget, 2015). σA share four main domains (Fig 4.12.*b* and 6.12) and in *M. tuberculosis*, the *C*-terminal domain is proposed to interact with WhiB proteins through binding the -35 elements of their promoters (Feng *et al.*, 2016).

The importance of Wbl:σ interactions is illustrated by the attenuation of *M. bovis* by the R515H σA-CTD mutation or deletion of *whiB3*, which suggests stimulation of unknown virulence genes require the WhiB3:σA-CTD interaction (Steyn *et al.*, 2002). In *M. tuberculosis* WhiB7, AT-hook domain binds σA-CTD and abolishes WhiB7:σA-CTD interaction when R515H mutation occurs in σA-CTD and WhiB7 multi-drug sensitivity effect due to R515H mutation, that is similar to the AT-hook deletion effect. Those findings indicate that WhiB7 acts to stabilise the σA-DNA binding and provides an addition selectivity for specific promoters in specific conditions (Burian *et al.*, 2013). Therefore, it is likely that *K. sedentarius* MBB13 WhiB1, WhiB2, WhiB3 and WhiB7 act similarly to *M. tuberculosis* WhiB7 via its binding with σA and DNA interaction. σA of *M. tuberculosis* and *M. smegmatis* is a major vegetative sigma factor that essential for driving the housekeeping genes expressions (Manganelli *et al.*, 2004). It is noteworthy that, infection by *M. tuberculosis* results in overexpression of σA in either human macrophages or mice lungs, and also modulates essential genes for virulence which leads to enhance intracellular growth (Wu *et al.*, 2004). Therefore it is unlikely that Wbl proteins act as anti-σA factors to abolish σA-RNAP interaction due to essentiality of σA, instead this binding is possibly essential for specific genes regulation by stabilizing interactions of σA with DNA, which provide additional promoter selectivity, especially since *M. tuberculosis* WhiB1 C-terminal domain is rich in positive amino acids, which are important for DNA-binding (Smith *et al.*, 2012). During macrophage infection σA is either be upregulated and interact with WhiB1 to induce other sigma factors that involve adaptation or virulence, besides that, some free σA at basal level is free for regulating housekeeping genes. Furthermore, it has been reported that whiB1 is possibly directly controlled by σF in *M. smegmatis* (Hümpel *et al.*, 2010). Production of NO in activated macrophages is the first antibacterial defence against *M. tuberculosis* that needs to adapt through switching on or off a number of transcription pathway genes (Yang *et al.*, 2009). Also sigma factors are sensory proteins that respond to external stimuli (Wösten, 1998). The *K. sedentarius* Wbl:σ interactions were then subjected to bipyridyl as an iron chelator, erythromycin and NaCl. It is obvious that bipyridyl effected on WhiB:σA-CTD but not on the control, unlike erythromycin and NaCl which have an effect on both WhiB:σA-CTD and the positive controls (Fig 6.12). It can be concluded that bipyridyl and erythromycin decreased impaired the activity of β-galactosidase of all interactions and it is possible to concluded that WhiB2:σA-CTD impaired more that WhiB3:σA-CTD in comparison to the same interaction under normal condition which indicated that each interaction may react differently under different stresses.

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| C:\Users\mbb\Desktop\SigA.JPG |
| Fig. 6.12: Ribbon diagram of *K. sedentarius* MBB13 SigA (σA) three dimensional structure. The domains are indicated as follows: ND1, *N*-terminal 1; ND2, *N*-terminal 2; LD, linker domain; CD, *C*-terminal domain. The structure was obtained from the following webpage ([swissmodel.expasy.org](file:///C:\Users\mbb\Desktop\My%20thesis%20June2019\swissmodel.expasy.org)). |

Expression of *wbl* genes under various conditions has been reported previously (Geiman *et al.*, 2006). By using qRT-PCR, it is possible to claim that all *whiB1*, *whiB2* and *whiB7* were upregulated significantly during the late exponential phase. These patterns could be relevant to the *in vivo* chronic infection and similar to those found in *M. tuberculosis whiB4*, *5* and *7* which were upregulated during the late exponential phase (Geiman *et al.*, 2006). Further assessment for adaptation for antibiotics response due to an evidence suggested that, *M. tuberculosis whiB7* governs a resistance mechanism for various antibiotics (Morris *et al.*, 2005). The gene *whiB7* is upregulated significantly by Ciprofloxacin which is the only antibiotic that inhibits cell division, moreover, protein synthesis inhibitor antibiotics gentamicin induces *whiB3* and *whiB7*, and tetracycline induces *whiB1*. Up to date, there is no evidence that explain the expression of *whiB1* and *whiB3* are stimulated by antibiotics.

In *Streptomyces*, the initiation of sporulation and the cessation of aerial growth is cocontrol by WhiB (homolog of *K. sedentarius* WhiB2) and WhiA (non-Wbl protein). By using BACTH system, the interaction between Wbl and σ proteins against WhiA were investigated, however, no evidence for binding were detected.

Results presented in this chapter show that the four Wbl proteins of *K. sedentarius* interact directly with the major sigma factor A (σA) through the *C*-terminal domain but not with σB, σH and σJ. No evidence for an interaction between WhiB proteins or sigma factors with WhiA. The genes *whiB1*, *whiB2* and *whiB7* were upregulated significantly during the late exponential phase, and *whiB7* was induced by ciprofloxacin, *whiB3* and *whiB7* are induced by gentamicin, and *whiB1* was induced by tetracycline.

# ***Chapter Seven***

## 7 General Discussion

### **7.1 Effects of sampling methodology**

The work presented here suggests that airborne bacteria contaminate the air within a lecture theatre. While this does not represent a problem to healthy individuals, airborne bacteria can be harmful for immunocompromised individuals (Folayan *et al.*, 2018). Results in Chapter 3 indicated that isolating airborne bacteria by more than one method increases the chances to estimate the variety of airborne bacterial species accurately around individuals in indoor environments. This was assessed by visual appearance of colonies on Luria-Bertani plates, it is clear that dehumidifier collected a higher number of bacteria (Fig 3.1). It is likely that using an active sampling method such as a dehumidifier that depends on air suction may help to collect more airborne bacteria that are not detected using the passive monitoring such as settle plates, which only rely on gravity to draw airborne bacteria to the medium (Sayer *et al.*, 1972) rather than using a specific fitted device to drag air (Zhang, 2006). The results matched those of Sayer *et al.* (1972) who claimed that applying different methods to isolate airborne microbes can simply produce different group of species. Elsewhere it has also been reported that active monitoring can collect more airborne bacteria especially for those considered inhalable viable particles (Napoli *et al.*, 2012). However the design of each building could have the ability to control which species of airborne bacteria can or cannot grow within those built environments (BI) due to the materials used (Kelley and Gilbert, 2013). To conclude, the assessment of the diversity of airborne bacteria in each BI depends on method of isolation, materials used to build BI and the season of isolation (Sayer *et al.*, 1972; Kelley and Gilbert, 2013). Therefore is possible to suggest that each BI may need its own investigation to identify the airborne microbes that may able to survive, especially in environments that are near to potentially hazardous laboratories which could enhance unusual organisms to transit from those laboratories to other non-clinical area such as waiting area room in hospitals which can pass individuals with various occupational hazards (Verde *et al.*, 2015).

### **7.2 *Kytococcus sedentarius***

It is obvious that bacteria isolated from dehumidifier were abundant (Fig. 3.1) and belonged to different families (Table 3.1) which encouraged this study to focus on these bacteria. Many reports employed the study of the bacterial resistance against antibiotics as an initial test to determine the infection control strategy for each isolated strain, and this step is due to excessive use of antibiotics that have led to the development of bacterial isolates that are multidrug resistant (Adzitey, 2015). The initial screening of bacteria isolated by dehumidifier against a selection of antibiotics that were suggested by BCAS (2013) indicated that *K. sedentarius* MBB13 is resistant to erythromycin, gentamicin and ciprofloxacin (Table 3.2). The work described here and elsewhere resulted in isolation of multi drug resistant *K. sedentarius* (Greene *et al.*, 1980; Marples and Richardson, 1980; Nordstrom *et al.*, 1987; Levenga *et al.*, 2004; Chaudhary and Finkle, 2010; Folayan *et al.*, 2018). However, up to date, resistance to erythromycin has not been reported; furthermore, erythromycin has been used as a first-line treatment against *K. sedentarius* infections (Singh and Naik, 2005; Pranteda *et al.*, 2014).

Due to the above facts, it was important to investigate *K. sedentarius* MBB13 at the molecular level, although, the genome of *K. sedentarius* was sequenced previously (Sims *et al.*, 2009). Therefore, the whole genome of *K. sedentarius* MBB13 was sequenced in Chapter four. *Kytococcus sedentarius* is known to cause pitted keratosis (PK), likely through the ability of the cocci to produce two proteases, P1 and P2 that are active against keratin extracted from human callus (Longshaw *et al.*, 2002). Brandelli *et al.* (2010) reported a comprehensive investigation of keratinase producers and suggested several conserved regions that are normally located in the *N*-terminal regions of some keratinases and similar proteases. The latter mentioned conserved regions however were not present in the *K. sedentarius* MBB13 genome which led to an investigation this bacterium *in vivo*. After growing *K. sedentarius* MBB13 on azure-based culture media assay, the cocci of MBB13 was unable to degrade the keratin dissolved within the Luria-Bertani plates, which indicates that probably not all *K. sedentarius* strains including MBB13 (Fig 4.11) are able to degrade callus that led to the infection of PK.

