Investigation of STM3071 as a potential regulator of cobalt transport in *Salmonella enterica*

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Abstract

Using bioinformatics we have identified *stm3071* as a possible regulator of anaerobically induced genes involved in metal homeostasis (Price-Carter *et al.,* 2001) and the aim of this study is to determine the function of *stm3071* and define the conditions that induce its expression. Cobalt is required for incorporation into cobalamin (vitamin B12) which is important during *S*. Typhimurium infection. Vitamin B12 is synthesised *de novo* under anaerobic conditions and is required for metabolism of 1,2-propanediol and ethanolamine which act as sources of carbon and nitrogen when *Salmonella* is in the gut (Raux *et al*., 1996; Thiennimitr *et al*., 2011). Therefore, sensing Co^{2+} from the environment, and maintaining Co^{2+} homeostasis, to avoid metal-mediated toxicity, is required for vitamin B12 biosynthesis.

Using λ-red based mutagenesis we have constructed a deletion mutant in order to investigate the function of *stm3071*. We examined the effect of mutation on the utilisation of 1,2-propanediol under anaerobic conditions and ability to produce vitamin B12. We have also tested the effect of mutation on tolerance to cobalt both aerobically and anaerobically. In order to monitor conditions in which $P_{stm3071}$ is switched on, a $P_{stm3071::lacZ}$ transcriptional fusion was constructed in plasmid pRS415. Levels of β-galactosidase activity were measured in the presence of cobalt in both *Δstm3071* and SL1344 (wild type strain) under anaerobic conditions.

Anaerobic growth experiments and B12 assays showed that *stm3071* is not essential for growth or synthesis of vitamin B12. In addition, cobalt tolerance in both aerobic and anaerobic conditions was unaffected. However, as measured by β-galactosidase assay, our data suggests that $P_{stm3071}$ expression is induced in the presence of cobalt in the deletion mutant. In contrast, we observed no difference in expression of $P_{stm3071}$ in the presence or absence of cobalt in SL1344.

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Abbreviations

°C Degree Celsius

µM Micromolar

amp ampicillin

ABC ATP-binding cassette

AMPs antimjicrobial peptides

ATP adenosine triphosphate

BCA bicinchoninic acid

BLAST Basic local Alignment Search Tool

bp base pair

BSA bovine serum albumin

CAMPs cationic host defence peptides

cat chloramphenicol

CBP Corticosteroid-Binding Protein

CFU Colony Forming Unit

CNB12 cyanocobalamin

COG cluster of orthologue

DNA Deoxyribonucleic acid

DTT Dithiothreitol

EDTA Ethylenediaminetetraacetic acid

ECF energy coupling factor

EMSA electromobility shift assay

h hour

HIF-α hypoxia inducible factor alpha

IPTG Isopropyl-β-Dthiogalactopyranoside

kb Kilo base

kDa Kilo Dalton

LB Luria-Bertani medium

Log Logarithmic phase

LPS lipopolysaccharide

M molar

min minute

ml millilitre

MW molecular weight

NCBI National Center for Biotechnology Information

NCE Non carbon essential medium

NO Nitric oxide

NRAMP Natural Resistance Associated pattern

OD Optical density

ONPG ortho-nitrophenyl-β-galactoside

ORF Open reading frame

PBS Phosphate buffered saline

PCR Polymerase chain reaction

pM Picomolar

PEP phosphoenolopyruvate

PPD 1,2-propanediol

PPC plants and prokaryotes conserved

RNA Ribonucleic acid

RPM Revolutions per minute

S second

SCV *Salmonella* containing vacuole

SDS PAGE Polyacrylamide gel electrophoresis

SPI *Salmonella* pathogenicity island

T1SS Type I secretion system

T3SS Type III secretion system

T5SS Type V secretion system

TCA cycle tricarboxylic acid cycle

tet tetracycline

TF transcription factor

TLCC Taylor Laboratory Culture Collection

TLRs Toll-like receptors

TNF Tumor necrosis factor

WT Wild type

1 Introduction

1.1 The genus *Salmonella*

The genus *Salmonella* is classified into two species, *Salmonella enterica* and *Salmonella bongori,* which are thought to have diverged from a common ancestor more than 100 million years ago (Doolittle *et al.*, 1996) . *Salmonella enterica* has been intensely studied and there are six known subspecies: *enterica, salamae, arizonae, diarizonae, houtenae and indica* (Brenner *et al*., 2000; Crosa *et al*., 1973). In contrast, there has been scant investigation of *S. bongori* and our knowledge of this species is therefore limited, although recent work suggests that *S. bongori* lies evolutionarily somewhere between *Salmonella* and *E. coli* (Fookes *et al*., 2011).

1.1.1 *Salmonella enterica*

S. enterica subspecies are further subdivided into at least 2500 serovars. Serovars are determined by serotype which are further categorised into six sub-groups using the Kauffman-White scheme. This scheme discriminates serotypes based on variations in the lipopolysaccharide (O antigen), capsular antigen (K or Vi antigen) and flagellar antigens (H antigen) (White 1926, Kauffman 1966). Notably, Vi antigens are only found in three pathogenic serovars: Typhi, Paratyphi C and Dublin (Eng *et al*., 2015). Given the complexity of subspecies and serovar naming, salmonellae are normally described by omitting the subspecies. For example, *Salmonella enterica* subspecies *enterica* serotype Typhimurium, is shortened to *Salmonella* ser. Typhimurium or *S.* Typhimurium (Brenner *et al*., 2000).

1.2 Importance of *Salmonella* as a pathogen

According to epidemiological analysis there are 93.8 million cases of human gastroenteritis caused by *Salmonella* species occurring globally each year, of which 80.3 million are foodborne and 155,000 are fatal (Majowicz *et al*., 2010). Approximately 99% of these infections are caused by *S. enterica* subsp. *enterica* (I).

Salmonella infections affect mostly elderly, very young and immunocompromised patients. *S.* Typhimurium experimental infection in mice can be used as a model for the life-threatening human pathogen *S.*Typhi that

causes typhoid fever. Although the incidence of *S.* Typhi infections worldwide is decreasing, the occurrence of non-typhoidal salmonellosis is increasing (Majowicz *et al*., 2010). In addition, due to the increasing incidence of antibiotic resistant isolates of *S.* Typhimurium (Yoke-Kqueen *et al.*, 2008) an understanding of how the pathogen survives and investigation of unknown virulence factors is crucial and urgently required for developing novel antimicrobial strategies.

1.3 Clinical manifestations

Given the clinical outcomes of *Salmonella* infections in humans, salmonellae are broadly grouped into either typhoid or non-typhoid *Salmonella* (NTS). There are four main clinical manifestations seen in humans which are gastroenteritis, bacteraemia, enteric fever, and chronic carrier state (Darby & Sheorey, 2008).

1.1.1 Gastroenteritis

Gastroenteritis is an inflammatory condition of the gastrointestinal tract, caused by NTS, usually accompanied by symptoms such as nausea, diarrhoea, vomiting and abdominal pain. Such infections are considered ‘mild’ with a short incubation period of (6 – 12 h) and are usually self-limiting (Crump *et al*., 2008). At risk groups are more susceptible to NTS infections and tend to experience more severe symptoms.

1.1.2 Bacteraemia

Bacteraemia can occur when salmonellae invade the intestinal barrier and end up in the bloodstream. If the infection is severe, septic shock can lead to death. Although almost all serotypes of *Salmonella* can cause bacteraemia, it is most commonly associated with NTS infection where approximately 5% of those infected will develop bacteraemia (Woods *et al*., 2008)

1.1.3 Enteric fever

Enteric fever is the collective term for typhoid fever, caused by *S.* Typhi, and paratyphoid fever, caused by *S.* Paratyphi A, B and C (Connor & Schwartz, 2005). Enteric fever is the exclusively human infection as the serovars involved only affect humans. Infection arises following ingestion of bacteria in contaminated water or food, or following exposure to contaminated faeces. Incubation period is usually at least one week and initial gastroenteritis-like

symptoms are followed by the onset of fever (Bhan *et al*., 2005). As well as developing high grade fever (> 38.2°C to 41.5°C) in the second week of infection, patients can go on to develop further complications such as slow heart rate, muscle pain, enlarged liver, enlarged spleen and rose spots which can appear on the chest and abdomen. In serious cases, haemorrhage can occur which result in bloody diarrhoea (Kuvandik *et al*., 2009).

1.1.4 Chronic carrier state

Approximately 4% of patients with enteric fever may become chronic carriers of *S.* Typhi *or* Paratyphi bacteria (Bhan *et al*., 2005). This is defined as the shedding of bacteria in the stools for more than a year after the infection. In contrast, NTS is associated with only a 0.1% likelihood of carrier state (Hohmann, 2001).

1.4 Pathogenicity, transmission and disease

Salmonella infects both human and animal hosts and is a major cause of diseases worldwide such as enteric fever, gastroenteritis, bacteraemia and systemic infection (Klumpp & Fuchs, 2007). Given the relevant safety of the cultivation and natural properties allowing rather uncomplicated genetic manipulation, *S. enterica* was extensively studied and served as a model to understand major mechanisms of the infection process.

Salmonella is able to penetrate the intestinal epithelial barrier and to disseminate to the underlying Peyer's patch (Figure 1.1) (Fields *et al*., 1986). After ingestion, bacteria adhere to ileum of an intestinal mucosa that is nearest to the ileocecal valve and becomes translocated via membranous epithelial (M) cells. M cells are located in the specialised follicle-associated epithelium (FAE) overlying lymphoid aggregates, for instance Peyer's patches (de Jong *et al*., 2012; Fields *et al.*, 1986). M cell features promote adherence and uptake of microbes within the lumen which are then translocated to the underlying epithelium lymphoid tissue and where the immune response is triggered (Kraehenbuhl & Neutra, 2000; Sansonetti, 2004). It was previously demonstrated that *Salmonella* preferentially invades the host through the M cells (Clark *et al.*, 1994; Jones *et al*., 1994; Kohbata *et al*., 1986; Pascopella *et al.*, 1995). The uptake of the bacterium also takes place via engulfment. Antigen presenting cells, such as macrophages can recognise and engulf *Salmonella*

which describes an alternative way of entry. *Salmonella* also evolved the ability to invade the enterocytes where it also manipulates cellular structure to cross epithelial barriers via dendritic cells (Figure 1.1) (Guo *et al.*, 2007; Sansonetti, 2004). Once the bacteria cross the mucosal barrier and invade the organism reaching the intestinal lymphoid follicles and lymph nodes, some pass through to the reticuloendothelial cells and then are spread to liver and spleen (de Jong *et al*., 2012).

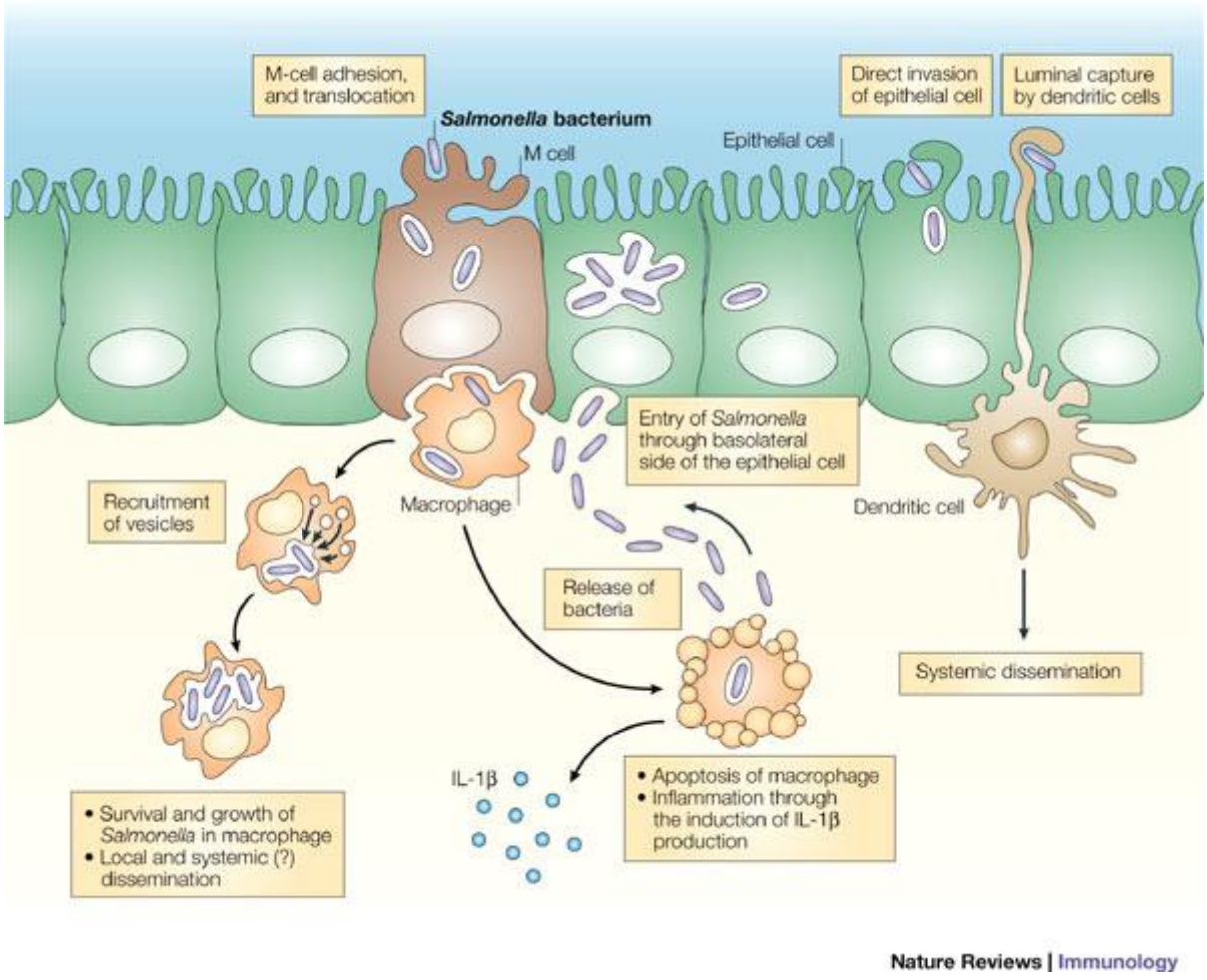


Figure 1.1. Schematic representation of strategies that allow *Salmonella* spp. to invade and survive within the host (Sansonetti, 2004). *Salmonella* can cross the epithelial barrier via M cells localised in the Peyer's patches in the ileum and possibly colon. Then in subepithelial location can cause macrophage apoptosis using injector effectors of the T3SS-1 expressed from SPI-1 and at the same time induce inflammation. *Salmonella* can also switch and express SPI-2 which encodes T3SS-2 effectors which allow injection of effector proteins and formation of the SCV. That in turn enables bacterium to survive and multiply intracellularly. These strategies can trigger infection and systemic dissemination at the same time. Alternatively, *Salmonella* can cross the epithelial barrier through the apical pole or pseudopods of the dendritic cells, which also supports systemic dissemination of *Salmonella* (Boumart & Velge, 2014; de Jong *et al*., 2012; Sansonetti, 2004).