Another fact about *K. sedentarius* is that it was first isolated from a marine environment and uses the skin, mucosae and oropharynx of human beings as a habitat (Sims *et al.*, 2009), which indicates that *K. sedentarius* is salt tolerant. The genome of *K. sedentarius* MBB13 reveals a combination of several strategies used by other organisms to adapt and grow in saline environments (Fig 4.9), and in this study, adaptation to different concentrations of NaCl was confirmed after an NMR examination of the cocci showing glycine betaine as the main compatible solute alongside ectoine, proline, acetate, lactate and glutamate (Fig 4.10), which indicate that the mentioned solutes could be beneficial for adaptation to salty environments.

In this study and elsewhere *K. sedentarius* is classified as a strict aerobe (Fig 4.6) (Sims *et al.*, 2009), thus, for better understanding, the analysis of the MBB13 strain reveals various factors for its dependence on oxygen for survival. For example, the strain MBB13 can make 41 reactions that require molecular oxygen (Table 4.2), RNR systems and absence of fermentation and anaerobic respiratory pathways. Also the existence of cytochrome *c* pathway that contains (cytochrome *bc1-aa3* oxidase and cytochrome *bd* oxidase) suggested its oxygen dependence.

The genome of *K. sedentarius* MBB13 also possesses the major sigma factor (σA) that has a high similarity with σA of *M. tuberculosis* (Fig 4.12). In bacteria, to initiate a transcription from promoters requires core RNA polymerase (RNAP) to acquire a dissociable specificity subunit, the sigma factor (Paget, 2015). Feng *et al.* (2016) reported that the *M. tuberculosis* σA interacts though its *C*-terminal domain (CTD) with the seven Wbl transcription factors. *Kytococcus sedentarius* MBB13 possesses four Wbl proteins, and in *M. tuberculosis* and other actinomyces members, Wbl proteins play essential roles (Bush, 2018).

### **7.3 Wbl proteins**

The work presented in Chapter five partially characterized two of the four Wbl proteins of *K. sedentarius* MBB13. WhiB1 and WhiB2 of *K. sedentarius* MBB13 are iron-sulfur cluster proteins and NO-reactive; WhiB1 is O2-insensitive but not WhiB2; the cluster of WhiB1 is essential for protein folding. *Mycobacterium tuberculosis* WhiB1 is essential (Smith *et al.*, 2010). The purified recombinant WhiB1 of *K. sedentarius* MBB13 was found to carry a [4Fe-4S] cluster (Fig 5.6). Iron-sulfur clusters are proposed to be one of the oldest and most versatile cofactors for several protein classes and are implicated in many functions (Alam *et al.*, 2009). This work and others propose a question about the nature of WhiB1. It was been proposed that WhiB1 play a role in switching between dormant and replicative states in *M. tuberculosis* (Smith *et al.*, 2010). Here all four Wbl proteins interacted with σA and all the *wbl* genes, except *whiB2*, are upregulated in late exponential growth phase and with different antibiotics. Also the interaction of WhiB1 with σA could lead to an answer as to why WhiB1 is essential. σA is required for housekeeping gene transcription (Wu *et al.*, 2004), therefore it is unlikely that WhiB1 acts as anti-sigma factor. Beside that several bacteria including actinomyces members showed a presence of anti-sigma factors (Beaucher *et al.*, 2002; Lee *et al.*, 2004). It is possible to hypothesise that switching WhiB1 release and binding to RNA polymerase depends on a WhiB1-partner which can have a specific role during the growth development in *K. sedentarius* MBB13. Another possibity is that essential genes can be regulated in response to specific stimuli for example, Spermine NONOate (NO donor). Here and others reported that WhiB1 did not respond to O2 but reacted to NO and most likely formed an octa-nitrosylated form (Kudhair *et al.*, 2017). This reaction switches the form of WhiB1 from non-DNA binding to DNA-binding (Smith *et al.*, 2010). Kudhair *et al.* (2017) reported NO triggers WhiB1 to release from the complex of WhiB1:σA. Thus it is possible to conclude that switching between binding with DNA and σA in response to NO plays a regulatory role in *K. sedentarius* MBB13.

Although, the sensitivity of WhiB1 to NO and O2 are studied here and by others; other external signals could play a role in reacting or regulating with WhiB1 cluster in response to infections. In *M. tuberculosis*, cyclic AMP is a signal that important to regulate WhiB1, which is present in macrophages during the infection at high levels (Bai *et al.*, 2009). The cAMP receptor protein (CRP) regulates *whiB1* and binds the upstream of *whiB1* directly (Agarwal *et al.*, 2006). At high levels of cAMP, *whiB1* is downregulated by two binding sites that are occupied by CRP, but at low levels of cAMP, only one site is occupied by CRP which results in upregulation of *whiB1* (Stapleton *et al.*, 2010). Thus it is possible to conclude that, presence of low NO and high level of cAMP could enter *M. tuberculosis* into dormancy state in which the nitrosylated-WhiB1 down regulates the *whiB1* gene (Kudhair *et al.*, 2017). The downregulation may change the transcription state of other regulators which important for dormancy such as Dos regulon (Kudhair *et al.*, 2017). *Kytococcus sedentarius* was isolated from 400 atmospheric pressure ‘atm’ at deep seawater in active state, but at 600 atm, it was in the no multiplication state (but was not killed) (Oppenheimer and ZoBell, 1952). Entering *K. sedentarius* into no multiplication state may raise a question, was it in dormancy state and was *whiB1* playing any role in that no multiplication state.

*Mycobacterium smegmatis* WhmD and *Streptomyces coelicolor* WhiB(homolog of *M. tuberculosis* WhiB2) are essential genes involved in cell division (Raghunand and Bishai, 2006; Bush *et al.*, 2016). *Streptomyces coelicolor* WhiB and WhiA proteins function cooperatively to control the expression of set of genes that targeted by WhiBA, and a mutant of WhiB (within the cysteine residues) prevents DNA binding by both WhiA and WhiB (Bush *et al.*, 2016). WhiA does not belong to the family of WhiB proteins and is present in Gram-positive bacteria, including non-actinomycetes (Bush *et al.*, 2016). In *K.* *sedentarius* MBB13, no interaction occurred between WhiB2 and WhiA, which indicating the WhiBA may not function as same as in *S. coelicolor*.

The biochemical evidence in this work showed that WhiB2 of *K. sedentarius* MBB13 is sensitive to O2 which agreed with Rybniker *et al.* (2010), and is also sensitive to Spermine NONOate (NO donor) in agreement with the general features of Wbl proteins (Bush *et al.*, 2016); however, up to date, WhiB2 has not been examined in the presence of NO yet. NO reacts with iron–sulfur cluster proteins and the consequent toxic effects are used by the immune system of the host in the response to pathogenic bacteria (Smith *et al.*, 2010). This reaction of WhiB2 to Spermine NONOate could lead to change in the transcription state that may be important for the function of WhiB2, however, for better understanding, it is necessary to determine the function of WhiB2 in *K. sedentarius.*

In conclusion, the isolation of bacteria from the lecture theatre MBB-F02 revealed an antibiotic resistant bacterium *K. sedentarius* MBB13. The genome sequence and *in vivo* examination showed that, *K. sedentarius* MBB13 is strictly aerobic, unable to degrade keratin and salt tolerant. The nature of WhiB1 and WhiB2, their response to NO and the ability of WhiB1, 2, 3 and 7 to bind σA could provide new information about the significance of these regulators. Thus WhiB1 and WhiB2 are important NO sensor proteins that may play roles in *K.* *sedentarius* during adaptation to harsh environments or cell division respectively. Therefore, WhiB1 and WhiB2 are potential factors that can be targeted for more research which could lead to new treatment against this organism.

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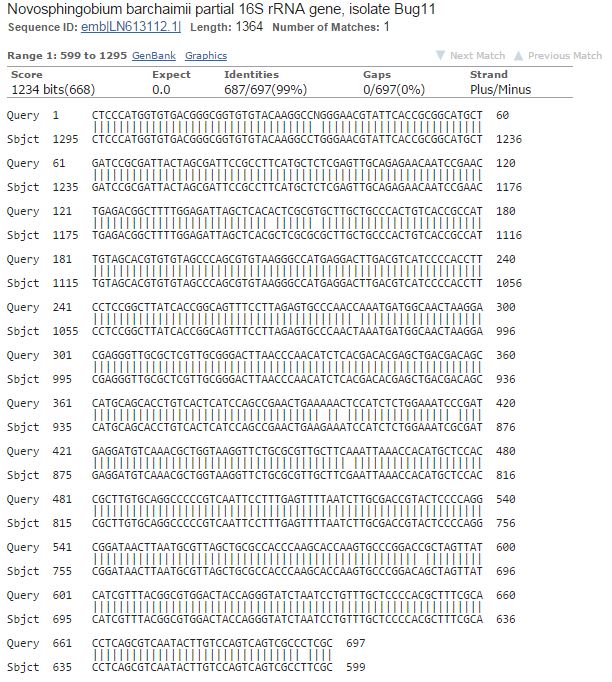
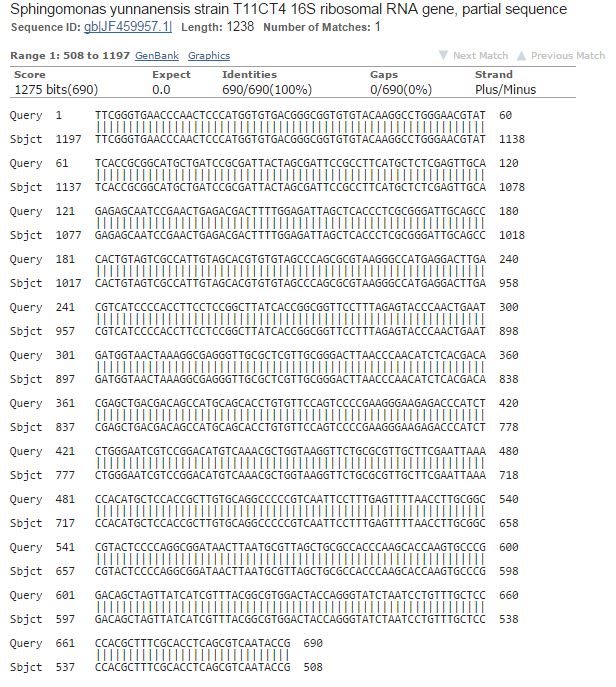
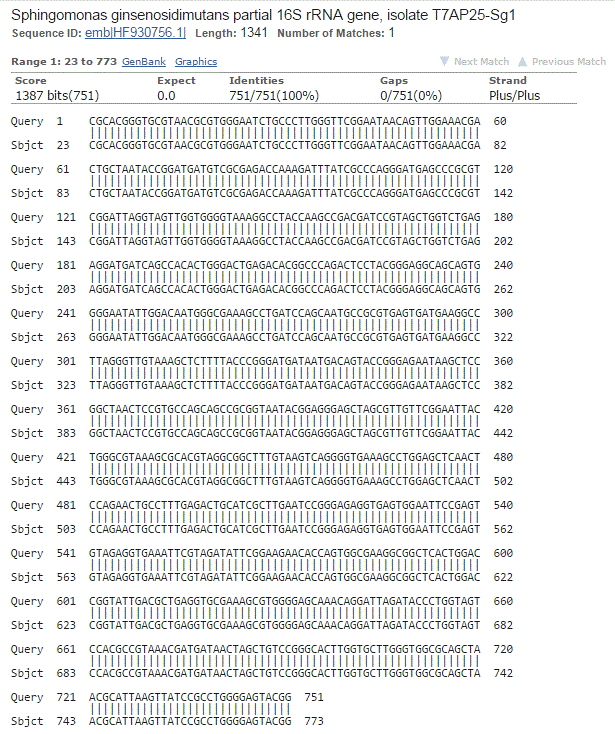
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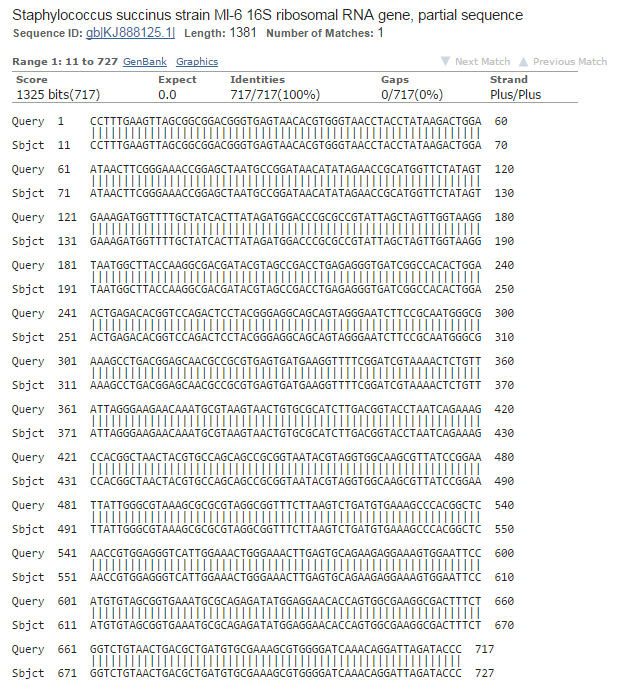
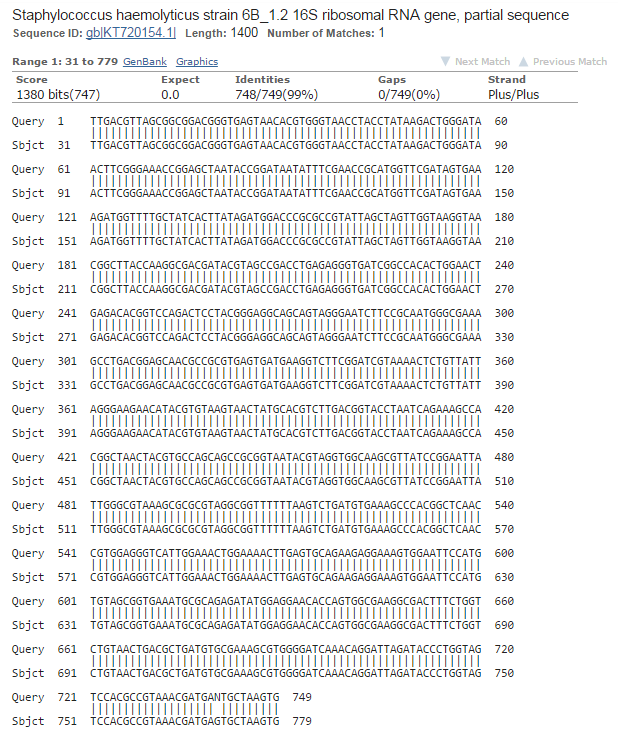
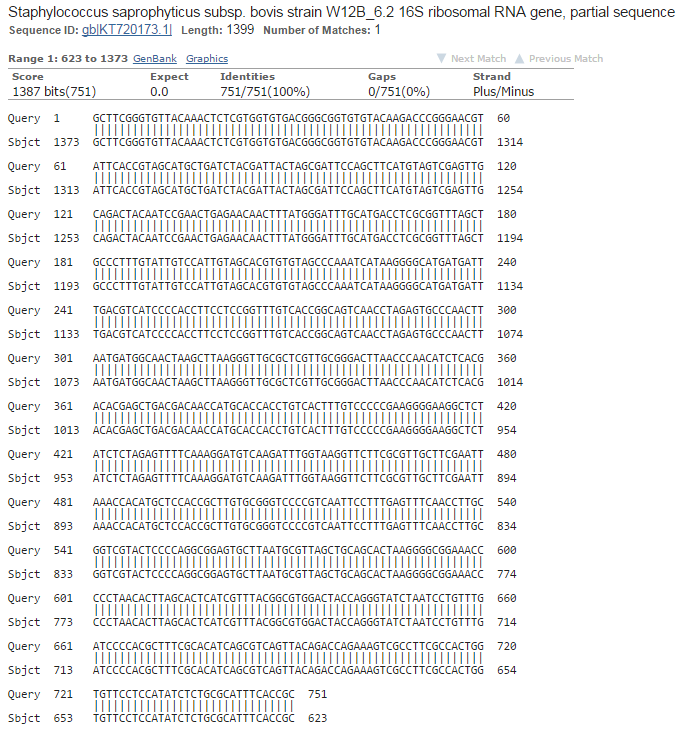
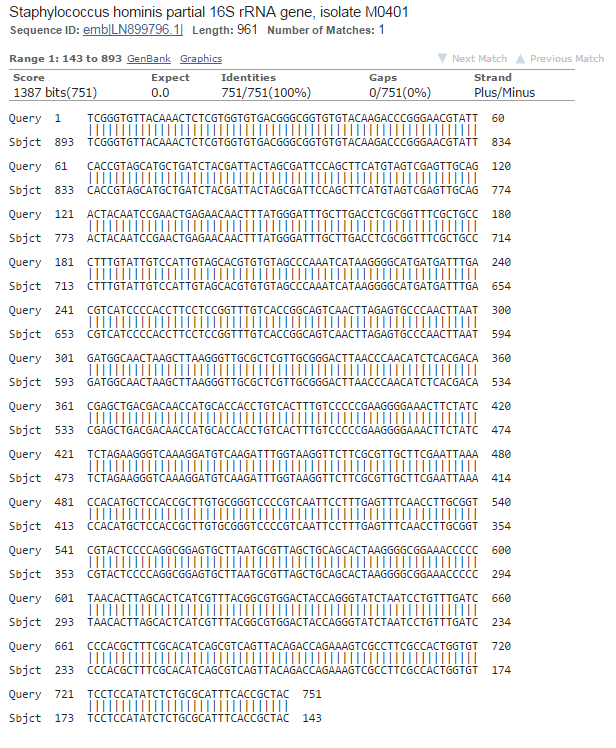
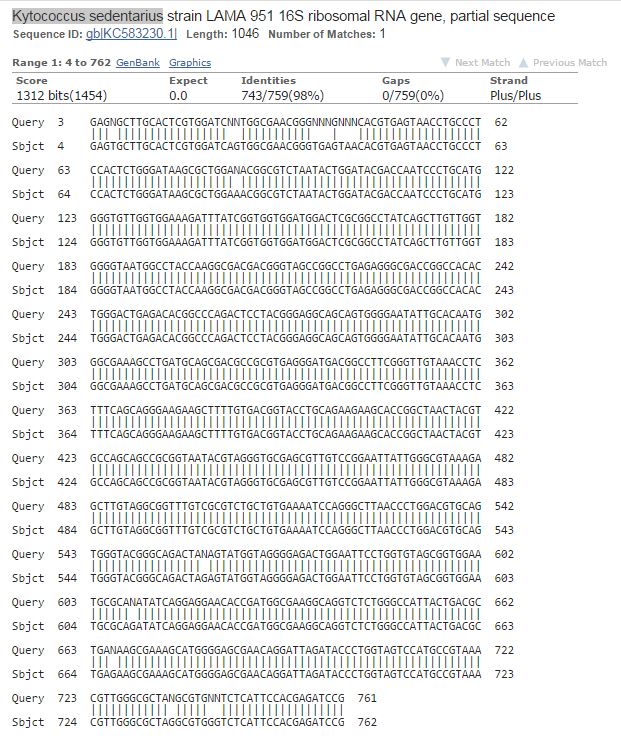
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# **9 Appendix**