1.1.5 Virulence factors

Salmonella are thought to have evolved as a pathogen some 100 – 150 million years ago (Doolittle *et al*., 1996) and the genome encodes a variety of factors which determine virulence and therefore pathogenicity. Several of these are located at discrete genomic loci while several are encoded on the pSLT virulence plasmid (Gulig & Doyle, 1993).

1.1.6 Salmonella pathogenicity islands

Virulence factors of *Salmonella* are determined by virulence genes located within pathogenicity islands known as *Salmonella*-pathogenicity islands or SPIs (Schmidt & Hensel, 2004). It was established that the presence of virulence determinants was acquired by the horizontal gene transfer in the process of evolutionary adaptation to the host (A J Bäumler, 1997; Rabsch *et al*., 2002). At least 21 SPIs have been identified in the salmonellae to date (Sabbagh *et al.*, 2010). However, *S*. Typhi and *S*. Typhimurium are known to share eleven islands (SPIs1-6, 9, 11, 12, 13 & 16) (Table 1.1) and some of these key SPIs (SPIs1-5) will be discussed briefly.

SPI-1 genes encodes a TS33 along with the *sitABCD* metal transport operon (Mills *et al*., 1995). The T3SS is required for invasion of non-phagocytic cells and mediation of the translocation of effector proteins into eukaryotic host cells (de Jong *et al*., 2012; Galán, 2001) while the sit genes are required for later stages of infection. SPI-1 genes are expressed under conditions determined by the host microenvironment, such as pH, osmolarity, and growth phase. According to Schmidt & Hensel, 2004 low oxygen and high osmolarity induce invasiveness; while in contrast conditions of high oxygen silence invasiveness (Schmidt & Hensel, 2004). There are two-component system global regulators such as PhoPQ, EnvZ/OmpR, BarA/SirA, PhoRB and FliZ and Hha, which are involved in activation of the virulence cascade (Figure 1.2) (Schmidt & Hensel, 2004.)

SPI-2 encodes another T3SS required for intracellular survival which is needed for the systemic phase of the disease and proliferation and survival within host cells (Ochman *et al.*, 1996). T3SS components encoded on SPI-2 are used by bacteria inside the phagosome to prevent phagosome-lysosome fusion and enable *Salmonella* containing vacuole formation (SCV) (Coburn *et al*., 2007).

Major effectors of T3SS-2 encoded on SPI-2 involve SpiC, an actin-binding protein that interferes with the hosts cell's actin cytoskeleton (Hayward & Koronakis, 1999), SseF and SseG that interact to ensure intracellular positioning of SCV (Deiwick *et al*., 2006). There are also effectors of T3SS-2 involved in the process, which are not encoded on SPI-2. These are SifA, that maintains phagosomal integrity of the SCV (Ohlson *et al.*, 2008), SifB, PipB, PibB2, SseI, SseJ, SseL, SspH2, and GogB (Lavigne & Blanc-Potard, 2008). In addition, SPI-2 encodes genes for tetrathionate reductase to enable anaerobic growth. However, these are not essential for virulence (Sabbagh *et al*., 2010).

Island	Size (kb)	Virulence function(s)
SPI-1	38.8	T3SS-1, invasion, proinflammatory responses, divalent metal cation uptake
SPI-2	39.8	T3SS-2, intracellular survival, tetrathionate respiration
SPI-3 (I)	17.3	Colonisation of GI tract, magnesium uptake (SPI-3 (II) & (III) distributed in *S. bongori* and subspecies)
SPI-4	23.4	T1SS, colonisation of cattle GI tract
SPI-5	7.6	T3SS-1 and T3SS-2 effectors, enteropathogenesis
SPI-6	47/58.9	Saf and Tcf fimbriae
SPI-7	133.6	Vi antigen, SopE prophage, invasion, enteropathognesis, type IV pili
SPI-9	16.3	Biofilm formation, intestinal colonisation
SPI-11	14.0	Intramacrophage survival, serum resistance
SPI-12	6.3	TTSS-2 effector
SPI-13	19.5	Virulence in chicks
SPI-16	4.5	Serotype conversion

Table 1.1 Pathogenicity islands shared by *S.* Typhiumurium and *S.* Typhi and roles in virulence. Adapted from (Gerlach & Hensel, 2007).

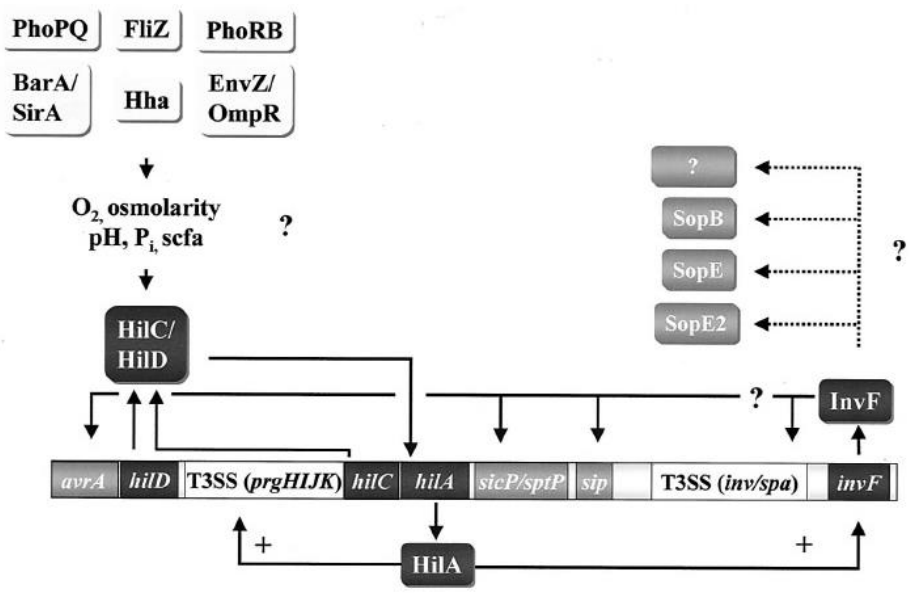


Figure 1.2. Schematic representation of virulence genes expression regulation in *S. enterica*. Environmental factors have been described to modulate expression of SPI-1 genes. Virulence genes are also co-regulated by regulatory systems PhoPQ, BarA/SirA, EnvZ/OmpR, PhoRB, FliZ and Hha. HilC/HilD displaces a repressor from *hilA* promoter site which cause HilA to bind to *invF* and *prgH* promoter site, which induces transcription of InvF and PrgH regulating the expression of T3SS virulence genes encoded on SPI-1. Adapted from (Schmidt & Hensel, 2004).

SPI-3 genes encode for factors important for intracellular survival. This pathogenicity island encodes a magnesium transporter MgtCB which is commonly present in other intracellular pathogens (Belon *et al.*, 2015). The *mgtCB* operon encodes MgtC, macrophage survival protein, and magnesium transport protein MgtB, whose expression is controlled by PhoPQ regulatory system (Blanc-Potard *et al.*, 1999). SPI-3 was found to also encode for MisL representing a T5SS (type V secretion system) which is known to be similar to VirG protein from *Shigella flexneri* and AIDA-1 protein from entropathogenic *E. coli*. This suggests autotransporter functios responsible for cell-to-cell spread are found to enable *S.* Typhimurium to bind to fibronectin (Blanc-Potard *et al.*, 1999; Dorsey *et al.*, 2005).

SPI-4 forms an insertion region of 25 kb similar to those encoding for T1SS toxins and effectors required for survival in macrophages (Schmidt & Hensel, 2004). SPI-4 encodes for SiiE which describes a giant non-fimbrial adhesion protein (600 kDa) that was found to mediate binding to the apical side of the polarised epithelial cells (Wagner *et al.*, 2014).

SPI-5 mostly encodes for effector proteins for SPI-1 T3SS-1 including SopA, SopB, SopD, SopE, SopE2 and Slrp (Lavigne & Blanc-Potard, 2008; Schmidt & Hensel, 2004). This locus is relatively small (7.6 kb) but encodes important effectors that take part in key pathogenic events such as membrane ruffling followed by bacterial internalisation (McGhie *et al.*, 2009).

1.1.7 Invasion

For some time it was considered that the only invasion mechanism occurred via a T3SS-1 route also known as a trigger mechanism which involves T3SS-1 and bacterial effector proteins (Coburn *et al.*, 2007). However, it is now known that another mechanism, called the zipper mechanism is involved in invasion of non-phagocytic host cells. Trigger mechanism involves cytoskeletal changes that are described as ‘membrane ruffling’ (Schlumberger & Hardt, 2006). Effector proteins involve SipA, SipC, SopB and SopE2. SipA together with SipC bind actin and prevent its depolymerisation (McGhie *et al*., 2009). Then the effector proteins SopB, SopE and SopE2 stimulate Rho GTPases family Rac1 and Cdc42, which in turn initiate actin polymerisation via the Arp2/3 complex (Zhou & Galán, 2001). Then, once inside the cell, effector SptP inactivates Rho

GTPases allowing restoration of actin and at the same time bringing the invaded cell to its original state (Murli *et al*., 2001). Other T3SS effectors subsequently modulate the immune response and survival within the host cell. In contrast, the zipper mechanism is initiated by the interaction of a bacterial ligand and host cell receptor, which in turn activates a signalling cascade leading to localised accumulation of actin and as a result, minor cytoskeletal rearrangements (Rosselin *et al.*, 2010).

1.5 Adaptation to intracellular lifestyle

1.1.8 *Salmonella*-containing vacuole formation

Salmonella is taken up by host cells either by T3SS-1 triggered micropinocytosis or via phagocytosis. Effector proteins are then translocated via T3SS-2 into the host cell. Following internalisation, the bacterium is enclosed in the phagosome which then fuses with the lysosome, acidifies and shrinks down to become adherent to the bacterium – a process known as *Salmonella* containing vacuole formation and described in the Figure 1.4 (Haraga *et al*., 2008).

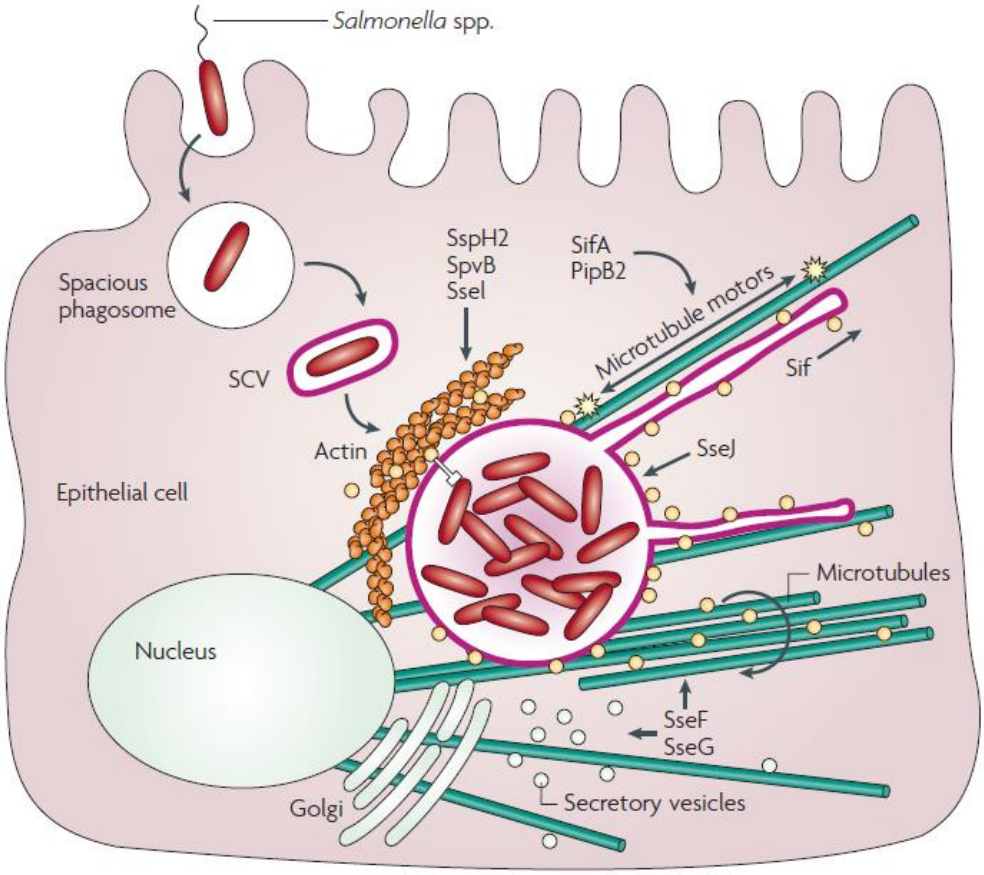


Figure 1.4. SCV formation and maturation process (Haraga *et al*., 2008)**.** Upon micropinocytosis, a phagosome fuses with a lysosome which results with SCV. Then SCV undergoes the maturation process, which is characterised by specific endosomal markers. Lysosomal associated membrane proteins LAMP-1, LAMP-2 and LAMP-3 endosomal markers and GTPase Rab7 as well as vacuolar ATPase are associated with trafficking and later time points of SCV maturation. SPI-2 T3SS-2 is induced within the SCV and triggers expression of the afore mentioned effectors. SifA and BipB2 induce formation of *Salmonella*-induced filament (Sif) and regulation of the microtubules – motor accumulation of the Sif and SCV. SseJ is a deacylase and SseF together with SseG act on microtubules. SCV can persist intracellularly from hours to days. SifA, SifB and SseJ proteins localise to SCV and to *Salmonella*-induced filaments (Sifs).

The bacterium senses the acidic environment of the SCV which initiates the cascade of various regulatory systems that promote intracellular survival including OmpR/EnvZ, PhoP/PhoQ, RpoS/RpoE, PmrA/PmrB, Cya/Cyp as well as cyclic diGMP which are involved in resistance to oxidative stress and to antimicrobial peptides (Sarnacki *et al.,* 2013). Acidic environment of the SCV and concentration of magnesium and calcium ions at 1 mM have been observed to influence *Salmonella* virulence (Hicks *et al.*, 2015; Mouslim & Groisman, 2003). There are sensory systems that determine the response to the phagosomal environment and trigger the cascade events implicated in intracellular survival. PhoQ sensor promotes resistance to the antimicrobial peptides (Prost *et al*., 2007), the domain is bridged to the inner membrane aided with metal ion interactions that are responsible for antimicrobial peptide sensing (Gunn & Miller, 1996; Prost *et al*., 2007). Upon phagocytosis bacterial surface components undergo serious remodelling and synthesis of enzymes responsible for dealing with oxidative and nitrogenous radicals also take place (Haraga *et al*., 2008).

1.1.9 Adaptation within the SCV

Microarray studies carried out by Eriksson and colleagues (2003) identified changes in expression of 919 out of 4451 coding sequences of *S.* Typhimurium of which 384 were upregulated and 535 downregulated (Eriksson *et al*., 2002). In the study, expression profiles of bacterial genes from 4 h after infection in murine macrophages were compared to expression profiles of bacteria grown to stationary phase *in vitro*. Interestingly, results showed that only a small proportion of genes were upregulated suggesting that intracellular bacteria were not in a classical stationary phase (Eriksson *et al*., 2002a). These changes in expression strongly suggest that during transition from the nutrient rich environment to the SCV *Salmonella* undergoes dramatic changes. Additionally, 4 h post-infection most genes associated with the *sifA* gene were upregulated; SPI-2 regulatory genes were observed to be slightly induced, genes encoding for SPI-2 secretion components were moderately induced and genes responsible for encoding SPI-2 effectors were strongly upregulated. Conversely, SPI-1 genes and LPS encoding genes were found to be down-regulated. Results from Eriksson microarray study only confirm, that a strict switch in the

regulation of gene expression takes places in *Salmonella* upon phagocytosis events leading to SCV formation.