Appendix 9.1 Partial sequences of the 16S rRNA of isolated strains 



## Appendix 9.2 Comparative interpretation of disc diffusion test of *K. sedentarius*

|  |  |  |
| --- | --- | --- |
| C:\Users\mbb\Desktop\best collection\edited\Vancomycin.jpg | C:\Users\mbb\Desktop\best collection\edited\Genta.jpg | C:\Users\mbb\Desktop\best collection\edited\Amikacin 30μg.ml.jpg |
| C:\Users\mbb\Desktop\best collection\edited\Imipenem 10 mg.ml.jpg | C:\Users\mbb\Desktop\best collection\edited\chloramphenicol 10μg.ml.jpg | C:\Users\mbb\Desktop\best collection\edited\Rifampin 2μg.ml.jpg |
| C:\Users\mbb\Desktop\best collection\edited\Dor.jpg | C:\Users\mbb\Desktop\best collection\edited\Tetra.jpg | C:\Users\mbb\Desktop\best collection\edited\Ery, cipro, pip.jpg |
| Fig shows the susceptibility pattern of *Kytococcus sedentarius* MBB13 to the follow antibiotics; Doripenem(DOR10), Imipenem (IMP10), Piperacillin-tazobactam(TZP85) Amikacin(AK30), Gentamicin(CN10), Ciprofloxasin(CIP1), Rifampin(RD5), Erythromycin(E5), Vancomycin(VA30), Tetracycline(TE10) and Chloramphenicol(C10). Three control strains are used to test the antibiotics performance, *Staphylococcus aureus* NCTC 12981, *Escherichia coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 10662 (not shown). | | |

## Appendix 9.3 Relative MIC response levels of *K. sedentarius* MBB13 against the tested antibiotics

## Appendix 9.4 Processing genome files after sequencing

**(*a*)**

Processing genome files from MicrobesNG Company

Open the following website and register an account (it might work without registration, haven't tried

it).

http://hactar.shef.ac.uk/galaxy

Click on "Get Data" at the left of the page, and select "Upload File", then "Choose local file", and

upload the reference genome and the Birmingham assembly file. Accepted formats are Fasta,

Genbank and EMBL.

Next click on "Genome assembly" (again on the left) and select "Actcompare".

Select the appropriate datasets as the reference and assembled contigs. Switch on the repeat filter.

Press "Execute".

This should produce a PNG image of the comparison, an ACT-compatible comparison file which you

can download, a log file and Genbank files representing the reference and contig genomes, each

with all replicons/contigs concatenated into a single pseudo-chromosome.

Save concatenated contig file and ACT comparison file.

Open concatenated contig file in Notepad and remove header/spaces/numbers (use replace tool).

Add a FASTA header e.g. >Identifier

Save as txt file

Back to Galaxy

On left Genome Annotation

Proka program run on concatenated contig txt file

Save genbank file

In ACT use reference genbank file

ACT comparision file

Proka genbank file

**(*b*)**

The following tracks is to how to extract data from ACT software:

Find gene sequence or amino acid or to search by piece of amino acid:

Goto → Choose the genome → Navigator → find the gene → right click → view → choose amino acid or bases

Codon usage:

File → Choose the genome → Write → Codon Usage of all Selected Features

All genes:

View → Choose the genome → SDS Genes and Products

## Appendix 9.5 Effect of bipyridyl, erythromycin and NaCl on Wbl:σ BATCH system

|  |  |  |
| --- | --- | --- |
| (*a*) | σ A σB σH σJ | (*b*) |
| WhiB1  WhiB2  WhiB3  WhiB7  controls | C:\Users\mbb\Desktop\BACTH system\Oct 2018 results نتائج الثيسس هنا\2HS +SIGJ\Bip2 done.jpg |  |
| (*c*) |  | (*d*) |
| WhiB1  WhiB2  WhiB3  WhiB7  controls | C:\Users\mbb\Desktop\BACTH system\Oct 2018 results نتائج الثيسس هنا\2HS +SIGJ\Ery done.jpg |  |
| (*e*) |  | (*f*) |
| WhiB1  WhiB2  WhiB3  WhiB7  controls | C:\Users\mbb\Desktop\BACTH system\Oct 2018 results نتائج الثيسس هنا\2HS +SIGJ\Salt done.jpg |  |
| Fig shows the analysis by BACTH and β-galactosidase assays after disruption by stress factors between *K. sedentarius* MBB13 Wbls and Sigma factors (σ) fused into the domains, pKT25 and pUT18 of *Bordetella pertussis* respectively in *E. coli* BTH101. The interactions detection were by (*a, c* and *e*) LB medium in the presence of X-gal and IPTG as an inducer with different stress, 0.05 mM 2,2’ Bipyrdyl, 64 µg/ml Erythromycin and Salt: 5 % NaCl respectively. (*b*, *d* and *f* ) Enhancing of the β-galactosidase activities between the fused partners interactions with different stress as indicated. Control cultures were uncut from the same domains for negative control, and fused with GCN4 leucine zipper (Zip-Zip) for positive control. Both plates and liquid cultures were supplemented with ampicillin, kanamycin and streptomycin. Dara points means of four replicates plus or minus standard deviation, signifies (t-test) is indicated. | | |

## Appendix 9.6 Growth curve of *K. sedentarius*

|  |
| --- |
|  |
| Fig shows the growth curve of *K. sedentarius* MBB13 in LB medium incubated at 37 °C over 30 h. Red arrows indicate the phase of growth that used to extract the RNA from cells as follows, ~ 0.7 = early exponential, ~ 1.8 = late exponential and ~ 2.3 = stationary. |

## Appendix 9.7 NCBI numbers of *M. tuberculosis* H37Rv, *S. coelicolor* A3, *C. glutamicum* MB001*, M. smegmatis* MC2 155 and *Mycobacteriophage* TM4

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Gene name in *K. sedentarius* MBB13 | *Mycobacterium tuberculosis* H37Rv (taxid:83332) | *Streptomyces coelicolor* A3(2) (taxid:100226) | *Corynebacterium glutamicum* MB001 (taxid:1310161) | *Mycobacterium smegmatis* MC2 155 | *Mycobacteriophage*  TM4  (taxid:88870) |
|  | Percentage / NCBI ID | | | | |
| KS-WhiB1 | 73.42%/  NP\_217735.1 | 65%/  NP\_631361.1 | 83%/  AGT04761.1 | 74.68/  YP\_886285.1 | No similarity |
| KS-WhiB2 | 76.32%/  NP\_217777.1 | 85.51%/  NP\_627256.1 | 85.96%/  AGT04734.1 | 77.63 %/  YP\_886201.1 | 53.12%  NP\_569784.1 |

## Appendix 9.8 Purification of WhiB1 in the presence of DTT

|  |  |
| --- | --- |
| (*a*) |  |
| (*b*) | 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 |
| 72~ kDa  24~ kDa  8~ kDa | C:\Users\mbb\Desktop\50mM tris, 500mM NaCl, 10mM DTT gel cut.jpg |
| Fig shows isolation of *K. sedentarius* MBB13 WhiB1 from *M. smegmatis* MC2 155 using the buffer indicated. (*a*) HiTrap chelating column of elution profile of cell lysate containing WhiB1, the blue trace shows the protein absorbance as indicated of the injected cell lysate, and the red trace shows the concentration of the imidazole gradient from 0-0.5 M. Eluted WhiB1 appears in the form as indicated. (*b*) Coomassie stained SDS-PAGE gel of the elution profile fractions. Lane 1, protein ladder (size indicated); lanes 2-5, invisible of non-specific bound proteins usually contain high histidine contents; lanes 6-15, fraction of [4Fe-4S]-, [2Fe-2S]- and Apo- WhiB1. | |

## Appendix 9.9 Purification of WhiB2 in the presence of DTT

|  |  |
| --- | --- |
| (*a*) |  |
| (*b*) | 1 2 3 4 5 6 7 8 9 10 11 |
| 72~ kDa  24~ kDa  9~ kDa | C:\Users\mbb\Desktop\OneDrive - sheffield.ac.uk\(100 mM Tris, 150 mM NaCl, 10 mM DTT, pH 7.4_24.8.18.jpg |
| Fig shows isolation of *K. sedentarius* MBB13 WhiB2 from *M. smegmatis* MC2 155 using the buffer indicated. (*a*) HiTrap chelating column of elution profile of cell lysate containing WhiB2, the blue trace shows the protein absorbance as indicated of the injected cell lysate, and the red trace shows the concentration of the imidazole gradient from 0-0.5 M. Eluted Apo-WhiB2 appears in the form as indicated. (*b*) Coomassie stained SDS-PAGE gel of the elution profile fractions. Lane 1, protein ladder (size indicated); lanes 2-6, visible and invisible of non-specific bound proteins usually contain high histidine contents; lanes 7-15, fractions of Apo- WhiB2. | |

## Appendix 9.10 Draft of the CD analysis of secondary structure of Holo- and Apo-WhiB1

|  |
| --- |
|  |
| Fig show a CD analysis of secondary structure of Holo- and Apo-WhiB1. The spectra of both samples (~35 µM cluster) were recorded in 20 mM NaH2PO4, 0.5 M NaCl, pH 7.4 at room temperature. The concentration affects negatively on figure reading in comparison to figure 6.10. |

## Appendix 9.11 Gene sequences after cloning

For the two hybrid system:

*whiB* (*wbl*) are fused in 25 fragment between XbaI (TCTAGA) and KpnI (GGTACC).