The alternative sigma factors were found to also play role in the response to nutritional stress associated with the transition to the SCV. Sigma factor RpoS controls the expression of *Salmonella* plasmid virulence *spv* genes that are important in establishing systemic infection (Nickerson & Curtiss, 1997). It was revealed that deletion of RpoS resulted in 10-fold decreased virulence in comparison to the WT *S*. Typhimurium (Kazmierczak *et al*., 2005). It was also found that RpoE was responsible for bacterial homeostasis and membrane integrity in response to extracytoplasmic stress (such as oxidative stress) and deletion of *rpoE* resulted in *S.* Typhimurium lethal phenotype for intracellular survival in macrophages (Li *et al.*, 2015). It can however be summarised that RpoE and RpoS somewhat overlap functionally; both are important for virulence and loss of one of them can be functionally compensated by the other (Du *et al*., 2011; Humphreys *et al.*, 1999).

Aside from the importance of sigma factors, two-component systems such as PhoPQ play a crucial role in SCV transition. The acidic environment of the SCV switches PhoP and PmrA (Richards *et al.*, 2012). Two-component regulatory systems respond in general to environmental changes such as ion concentration (low magnesium, high iron), acidic pH, and presence of cationic host defence peptides (CAMPs), also known as antimicrobial peptides (AMPs). Those altogether induce major changes and switches the signalling cascade in intestinal lumen cells (Bader *et al*., 2005; Richards *et al.*, 2012a).

1.1.10 Metabolism in the SCV

In the SCV compartment glucose serves as a major carbon source for the bacterium and it is known to be utilised in a complete TCA cycle (Dandekar *et al*., 2012). Studies show that growth on glucose as the sole carbon source switches *ppc* encoding for phosphoenolopyruvate (PEP) carboxylase that plays a vital role in directing the flux into the TCA cycle. However, when *ppc* was missing, the loss was compensated with alternative carbon sources utilisation pathways and at the same time no reduced virulence was observed. This, in turn indicates that *Salmonella* has evolutionarily adapted with the ability to

switch its metabolism completely upon environmental changes (Tchawa Yimga *et al*., 2006). It also indicates availability of other carbon sources in the intracellular environment. Those other carbon sources can also be fed into the TCA cycle though transaminase activity. In the intracellular lifestyle, the host provides all the nutrients which are acquired via specific uptake systems (Fig. 1.5).

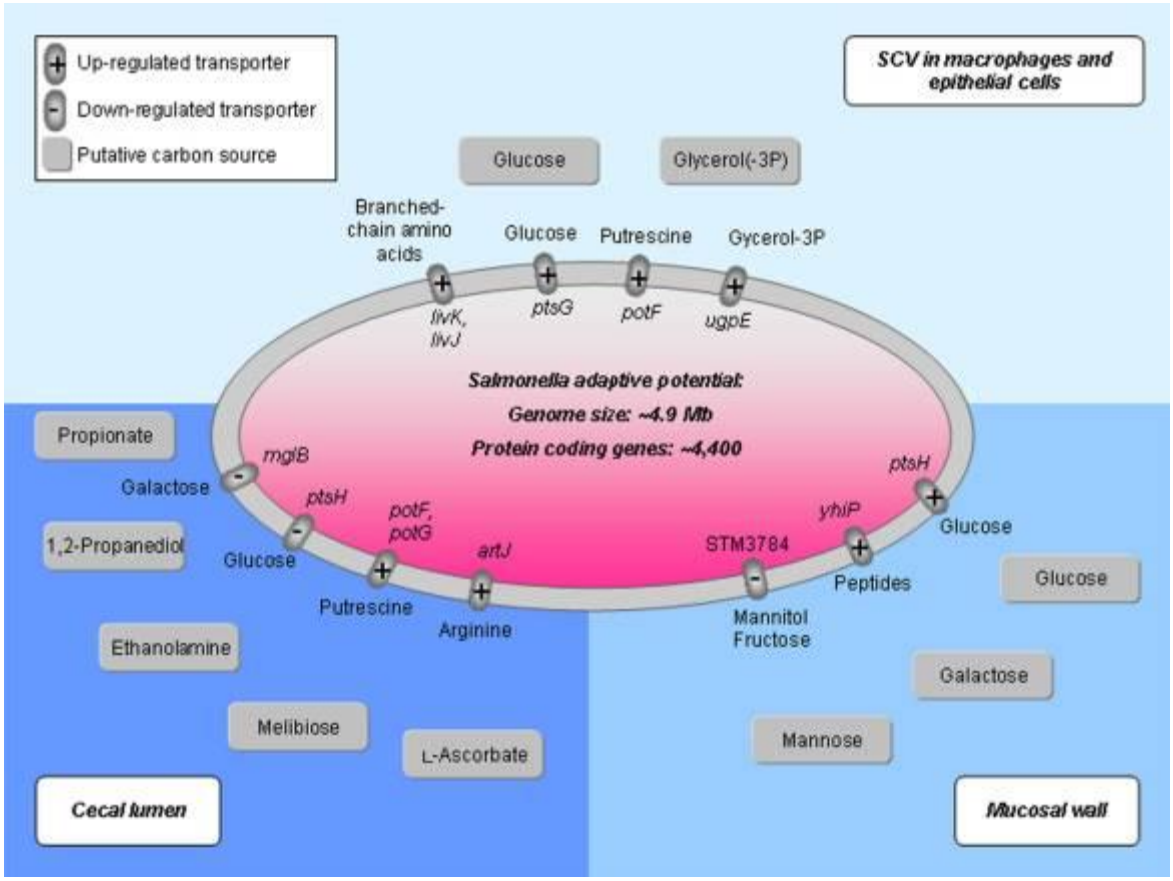


Fig. 1.5. Schematic representation of various host niches colonised by *Salmonella* and carbon sources utilised (Dandekar *et al.*, 2012)**.** Upon ingestion, bacteria first colonise cecal lumen of the gut where L-ascorbate, melibiose, propionate, ethanolamine and 1,2-propanediol are available. Then bacteria attach to the mucosal layer (where mannose, galactose and glucose are available) until they invade macrophages and epithelial cells forming SCV (glucose and glycerol). Grey boxes represent predicted carbon sources utilised in each environmental niche of the host. Small, grey ovals represent transported metabolites at the outside of the bacterial cell and transporters.

This survival battle from bacterial side meets the defensiveness of the host which leads to dynamics in gene expression as well as the evolutionary processes from both sides of the game. Interestingly, carbon source metabolism in this aspect serves as a target for not only better understanding of the survival strategy but also can be a key to better understanding the virulence and aid the search for novel antimicrobial approaches. Microbial defence and survival are likely to be limited by the environmental factors of the niche as well as the phenotypic traits of the invading bacteria and potential competing microbiota (Becker *et al*., 2006a). *Salmonella* shows very specific adaptations to the environment and the usage of different carbon sources is determined with the colonised niche and activated expression of specific transporters (Figure 1.5).

Crp as mentioned earlier, acts as a major gene expression global regulator in *Salmonella*. Interestingly Crp through the Csr system was found to also connect nutrients and carbon metabolism regulation with virulence factors. It was shown that the absence of *csrA* encoding for regulatory RNA-binding protein lowered expression of genes responsible for the utilisation of maltose, 1,2-propanediol and ethanolamine, metabolism of tetrathionate and production of hydrogen sulphite (Lawhon *et al*., 2003). It was described by Winter and colleagues that in the presence of tetrathionate *Salmonella* is able to use alternatives to glucose as a source of energy (Winter *et al*., 2010). Another global regulator gene *cya* that encodes for enzyme adenosyle cyclase together with *crp* (encoding cAMP receptor protein CRP) they form cAMP-CRP complex that regulate starvation response and thus regulates carbon utilisation genes (Eriksson *et al.*, 2002).

In 2010, Bowden and colleagues conducted research showing that although complete TCA cycle takes place in the *Salmonella* cell, in the event when incomplete TCA cycle takes place it may give bacterium higher rate of survival in macrophages suggesting that intermediates can be used in an alternative ways. However, within specific host environments complete TCA cycle may be advantageous (Bowden *et al.*, 2010). Overall, *Salmonella* is able to utilise glucose combining various pathways to supply both carbon and nitrogen. The combination of different pathways provides well balanced management of internal metabolites. Additionally, it makes it more difficult to target *Salmonella* infection by blocking key metabolic enzymes (Becker *et al.*, 2006b).

Salmonella however, as mentioned earlier, has also evolved with the ability to utilise different carbon sources (such as 1,2-propanediol and ethanolamine, figure 1.5) and incorporate metabolites into the TCA cycle. In the presence of vitamin B12 ethanolamine and 1,2-propanediol can be degraded into acetyl coenzyme A that can be directly incorporated into TCA cycle. This takes place, in the presence of oxygen or when an alternative electron acceptor is provided (Price-Carter *et al.*, 2001). In the event where final electron acceptor is not provided ethanolamine and 1,2-propanediol cannot be utilised. Although, the spectrum of nutrients available in the gut is complex, it is likely that competing microbes limit the accessibility of the substrates that can support fermentative growth in the anaerobic environment. Therefore, the ability to utilise non-fermentable carbon sources gives the bacterium potential advantage over the existing intestinal microbiota that only rely on fermentative metabolism (Thiennimitr *et al.*, 2011).

1.1.11 Role of inflammation in nutrient release

In order to establish the disease *Salmonella* had to evolve with numerous mechanisms that enable the bacterium to overcome many environmental factors such as competition, hosts immune system and nutrient stress. Throughout its intracellular life *Salmonella* remains in SCV which is termed as a nutritionally deprived environment therefore, the fact that the bacterium is able to replicate within the SCV indicates remarkably successful adaptation to this intracellular setting (Dandekar *et al.,* 2012*)*. Consequently, an understanding how the bacterium has adapted to acquire nutrients is important for developing novel strategies for targeting *Salmonella* infections.

Neutrophils control *S.* Typhimurium dissemination which suggest data obtained from clinical observations in patients with defects in neutrophil killing mechanisms or neutropenia (Winkelstein *et al.*, 2000). Among these patients, *S.* Typhimiurium infection disseminates from the gut which results with bacteraemia and often mortality. As a consequence, both neutrophils and epithelial cells of the host secrete potent antimicrobial proteins directly into the intestinal lumen which might be also responsible for drastic changes of the gut microbiota that is associated with the salmonellosis (Barman *et al*., 2008). Interestingly, many studies suggest that inflammatory response in the host is beneficial to *Salmonella* and facilitates its colonisation (Barman *et al*., 2008;

Lawley *et al*., 2008; Stecher *et al.*, 2007). This inflammatory environment is most likely difficult as it requires the bacterium to battle out entire host protection mechanisms (including antimicrobial proteins, different nutrient availability, and inhibition mechanisms) but might be beneficial to this pathogen. It potentially allows the bacterium to anaerobically respire tetrathionate and therefore utilise 1,2-propanediol and ethanolamine in a cobalamin-dependent manner to successfully outgrow other microbial communities (Winter *et al.*, 2010).

1.6 Acquisition of metals to support intracellular survival

Intracellular existence of *Salmonella* and its ability to survive and establish infection not only depends on the availability of the carbon sources.

Certain metals are essential for multiple functions in the biological systems and they cannot be displaced by any other molecules. Metals that play known biological roles have been classified as essential metals, and these include: Na, Mg, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Mo (Waldron & Robinson, 2009). Other metals that have not yet been described as playing any biological role have been listed as non-essential metals (Lemire *et al*, 2013). However, all the essential metals, despite their importance, when present in the excess are toxic to all living cells.

Nutrients, such as metals take part in many important processes such as bacterial sensing, gene expression regulation and regulation of metabolic pathways. Particularly of importance are divalent metal cations that play a role in gene responses and act as cofactors in many enzymatic reactions *(Eriksson et al.*, 2002a). Some of which are essential, yet their levels are limiting, which in turn is strictly associated with expression of the transporting systems. For instance Mg^{2+} was found to be limiting in the SCV. It was reported that 4 h after infection in macrophage cells *mgtBC* involved in Mg^{2+} transport were highly upregulated (Smith *et al*., 1998). *Salmonella* has three Mg^{2+} transporters, constitutively expressed CorA, and MgtA and MgtB that were found to be expressed when magnesium was limiting (Smith *et al.*, 1998; Snavely *et al.*, 1989). Interestingly, similar increase in expression of MgtA and MgtB was also

observed during *S.* Typhimurium invasion in epithelial cells which is mediated by major virulence regulator PhoPQ system. PhoPQ is known to be involved in controlling reaction to antimicrobial peptides, low pH, and response during invasion and overall controlling the activity of many genes in response to environmental signals (Hicks *et al.*, 2015; Richards *et al.*, 2012 b). Inorganic phosphate was also reported to be limiting to *Salmonella*. It was reported that 4 h after infection *pst* genes responsible for phosphate transportation were upregulated. Additionally, it is known that SPI-2 genes responsible for establishing systemic infection are induced under low phosphate conditions which indicates a link to *Salmonella* virulence (Deiwick *et al*., 1999). Therefore it might be that inorganic phosphate starvation leads to decreased LPS synthesis or decreased replication in macrophages (Eriksson *et al*., 2002a).

1.1.12 Iron

Many bacteria, like all living organisms, require transition metals for growth and survival. Nearly one third of all proteins and half of all enzymes require at least one metal ion in their structure (Andreini *et al*., 2008; Waldron & Robinson, 2009). Among those, one of the most important metals is iron. It plays a crucial role as a cofactor in many metabolic pathways of both *Salmonella* and the host (incorporated into transferrin, lactoferrin, haem, haemoglobin), therefore both pathogen and host compete over it (Schaible & Kaufmann, 2004). Iron however, like many other transition metals when unbound appears extremely toxic. Owing to its ability to catalyse Fenton chemistry it generates highly reactive hydroxyl radicals that are damaging to proteins, membranes and DNA (Lemire *et al.*, 2013). Iron intensifies oxygen toxicity by catalysing electron transfer from a donor molecule to H_2O_2. Redox-sensing transcription factors regulate reactive oxygen species (ROS) and at the same time metal transport systems to ensure homeostasis whilst avoiding metal-aided toxicity (Taylor *et al*., 2009). In the presence of high iron concentration Fur protein binds Fe^{2+} and represses transcription of genes involved in iron uptake (Andrews *et al.*, 2003). A genomic profiling study showed that around 7% of the *Salmonella* genome was regulated by iron – either directly or indirectly (Bjarnason *et al*., 2003). Iron is essential for *Salmonella* and as an intracellular pathogen it occupies an iron-rich niche as most of iron is available intracellularly due to the Nramp1 (Natural Resistance Associated Protein 1) activity (Atkinson & Barton, 1999). During iron starvation

repression of Fur is relieved and transcription of uptake genes is activated. Fur also stimulates iron storage by regulating expression of ferritins FtnA, Bfr or Dps through small RNA *ryhB* (Massé & Gottesman, 2002). Fur is involved in regulation of virulence genes known to be involved in iron transport (Ikeda *et al*., 2005). In *S.* Typhimurium iron is transported with ABC-type Sit transporting system. SitABCD encoding genes are located within SPI-1 and were found to be required for mouse systemic infection (Andrews *et al.*, 2003a). Interestingly the system was also found to have affinity towards Mn^{2+}, hence it was also characterised as MntABCD (Ikeda *et al.*, 2005; Kehres *et al.*, 2002). MntH is another regulator that is involved in transporting Mn^{2+} and is also involved in iron uptake, which in the presence of iron is repressed by Fur (Kehres *et al*., 2002a). Transporters which in principle have preference towards zinc are also known to be involved in iron transport. ZIP transporter is induced both by zinc or iron deficiency, ZupT was found to be involved in iron and manganese transport in addition to zinc (Cerasi *et al.*, 2014).