*Sigma factor* (σ) are fused in 18 fragment between HindIII (AAGCTT) and KpnI (GGTACC).

*whiA* was fused in both fragments (25 and 18).

Stop codon of gene fused in fragment T18 were removed.

*whiB3*, *σA*, *σB*, *σJ* and *whiA* are optimised and synthesised.

For the overexpression:

*whiB* (*wbl*) 1,2 and 7 are fused between NcoI (CCATGG) and HindIII (AAGCTT).

Base pairs in red are added to be in frame, in green are modified and in blue are the restriction enzymes.

(*a*) Plasmid sequence of the two hybrid system

WhiB1 ------------------------------------------------------------

WhiB1\_pKT25 TGACCAGCGGCGATTCGGTGACCGATTACCTGGCGCGCACGCGGCGGGCTGCAGGGTCGA

WhiB1 --------ATGGACTGGCGAAGCAAAGCGGCCTGTCTCGACGAGGACCCGGAACTGTTCT

WhiB1\_pKT25 CTCTAGAGATGGACTGGCGAAGCAAAGCGGCCTGTCTCGACGAGGACCCGGAACTGTTCT

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB1 TCCCCATCGGGAACACGGGCCCGGCCATCGAGCAGATCGAGAAGGCCAAGAAGGTCTGCG

WhiB1\_pKT25 TCCCCATCGGGAACACGGGCCCGGCCATCGAGCAGATCGAGAAGGCCAAGAAGGTCTGCG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB1 CGCGGTGCGAGGTCACAGAGACCTGCCTGCAGTGGGCGATCGAGACCGGCCAGGACGCCG

WhiB1\_pKT25 CGCGGTGCGAGGTCACAGAGACCTGCCTGCAGTGGGCGATCGAGACCGGCCAGGACGCCG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB1 GCGTGTGGGGCGGTCTCTCCGAGGACGAGCGCCGCGCCCTGAAGCGACGCAACGCGCGCG

WhiB1\_pKT25 GCGTGTGGGGCGGTCTCTCCGAGGACGAGCGCCGCGCCCTGAAGCGACGCAACGCGCGCG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB1 CCCGCCGCGCCAGCTGA-------------------------------------------

WhiB1\_pKT25 CCCGCCGCGCCAGCTGAGGTACCTAAGTAAGTAAGAATTCACTGGCCGTCGTTTTACAAC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB2 ----------GTGCACGAACTTCAGATCGTCGGACATCCCGAGCAGGCCGTTCCCAGTTG

WhiB2\_pKT25 GACTCTAGAGATGCACGAACTTCAGATCGTCGGACATCCCGAGCAGGCCGTTCCCAGTTG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB2 GCAGGAGCGCGCCCTGTGCGCCCAGACCGACCCCGAGGCCTTCTTCCCCGAGAAGGGTGG

WhiB2\_pKT25 GCAGGAGCGCGCCCTGTGCGCCCAGACCGACCCCGAGGCCTTCTTCCCCGAGAAGGGTGG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB2 CTCCACGCGCGAGGCCAAGCGCGTCTGCACCGGCTGCGACGTGCGTTCCGAGTGCCTCGA

WhiB2\_pKT25 CTCCACGCGCGAGGCCAAGCGCGTCTGCACCGGCTGCGACGTGCGTTCCGAGTGCCTCGA

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB2 CTACGCCCTCGAGAACGATGAGCGCTTCGGCATCTGGGGTGGCCTGTCCGAGCGGGAGCG

WhiB2\_pKT25 CTACGCCCTCGAGAACGATGAGCGCTTCGGCATCTGGGGTGGCCTGTCCGAGCGGGAGCG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB2 CCGCAAGCTCAAGCGGCGCGCGGTCTTCACCGCCTGA-----------------------

WhiB2\_pKT25 CCGCAAGCTCAAGCGGCGCGCGGTCTTCACCGCCTGAGGTACCTAAGTAAGTAAGAATTC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB3 ------------------------------------------------------------

WhiB3\_pKT25 CAGCGGCGATTCGGTGACCGATTACCTGGCGCGCACGCGGCGGGCTGCAGGGTCGACTCT

WhiB3 ----ATGGACAGCACGGCTCGCCAACCGGGCCCGGTTGCCGACTTGTGGGACTGGCAATT

WhiB3\_pKT25 AGAGATGGACAGCACGGCTCGCCAACCGGGCCCGGTTGCCGACTTGTGGGACTGGCAATT

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB3 TGAGGGATTGTGCCGTACCACAGACCCGGAAGAGTTCTTCCATCCGGAAGGTGAACGTGG

WhiB3\_pKT25 TGAGGGATTGTGCCGTACCACAGACCCGGAAGAGTTCTTCCATCCGGAAGGTGAACGTGG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB3 CTCTGCCCGTCGTCTGCGCGACGAGCGTGCTAAACGTGTCTGCCAACGCTGTCCTGTGAT

WhiB3\_pKT25 CTCTGCCCGTCGTCTGCGCGACGAGCGTGCTAAACGTGTCTGCCAACGCTGTCCTGTGAT

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WhiB3 TCTTGAGTGCCGTGAACACGCATTAGCAGCGAAGGAACCTTATGGAGTCTGGGGGGGCTT

WhiB3\_pKT25 TCTTGAGTGCCGTGAACACGCATTAGCAGCGAAGGAACCTTATGGAGTCTGGGGGGGCTT

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WhiB3 GAGCGAAGACGAACGTGAACAAGAACTGGCACGTCGTTCGCGTCGCGGGCTTCGTGGTGC

WhiB3\_pKT25 GAGCGAAGACGAACGTGAACAAGAACTGGCACGTCGTTCGCGTCGCGGGCTTCGTGGTGC

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WhiB3 ATGA--------------------------------------------------------

WhiB3\_pKT25 ATGAGGTACCTAAGTAAGTAAGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGA

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WhiB7 ------------------------------------------------------------

WhiB7\_pKT25 CCAGCGGCGATTCGGTGACCGATTACCTGGCGCGCACGCGGCGGGCTGCAGGGTCGACTC

WhiB7 -----ATGTTGGCCCTGGCAGATCACCAGACACTCATCGACCAAGCCCGTGTGGCCGGGC

WhiB7\_pKT25 TAGAGATGTTGGCCCTGGCAGATCACCAGACACTCATCGACCAAGCCCGTGTGGCCGGGC

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WhiB7 GCCCCCTGCCCTGCCTCGAGAACGACCCCGACACCTGGTTCGCCGACACCCCCACGGGTG

WhiB7\_pKT25 GCCCCCTGCCCTGCCTCGAGAACGACCCCGACACCTGGTTCGCCGACACCCCCACGGGTG

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WhiB7 TCGAGTACGCCAAGTCCCTGTGCCACGCCTGCCCCGTGCGCACCCTCTGCCTCGAGGGTG

WhiB7\_pKT25 TCGAGTACGCCAAGTCCCTGTGCCACGCCTGCCCCGTGCGCACCCTCTGCCTCGAGGGTG

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WhiB7 CCTTGGAACGCCGCGAGCCCTGGGGTGTGTGGGGCGGCGAGCTCATCGAGGCCGGCCGGG

WhiB7\_pKT25 CCTTGGAACGCCGCGAGCCCTGGGGTGTGTGGGGCGGCGAGCTCATCGAGGCCGGCCGGG

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WhiB7 TGCTGCCGCGAAAGCGTCCGCGAGGACGTCCGCGCAAGCACCCCATCGCGGCCTGA----

WhiB7\_pKT25 TGCTGCCGCGAAAGCGTCCGCGAGGACGTCCGCGCAAGCACCCCATCGCGGCCTGAGGTA

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WhiB7 ------------------------------------------------------------

WhiB7\_pKT25 CCTAAGTAAGTAAGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTG

whiA --------ATGACTGCCAGTCTGAAGGACGAGTTAAGCCGCGTTGTTGTGCCTACCGCAG

whiA\_pKT25 CTCTAGAGATGACTGCCAGTCTGAAGGACGAGTTAAGCCGCGTTGTTGTGCCTACCGCAG

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whiA AAGCTCGCAAAGCTGAAGTGAGTAGTTTGTTACGCTTCGCAGGTGCCCTTCATCTTGTCG

whiA\_pKT25 AAGCTCGCAAAGCTGAAGTGAGTAGTTTGTTACGCTTCGCAGGTGCCCTTCATCTTGTCG

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whiA GAGGCCGTGTAGTGATTGAAGCTGAAGTCGACACGGCAAGCGCAGCTCGTCGCACCCGCG

whiA\_pKT25 GAGGCCGTGTAGTGATTGAAGCTGAAGTCGACACGGCAAGCGCAGCTCGTCGCACCCGCG

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whiA CCGCGATTTCGGAATTGTACGGTCACGATTCAGAATTGTTAGTGTTATCAGCCGGCGGTC

whiA\_pKT25 CCGCGATTTCGGAATTGTACGGTCACGATTCAGAATTGTTAGTGTTATCAGCCGGCGGTC

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whiA TTCGCCGCACAACACGCTACGTAGTGCGTGTCGTTGAGGGAGGTCAAAGTTTAGCCCGTC

whiA\_pKT25 TTCGCCGCACAACACGCTACGTAGTGCGTGTCGTTGAGGGAGGTCAAAGTTTAGCCCGTC

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whiA AAACCGGGCTGCTGGACGCTCAGGGGCGCCCTGTTCGCGGGTTACCCGCACGCGTTGTTC

whiA\_pKT25 AAACCGGGCTGCTGGACGCTCAGGGGCGCCCTGTTCGCGGGTTACCCGCACGCGTTGTTC

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whiA GTGGACCGGTTGCCGCGGCGGAAGCTGCCTGGCGTGGCGCATTCTTGGCACGCGGGTCGC

whiA\_pKT25 GTGGACCGGTTGCCGCGGCGGAAGCTGCCTGGCGTGGCGCATTCTTGGCACGCGGGTCGC

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whiA TGACTGAGCCCGGCCGCTCCAGCTCGTTGGAGGTGACCTGCCCAGGTCCCGAAGCTGCTT

whiA\_pKT25 TGACTGAGCCCGGCCGCTCCAGCTCGTTGGAGGTGACCTGCCCAGGTCCCGAAGCTGCTT

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whiA TGGCTCTGGTTGGTGCCGCCCGTCGCTTGGATATTGCAGCGAAATCACGTGAAGTTCGTG

whiA\_pKT25 TGGCTCTGGTTGGTGCCGCCCGTCGCTTGGATATTGCAGCGAAATCACGTGAAGTTCGTG

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whiA GCGTGGACCGCGTCGTCGTACGTGACGGTGACGGCATTTCCGCATTATTAACTCGTATGG

whiA\_pKT25 GCGTGGACCGCGTCGTCGTACGTGACGGTGACGGCATTTCCGCATTATTAACTCGTATGG

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whiA GCGCGCACGACGCGGTCTTAGCGTGGGAAGAGCGTCGCATGCGTCGTGAAGTCCGTGGAA

whiA\_pKT25 GCGCGCACGACGCGGTCTTAGCGTGGGAAGAGCGTCGCATGCGTCGTGAAGTCCGTGGAA

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whiA CTGCCAATCGCCTTGCGAATTTCGATGACGCGAACTTGCGTCGTTCCGCGCGCGCCGCGG

whiA\_pKT25 CTGCCAATCGCCTTGCGAATTTCGATGACGCGAACTTGCGTCGTTCCGCGCGCGCCGCGG

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whiA TGGCTGCGAGTGCACGCGTGGCCCGCGCGATGGAGTTGCTTGGCGACGAAGTACCCGAAC

whiA\_pKT25 TGGCTGCGAGTGCACGCGTGGCCCGCGCGATGGAGTTGCTTGGCGACGAAGTACCCGAAC

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whiA ACCTTCGTGTAGCGGGTACATTACGCATGGAACACAAACAAGCCTCTCTGGAAGAATTGG

whiA\_pKT25 ACCTTCGTGTAGCGGGTACATTACGCATGGAACACAAACAAGCCTCTCTGGAAGAATTGG

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whiA GGCAGTTGGCGACTCCTCCTTTGACGAAGGACGCCGTCGCGGGTCGCATTCGCCGTCTGC

whiA\_pKT25 GGCAGTTGGCGACTCCTCCTTTGACGAAGGACGCCGTCGCGGGTCGCATTCGCCGTCTGC

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whiA TGGCAATGGCGGACCGCCGCGCTGCCGAATTGGGGGTTGATGATACCCGCGTCACCATCA

whiA\_pKT25 TGGCAATGGCGGACCGCCGCGCTGCCGAATTGGGGGTTGATGATACCCGCGTCACCATCA

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whiA GCGAAGCTGAGGTGGACGCCCTGTTAAGCGACGAAGCTGCGGGTGATGTTGCCCAGGCGA

whiA\_pKT25 GCGAAGCTGAGGTGGACGCCCTGTTAAGCGACGAAGCTGCGGGTGATGTTGCCCAGGCGA

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whiA CCGACACCCCGCAAGACTGA----------------------------------------

whiA\_pKT25 CCGACACCCCGCAAGACTGAGCGGTACCTAAGTAAGTAAGAATTCACTGGCCGTCGTTTT

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σA\_CTD -------------------------------ATGGACGCTGTCTCCTTCACCCTTTTGCA

σA\_CTD\_pUT18 AACAGCTATGACCATGATTACGCCAAGCTTGATGGACGCTGTCTCCTTCACCCTTTTGCA

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σA\_CTD GGAACAGCTTCATTCCGTGCTTGACACACTTAGCGAGCGTGAGGCGGGAGTAGTCTCAAT

σA\_CTD\_pUT18 GGAACAGCTTCATTCCGTGCTTGACACACTTAGCGAGCGTGAGGCGGGAGTAGTCTCAAT

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σA\_CTD GCGCTTTGGGTTAGCCGACGGCCAGCCGAAAACTCTGGATGAAATTGGGCGTGTCTATGG

σA\_CTD\_pUT18 GCGCTTTGGGTTAGCCGACGGCCAGCCGAAAACTCTGGATGAAATTGGGCGTGTCTATGG

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σA\_CTD CGTCACGCGCGAACGTATTCGTCAGATTGAATCCAAGACGATGTCTAAATTACGCCATCC

σA\_CTD\_pUT18 CGTCACGCGCGAACGTATTCGTCAGATTGAATCCAAGACGATGTCTAAATTACGCCATCC

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σA\_CTD TTCCCGTTCTCAAGTTCTTCGCGACTATTTAGACTGA-----------------------

σA\_CTD\_pUT18 TTCCCGTTCTCAAGTTCTTCGCGACTATTTAGACCGGGTACCGAGCTCGAATTCAGCCGC

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σB ------------------------------ATGATTCACGACGATTTTCCGGCCCGTACA

σB\_pUT18 ACAGCTATGACCATGATTACGCCAAGCTTGATGATTCACGACGATTTTCCGGCCCGTACA

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σB CATGGTGCATCTCATGCTGTACCGGACCAGGCTCTGGTCCGTCGCCGCTTAACGGCTGCC

σB\_pUT18 CATGGTGCATCTCATGCTGTACCGGACCAGGCTCTGGTCCGTCGCCGCTTAACGGCTGCC

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σB CACGCCTTGCCCGAACAACACCGCCAACAAGAACTTATTGATGTGATCGCAGATCATATC

σB\_pUT18 CACGCCTTGCCCGAACAACACCGCCAACAAGAACTTATTGATGTGATCGCAGATCATATC

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σB CCTTTCGCGCGCCGTCTTGGTCGTCGTTTCGCTCCCACTCCGTCCCTTGTAGACGATTGC

σB\_pUT18 CCTTTCGCGCGCCGTCTTGGTCGTCGTTTCGCTCCCACTCCGTCCCTTGTAGACGATTGC

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σB GAACAAGTTGCCTGTATGGCTCTGGTCTTAGCTGTTCAACGCTGGGATCCTGCGTTTGAC

σB\_pUT18 GAACAAGTTGCCTGTATGGCTCTGGTCTTAGCTGTTCAACGCTGGGATCCTGCGTTTGAC

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σB GCCAATTTGAGCAGTTACGCGCAGCCTACCATTCTTGGGGAATTGCGTCGCTTCTTACGT

σB\_pUT18 GCCAATTTGAGCAGTTACGCGCAGCCTACCATTCTTGGGGAATTGCGTCGCTTCTTACGT

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σB GACTCGACGTGGTGGGTCCGTCCTCCCCGTCGTATCCAGGAGTTGGCCGCGTTAGTACGC

σB\_pUT18 GACTCGACGTGGTGGGTCCGTCCTCCCCGTCGTATCCAGGAGTTGGCCGCGTTAGTACGC

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σB TCTACTGAGGAAGAACTTCGTCACAGTACTGGCCGTGAGCCCACTGCGCAGGAAGTCGCT

σB\_pUT18 TCTACTGAGGAAGAACTTCGTCACAGTACTGGCCGTGAGCCCACTGCGCAGGAAGTCGCT

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σB CGTGCTGTTGGGGCATCACCCGATGAGGTGTCGGAGGCTCGCGTCGCCGCCGCTGGGCGT

σB\_pUT18 CGTGCTGTTGGGGCATCACCCGATGAGGTGTCGGAGGCTCGCGTCGCCGCCGCTGGGCGT

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σB TACGTGGCTTCAGTTGACGAGGAAGACCCTGAAACAGGGCGTCTTGTACATCTGGTACAC

σB\_pUT18 TACGTGGCTTCAGTTGACGAGGAAGACCCTGAAACAGGGCGTCTTGTACATCTGGTACAC

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σB AGCCCTGCCGTCGATGAATGGGTATCGTTACACCCACATATCCAGGCCCTTGACCCTCGT

σB\_pUT18 AGCCCTGCCGTCGATGAATGGGTATCGTTACACCCACATATCCAGGCCCTTGACCCTCGT

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σB GACCGTTGCGTGCTGTTACGCCGCTATCTTGAAGATGAAACCCAAGCCTCCATTGCGCGT

σB\_pUT18 GACCGTTGCGTGCTGTTACGCCGCTATCTTGAAGATGAAACCCAAGCCTCCATTGCGCGT

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σB GCGCTTGGCATCAGTCAGGCACAAGTCTCACGCCGCTTGAAACGCGCACTGGATACGCTT

σB\_pUT18 GCGCTTGGCATCAGTCAGGCACAAGTCTCACGCCGCTTGAAACGCGCACTGGATACGCTT

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σB CGCGAGCAAGTTCCGGGAGGTTTATCGAATGCCTGA------------------------