In the event when intracellular iron is not available, bacteria are able to retrieve it from iron storage proteins, ferritins. Ferritins not only serve as iron reservoirs but also can function as a mechanism of protection from iron-mediated toxicity. In *S.* Typhimurium there are found four ferritins: bactoferritin (Bfr) that accounts for majority of the stored iron, ferritin A (FtnA), Ferritin B (FtnB), and DNA-binding Dps (Velayudhan *et al.*, 2007). Here, Fur also appears to play a role by positive regulation of *bfr* as well as *ftnA* under high iron conditions and negatively regulating *ftnB* and *dps* when iron is not available (Velayudhan *et al*., 2007).

Two component system PmrA/PmrB is another two-component system that senses and responds to the extracellular iron (Fe^{3+}) levels. This is mainly involved in inducing genes leading to an increased resistance to iron levels resulting in outer membrane modification (Wösten & Groisman, 1999). PmrA/PmrB is induced by high levels of intracellular iron, and downregulates *pmrA*, *pmrB*, *pmrD*, *pmrF* and *ugd* involved in LPS synthesis and antibiotic resistance; where *ugd* is also co-regulated by low magnesium induced PhoP/PhoQ (Mouslim & Groisman, 2003b). Intracellularly those genes were found to be repressed, suggesting that Fur repression occurring results with rich Fe^{2+} intracellular levels but not Fe^{3+}. Genes involved with iron acquisition

intracellularly were not induced in J774-A.1 infection model (Eriksson *et al*., 2002a). Overall it is hypothesised that Fur regulates gene expression in response to iron levels and genes that are controlled by Fur are not involved with iron but with other functions such as acid tolerance response or virulence (Bjarnason *et al.*, 2003; Riesenberg-Wilmes *et al.*, 1996).

1.1.13 Manganese

Manganese is another trace element that is required in many cellular mechanisms and as mentioned earlier it can potentially substitute iron functionally in metyloenzymes and the system responsible for transporting iron turned out to have greater affinity towards manganese (Ikeda *et al.*, 2005). Manganese is required for manganese superoxide dismutase enzyme SodA that plays a role in protection from ROS, and which not so surprisingly is also regulated by Fur (Tsolis *et al.*, 1995). Similarly to other transition metals, when present in excess, manganese becomes toxic. Metalloregulatory proteins are in control of the expression of genes involved in metal acquisition by either upregulating or repressing transcription (Que & Helmann, 2000). Here, at the same time by regulating virulence those metal ions often serve as an environmental signal for switching metalloregulation response strictly linked with host invasion. MntR is a manganese-dependent regulator, that just like those mentioned earlier regulates expression of both MntH involved in manganese transport as well as SitABCD (Ikeda *et al.*, 2005). It belongs to DtxR (diphtheria toxin repressor) family of metal-dependent repressors that was very well described in *B. subtilis* (Que & Helmann, 2000). MntR found in *S.* Typhimurium resembles similarities to Nramp2 which is a mammalian metal transporter that is known to take part in divalent metal cation transport through the gut epithelium based on proton-driven symport (Kehres *et al*., 2002a). As stated previously, MntH was found to have much greater affinity towards manganese. Its maximum activity was observed at only 0.1 mM manganese as opposed to 100 mM ferrous iron (Kehres *et al.*, 2000). As stated earlier SitABCD is involved in manganese transport and belongs to ABC-type transporter family. It consists of SitA which is a cation binding protein (Kehres *et al*., 2002b). MntH here is likely to function as a proton-coupled divalent cation transporter and SitABCD as an ATP-type of transporter, and gets energy for transport from ATP hydrolysis. MntH and SitABCD transcription is regulated by MntR and of course Fur

possibly simultaneously (Ikeda *et al.*, 2005; Kehres *et al.*, 2002b). Importantly both SitABCD and MntH were showed to be implicated in virulence. In Nramp1g169 mouse model *sitA* and *mntH* were upregulated and were both required for complete virulence suggesting that manganese is required for the intracellular survival of *S.* Typhimurium (Zaharik *et al.*, 2004).

1.1.14 Zinc

Zinc as another one of those metals that is essential for immunomodulation and immune system functioning (Stafford *et al.*, 2013). *S.* Typhimurium thrives in the inflamed gut by expressing ZnuABC described as high affinity zinc transporter that overcomes calprotectin mediated zinc chelation. Similarly to other metals bacteria compete over zinc with the host resulting in the development of zinc sequestration strategies (Liu *et al*., 2012). Zinc is required for many enzymatic reactions as a cofactor, takes part in regulation of replication, transcription, translation and also pH regulation and oxidative stress response (Katayama *et al*., 2002). It is suggested that SCV appears in zinc-restricted environment therefore it requires high affinity zinc uptake system (Ammendola *et al*., 2007). In *S*. Typhimurium zinc sensor Zur that belongs to the Fur family acts upon ZnuABC expression regulation. Zinc bound Zur represses ZnuABC transcription and coordinates with periplasmic protein ZinT. ZinT and ZnuA cooperate in the process of zinc acquisition during severe zinc shortage (Petrarca *et al*., 2010).

1.1.15 Nickel and cobalt

In *Salmonella* nickel and cobalt acquisition mechanisms are a lot less described. Nickel is known to be an essential trace element in many metalloenzymes such as Ni-Fe hydrogenase. Ni-Fe hydrogenase catalyses hydrogen oxidation serving as anaerobic way of gaining energy (Zhang *et al.*, 2009). In *E. coli* NikR acts as a repressor of anaerobically expressed *nikABCDE* operon known to transport nickel as a response to high zinc levels (Rowe *et al.*, 2005; Zhang *et al.*, 2009). NikR covers 99% identity of over 133 amino acids to the putative regulator found in *S.* Typhimurium giving strong evidence of its involvement in nickel acquisition especially as it is quite abundant across many organisms (Rowe *et al*., 2005). NikR is also predicted to be involved in NikMNQO and NiCoT transporter (Zhang *et al.*, 2009). NikR was described as ribbon-helix-helix type of transcription factor (Zhang *et al*., 2009). In the

presence of nicklel, NikR binds DNA resulting with suppression of transcription of *nikABCDE*. In the excess of nickel, there are found low affinity binding sites that are active and most likely serve as another way of protection from the metal excess (Phillips *et al*., 2010).

Cobalt is mainly found in the corrin ring of cobalamin and *S.* Typhimurium different from *E.* coli, requires cobalt for vitamin B12 *de novo* biosynthesis (Rodionov *et al.*, 2003) . Amongst genes involved in cobalamin biosynthetic pathway there are genes *cbiMNQO* that share similarities with other high affinity ABC-type metals transporters. It is likely that expression of these genes is regulated by B12 riboswich. It allows to selectively bind B12 and then to repress the expression of target genes. Therefore, levels of CbiMNQO expression might not be directly cobalt-dependent but rather be influenced by current B12 requirement (Dmitry A Rodionov et al., 2003).