σB\_pUT18 CGCGAGCAAGTTCCGGGAGGTTTATCGAATGCCCGGGGTACCGAGCTCGAATTCAGCCGC

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σH -------------------------------------ATGACACCCACCACCAGCGAGAA

σH\_pUT18 ACAGGAAACAGCTATGACCATGATTACGCCAAGCTTGATGACACCCACCACCAGCGAGAA

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σH GCCCCGCTCGGAGGTGAACAGCGACCCGGCAGAGGTCGACGTGGCCACCGAGACGCCCCA

σH\_pUT18 GCCCCGCTCGGAGGTGAACAGCGACCCGGCAGAGGTCGACGTGGCCACCGAGACGCCCCA

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σH GGAGCGCGCTGCGCGCTTCGAGCGCGAGGCGCTGCCCCATCTGGACCAGCTGTACAGCGC

σH\_pUT18 GGAGCGCGCTGCGCGCTTCGAGCGCGAGGCGCTGCCCCATCTGGACCAGCTGTACAGCGC

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σH GGCCCTGCGCACGACGCGTAACCCGACCGACGCCGAGGACCTGGTGCAGGAGACCTACGC

σH\_pUT18 GGCCCTGCGCACGACGCGTAACCCGACCGACGCCGAGGACCTGGTGCAGGAGACCTACGC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

σH CAAGGCGTACGCGGCGTTCCACCAGTACAAGCCGGGCACCAACCTCAAGGCGTGGATGTA

σH\_pUT18 CAAGGCGTACGCGGCGTTCCACCAGTACAAGCCGGGCACCAACCTCAAGGCGTGGATGTA

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σH CCGCATTCTCACCAACACGTACATCAACACCTACCGTAAGAAGCAGCGCCAGCCGCTGCA

σH\_pUT18 CCGCATTCTCACCAACACGTACATCAACACCTACCGTAAGAAGCAGCGCCAGCCGCTGCA

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σH GTCCGACGCCGCCGAGGTGGAGGACTACCAGTTGGCCGCAGCGGAGTCGCACACCGCCAA

σH\_pUT18 GTCCGACGCCGCCGAGGTGGAGGACTACCAGTTGGCCGCAGCGGAGTCGCACACCGCCAA

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σH GGGGCTGCGCTCCGCCGAGACCGAGGCGCTGGACCACATCGCCGACTCCCAAGTGACCGA

σH\_pUT18 GGGGCTGCGCTCCGCCGAGACCGAGGCGCTGGACCACATCGCCGACTCCCAAGTGACCGA

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σH CGCCCTGGCCCAGCTGAGTGAGGAGTTCCGCCTGGCGGTGTACCTGGCCGACGTGGAGGG

σH\_pUT18 CGCCCTGGCCCAGCTGAGTGAGGAGTTCCGCCTGGCGGTGTACCTGGCCGACGTGGAGGG

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σH CTTCGCCTACAAGGAGATCGCGGAGATCATGGACACGCCGATCGGCACCGTGATGTCTCG

σH\_pUT18 CTTCGCCTACAAGGAGATCGCGGAGATCATGGACACGCCGATCGGCACCGTGATGTCTCG

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σH CCTGCACCGGGGAAGAAAGCAGCTGCGGGAGCTGTTGGCCGAGTACGGAGCCGAACGCGG

σH\_pUT18 CCTGCACCGGGGAAGAAAGCAGCTGCGGGAGCTGTTGGCCGAGTACGGAGCCGAACGCGG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

σH CTTCGGCCGGCAGTCCCAAGGTAAGGAGGTCTCCTCGTGA--------------------

σH\_pUT18 CTTCGGCCGGCAGTCCCAAGGTAAGGAGGTCTCCTCGCGGGTACCGAGCTCGAATTCAGC

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σJ ---------------------------------------------------------ATG

σJ\_pUT18 AGATTGTACTGAGAGAAAGGCAATTGGGTACCGAGCTCGCGGCCGCAAGCAAGCTTGATG

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σJ GAAATGTCTCGTGTGGACCTGGAGCGTTTTGAGGTCGCCCGTGGACGTTTGGGTGCCGTC

σJ\_pUT18 GAAATGTCTCGTGTGGACCTGGAGCGTTTTGAGGTCGCCCGTGGACGTTTGGGTGCCGTC

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σJ GCTTATCGTCTGCTTGGGTCAGCTAGCGAAGCTGAAGATGTGGTACAAGAGTCCTTTGTC

σJ\_pUT18 GCTTATCGTCTGCTTGGGTCAGCTAGCGAAGCTGAAGATGTGGTACAAGAGTCCTTTGTC

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σJ CGTTGGCAGGCAGCCGACCGCGGTCGCATCGAAGTGCCCGTGGCGTGGTTGACTAAGGTT

σJ\_pUT18 CGTTGGCAGGCAGCCGACCGCGGTCGCATCGAAGTGCCCGTGGCGTGGTTGACTAAGGTT

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σJ GTTACCAATTTATGTTTAAACCAGCTTACCAGCGCACGTTCTCGTCGTGAAGAATACGTG

σJ\_pUT18 GTTACCAATTTATGTTTAAACCAGCTTACCAGCGCACGTTCTCGTCGTGAAGAATACGTG

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σJ GGTCAATGGCTGCCCGAGCCTCTGCTTGATGGGGATCCTATGTTAGGGCCTGCAGAAACC

σJ\_pUT18 GGTCAATGGCTGCCCGAGCCTCTGCTTGATGGGGATCCTATGTTAGGGCCTGCAGAAACC

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σJ TTGGAACAACGCGGGAGTGTTAGCTTAGCAATGTTGATGATCCTGGAAACTCTTAGTCCC

σJ\_pUT18 TTGGAACAACGCGGGAGTGTTAGCTTAGCAATGTTGATGATCCTGGAAACTCTTAGTCCC

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σJ ACCGAACGCGCAGTGTACGTGCTTCGCGAGGCGTTCGCTGTCCCTCATGGTGAAATTGCG

σJ\_pUT18 ACCGAACGCGCAGTGTACGTGCTTCGCGAGGCGTTCGCTGTCCCTCATGGTGAAATTGCG

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σJ GAAATTTTGGAAACTACGCCCGCTGCGACACAGCAAGCGTTATCCCGCGCTAAAAGCCGT

σJ\_pUT18 GAAATTTTGGAAACTACGCCCGCTGCGACACAGCAAGCGTTATCCCGCGCTAAAAGCCGT

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σJ ATCGCATCTTTGTCGCATCGTCACCGCACAGAAGCGGACCCTGTCGCTGCACGTGCTATC

σJ\_pUT18 ATCGCATCTTTGTCGCATCGTCACCGCACAGAAGCGGACCCTGTCGCTGCACGTGCTATC

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σJ GTAGAAGAGTTTTTGGCAGCCGCAACTAGCGGTCGCGTTGAAAATTTGGTCCGTCTGTTG

σJ\_pUT18 GTAGAAGAGTTTTTGGCAGCCGCAACTAGCGGTCGCGTTGAAAATTTGGTCCGTCTGTTG

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σJ ACTGATGACGCGTTCGGCATTGGGGACGGCGGAGGGGCGGTTCCCGCCCGTCCTAAACCA

σJ\_pUT18 ACTGATGACGCGTTCGGCATTGGGGACGGCGGAGGGGCGGTTCCCGCCCGTCCTAAACCA

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σJ GTCCTGGGGGCTCAGTCCGTCGCCAAGATGTTACGTGGATTAGCGGTTCCTTCAGCCGCC

σJ\_pUT18 GTCCTGGGGGCTCAGTCCGTCGCCAAGATGTTACGTGGATTAGCGGTTCCTTCAGCCGCC

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σJ AAGCGTGAGTTAGCAGGAGGCAGCTTAGATTGTCATTTCGCACTGGTCAATACATCCCCG

σJ\_pUT18 AAGCGTGAGTTAGCAGGAGGCAGCTTAGATTGTCATTTCGCACTGGTCAATACATCCCCG

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σJ GCCTTGGTGGCAGTAGTAGCGGGGCGCGTTGTAGGTGTAATCGTGCTTGATATTGCCGAC

σJ\_pUT18 GCCTTGGTGGCAGTAGTAGCGGGGCGCGTTGTAGGTGTAATCGTGCTTGATATTGCCGAC

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σJ GGACGTATCTCGGTGGTGCGTATTCAGGCCAATCCTCACAAACTTGACCGTGCGACTCGT

σJ\_pUT18 GGACGTATCTCGGTGGTGCGTATTCAGGCCAATCCTCCCAAACTTGACCGTGCGACTCGT

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σJ CGCTGGGCTGCTTCACCTCACGGTCGCCCGTTGCTGAGTGGGTGGTGA------------

σJ\_pUT18 CGCTGGGCTGCTTCACCTCACGGTCGCCCGTTGCTGAGTGGGTGGCGGGTACCACCTGCT

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whiA ------------------------------ATGACTGCCAGTCTGAAGGACGAGTTAAGC

whiA\_pUT18 ACAGCTATGACCATGATTACGCCAAGCTTGATGACTGCCAGTCTGAAGGACGAGTTAAGC

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whiA CGCGTTGTTGTGCCTACCGCAGAAGCTCGCAAAGCTGAAGTGAGTAGTTTGTTACGCTTC

whiA\_pUT18 CGCGTTGTTGTGCCTACCGCAGAAGCTCGCAAAGCTGAAGTGAGTAGTTTGTTACGCTTC

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whiA GCAGGTGCCCTTCATCTTGTCGGAGGCCGTGTAGTGATTGAAGCTGAAGTCGACACGGCA

whiA\_pUT18 GCAGGTGCCCTTCATCTTGTCGGAGGCCGTGTAGTGATTGAAGCTGAAGTCGACACGGCA

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whiA AGCGCAGCTCGTCGCACCCGCGCCGCGATTTCGGAATTGTACGGTCACGATTCAGAATTG

whiA\_pUT18 AGCGCAGCTCGTCGCACCCGCGCCGCGATTTCGGAATTGTACGGTCACGATTCAGAATTG

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whiA TTAGTGTTATCAGCCGGCGGTCTTCGCCGCACAACACGCTACGTAGTGCGTGTCGTTGAG

whiA\_pUT18 TTAGTGTTATCAGCCGGCGGTCTTCGCCGCACAACACGCTACGTAGTGCGTGTCGTTGAG

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whiA GGAGGTCAAAGTTTAGCCCGTCAAACCGGGCTGCTGGACGCTCAGGGGCGCCCTGTTCGC

whiA\_pUT18 GGAGGTCAAAGTTTAGCCCGTCAAACCGGGCTGCTGGACGCTCAGGGGCGCCCTGTTCGC

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whiA GGGTTACCCGCACGCGTTGTTCGTGGACCGGTTGCCGCGGCGGAAGCTGCCTGGCGTGGC

whiA\_pUT18 GGGTTACCCGCACGCGTTGTTCGTGGACCGGTTGCCGCGGCGGAAGCTGCCTGGCGTGGC

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whiA GCATTCTTGGCACGCGGGTCGCTGACTGAGCCCGGCCGCTCCAGCTCGTTGGAGGTGACC

whiA\_pUT18 GCATTCTTGGCACGCGGGTCGCTGACTGAGCCCGGCCGCTCCAGCTCGTTGGAGGTGACC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA TGCCCAGGTCCCGAAGCTGCTTTGGCTCTGGTTGGTGCCGCCCGTCGCTTGGATATTGCA

whiA\_pUT18 TGCCCAGGTCCCGAAGCTGCTTTGGCTCTGGTTGGTGCCGCCCGTCGCTTGGATATTGCA

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA GCGAAATCACGTGAAGTTCGTGGCGTGGACCGCGTCGTCGTACGTGACGGTGACGGCATT

whiA\_pUT18 GCGAAATCACGTGAAGTTCGTGGCGTGGACCGCGTCGTCGTACGTGACGGTGACGGCATT

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA TCCGCATTATTAACTCGTATGGGCGCGCACGACGCGGTCTTAGCGTGGGAAGAGCGTCGC

whiA\_pUT18 TCCGCATTATTAACTCGTATGGGCGCGCACGACGCGGTCTTAGCGTGGGAAGAGCGTCGC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA ATGCGTCGTGAAGTCCGTGGAACTGCCAATCGCCTTGCGAATTTCGATGACGCGAACTTG

whiA\_pUT18 ATGCGTCGTGAAGTCCGTGGAACTGCCAATCGCCTTGCGAATTTCGATGACGCGAACTTG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA CGTCGTTCCGCGCGCGCCGCGGTGGCTGCGAGTGCACGCGTGGCCCGCGCGATGGAGTTG

whiA\_pUT18 CGTCGTTCCGCGCGCGCCGCGGTGGCTGCGAGTGCACGCGTGGCCCGCGCGATGGAGTTG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA CTTGGCGACGAAGTACCCGAACACCTTCGTGTAGCGGGTACATTACGCATGGAACACAAA

whiA\_pUT18 CTTGGCGACGAAGTACCCGAACACCTTCGTGTAGCGGGTACATTACGCATGGAACACAAA

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA CAAGCCTCTCTGGAAGAATTGGGGCAGTTGGCGACTCCTCCTTTGACGAAGGACGCCGTC

whiA\_pUT18 CAAGCCTCTCTGGAAGAATTGGGGCAGTTGGCGACTCCTCCTTTGACGAAGGACGCCGTC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA GCGGGTCGCATTCGCCGTCTGCTGGCAATGGCGGACCGCCGCGCTGCCGAATTGGGGGTT

whiA\_pUT18 GCGGGTCGCATTCGCCGTCTGCTGGCAATGGCGGACCGCCGCGCTGCCGAATTGGGGGTT

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA GATGATACCCGCGTCACCATCAGCGAAGCTGAGGTGGACGCCCTGTTAAGCGACGAAGCT

whiA\_pUT18 GATGATACCCGCGTCACCATCAGCGAAGCTGAGGTGGACGCCCTGTTAAGCGACGAAGCT

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiA GCGGGTGATGTTGCCCAGGCGACCGACACCCCGCAAGACTGA------------------

whiA\_pUT18 GCGGGTGATGTTGCCCAGGCGACCGACACCCCGCAAGACGCGGTACCGAGCTCGAATTCA

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\* .

*(b)* Plasmid sequence of the overexpression system

whiB1 -------ATGGACTGGCGAAGCAAAGCGGCCTGTCTCGACGAGGACCCGGAACTGTTCTT

whiB1\_pMyNT GGGCGCCATGGACTGGCGAAGCAAAGCGGCCTGTCTCGACGAGGACCCGGAACTGTTCTT

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB1 CCCCATCGGGAACACGGGCCCGGCCATCGAGCAGATCGAGAAGGCCAAGAAGGTCTGCGC

whiB1\_pMyNT CCCCATCGGGAACACGGGCCCGGCCATCGAGCAGATCGAGAAGGCCAAGAAGGTCTGCGC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB1 GCGGTGCGAGGTCACAGAGACCTGCCTGCAGTGGGCGATCGAGACCGGCCAGGACGCCGG

whiB1\_pMyNT GCGGTGCGAGGTCACAGAGACCTGCCTGCAGTGGGCGATCGAGACCGGCCAGGACGCCGG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB1 CGTGTGGGGCGGTCTCTCCGAGGACGAGCGCCGCGCCCTGAAGCGACGCAACGCGCGCGC

whiB1\_pMyNT CGTGTGGGGCGGTCTCTCCGAGGACGAGCGCCGCGCCCTGAAGCGACGCAACGCGCGCGC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB1 CCGCCGCGCCAGCTGA--------------------------------------------

whiB1\_pMyNT CCGCCGCGCCAGCTGAAAGCTTATCGATGTCGACGTAGTTAACTAGCGTACGATCGACTG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB2 -------GTGCACGAACTTCAGATCGTCGGACATCCCGAGCAGGCCGTTCCCAGTTGGCA

whiB2\_pMyNT GGGCGCCATGGACGAACTTCAGATCGTCGGACATCCCGAGCAGGCCGTTCCCAGTTGGCA

\*\* \*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

WhiB2 GGAGCGCGCCCTGTGCGCCCAGACCGACCCCGAGGCCTTCTTCCCCGAGAAGGGTGGCTC

whiB2\_pMyNT GGAGCGCGCCCTGTGCGCCCAGACCGACCCCGAGGCCTTCTTCCCCGAGAAGGGTGGCTC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB2 CACGCGCGAGGCCAAGCGCGTCTGCACCGGCTGCGACGTGCGTTCCGAGTGCCTCGACTA

whiB2\_pMyNT CACGCGCGAGGCCAAGCGCGTCTGCACCGGCTGCGACGTGCGTTCCGAGTGCCTCGACTA

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB2 CGCCCTCGAGAACGATGAGCGCTTCGGCATCTGGGGTGGCCTGTCCGAGCGGGAGCGCCG

whiB2\_pMyNT CGCCCTCGAGAACGATGAGCGCTTCGGCATCTGGGGTGGCCTGTCCGAGCGGGAGCGCCG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB2 CAAGCTCAAGCGGCGCGCGGTCTTCACCGCCTGA--------------------------

whiB2\_pMyNT CAAGCTCAAGCGGCGCGCGGTCTTCACCGCCTGAAAGCTTATCGATGTCGACGTAGTTAA

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB7 --------ATGTTGGCCCTGGCAGATCACCAGACACTCATCGACCAAGCCCGTGTGGCCG

whiB7\_pMyNT AGGGCGCCATGGTGGCCCTGGCAGATCACCAGACACTCATCGACCAAGCCCGTGTGGCCG

\*\*\* \*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB7 GGCGCCCCCTGCCCTGCCTCGAGAACGACCCCGACACCTGGTTCGCCGACACCCCCACGG

whiB7\_pMyNT GGCGCCCCCTGCCCTGCCTCGAGAACGACCCCGACACCTGGTTCGCCGACACCCCCACGG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB7 GTGTCGAGTACGCCAAGTCCCTGTGCCACGCCTGCCCCGTGCGCACCCTCTGCCTCGAGG

whiB7\_pMyNT GTGTCGAGTACGCCAAGTCCCTGTGCCACGCCTGCCCCGTGCGCACCCTCTGCCTCGAGG

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB7 GTGCCTTGGAACGCCGCGAGCCCTGGGGTGTGTGGGGCGGCGAGCTCATCGAGGCCGGCC

whiB7\_pMyNT GTGCCTTGGAACGCCGCGAGCCCTGGGGTGTGTGGGGCGGCGAGCTCATCGAGGCCGGCC

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB7 GGGTGCTGCCGCGAAAGCGTCCGCGAGGACGTCCGCGCAAGCACCCCATCGCGGCCTGA-

whiB7\_pMyNT GGGTGCTGCCGCGAAAGCGTCCGCGAGGACGTCCGCGCAAGCACCCCATCGCGGCCTGAA

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

whiB7 ------------------------------------------------------------

whiB7\_pMyNT AGCTTATCGATGTCGACGTAGTTAACTAGCGTACGATCGACTGCCAGGCATCAAATAAAA