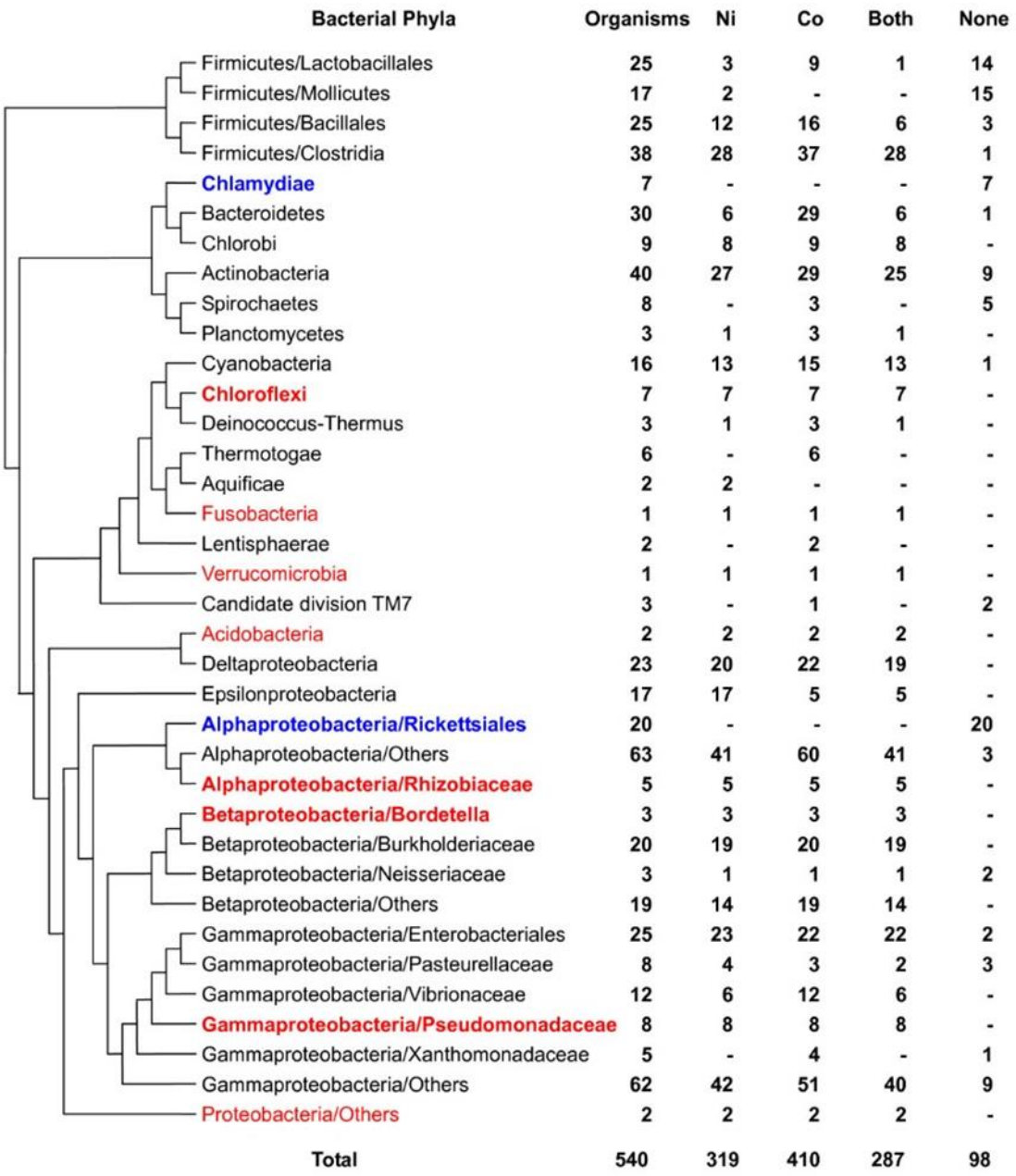
Overall, *S.* Typhimurium in order to survive developed strategies within the host that can be protected from metal mediated toxicity whilst effectively, at the same time, being completely able to compete over essential nutrients with the host as other gut microorganisms. As a result, this pathogen has evolved with vast metal-specific regulatory systems that enable control of the intracellular levels of metals, such as Fur, ZntR, MntR, NikR. Some of these regulators act upon a particular target gene and some act as global gene expression regulators and regulators of particular acquisition systems.

1.7 Contribution of cobalt to intracellular survival

Cobalt is an essential trace element for many living organisms as it plays a key biological role as the centrally coordinated ion in corrin rings. These corrinoids include coenzyme B_{12} and its cobalamin derivatives being important coenzymes in many metabolic reactions (Martens *et al.*, 2002). Zhang *et al*., (2009) carried out genomic analysis to determine that almost all bacterial phyla possess genes related to cobalt utilisation (Figure 1.6). Interestingly, bacteria which have adopted a parasitic lifestyle appear to have lost the ability to use both cobalt and nickel. In order to acquire sufficient cobalt for metabolism bacteria possess high affinity uptake systems to scavenge it from the environment (Eitinger *et al.*, 2005). However, when accumulation of cobalt becomes toxic the excess of the metal has to be removed from inside the cells by precisely regulated efflux systems (Cheng *et al.*, 2011).

1.1.16 Cobalt transport

The best characterised cobalt transport system is the NiCoT family which are found amongst a wide variety of bacteria. This family includes at least one nickel permease in addition to other proteins with affinities for either nickel or cobalt ions (Rodionov *et al*., 2006; 2003). The CbiMNQO family is also present in a number of bacteria, including *Salmonella*. These cobalt transport proteins are associated with cobalamin biosynthesis in *S.* Typhimurium and were found to possess conserved domains suggesting their function of tight cobalt regulation inside the cell (Raux *et al.*, 1996). According to Roth *et al.*, (1993) these proteins were found to share structural similarities to other members of energy dependent membrane transport systems. There were found homologous proteins that bore conserved sites suggesting an ABC-transport system. According to Blast analysis, CbiO protein appears to belong to an ATP-dependent membrane transport protein. It is suspected that CbiO is involved in the transport of cobalt as most similar proteins are involved in the active transport of metal ions in other microorganisms such as *Eschericha*, *Listeria*, *Citrobacter*, *Yersinia*, *Clostridium*, *Klebsiella*, *Staphylococcus, Shiglla, Enterobacter* (Blast analysis of the CbiQ). CbiQ protein is deducted to stabilise CbiO as it possesses membrane spanning domains that possibly serve as a CbiQ stabiliser. Roth *et al*., also explains that *cbiN* and *cbiO* are likely to encode for components of a cobalt active transport system; also they do not show significant similarities to other similar systems as anchor peptides with spanning membranes evolved more quickly than ATPases thus they are not obvious to characterise.



Bacterial Phyla	Organisms	Ni	Co	Both	None
Firmicutes/Lactobacillales	25	3	9	1	14
Firmicutes/Mollicutes	17	2	-	-	15
Firmicutes/Bacillales	25	12	16	6	3
Firmicutes/Clostridia	38	28	37	28	1
Chlamydiae	7	-	-	-	7
Bacteroidetes	30	6	29	6	1
Chlorobi	9	8	9	8	-
Actinobacteria	40	27	29	25	9
Spirochaetes	8	-	3	-	5
Planctomycetes	3	1	3	1	-
Cyanobacteria	16	13	15	13	1
Chloroflexi	7	7	7	7	-
Deinococcus-Thermus	3	1	3	1	-
Thermotogae	6	-	6	-	-
Aquificae	2	2	-	-	-
Fusobacteria	1	1	1	1	-
Lentisphaerae	2	-	2	-	-
Verrucomicrobia	1	1	1	1	-
Candidate division TM7	3	-	1	-	2
Acidobacteria	2	2	2	2	-
Deltaproteobacteria	23	20	22	19	-
Epsilonproteobacteria	17	17	5	5	-
Alphaproteobacteria/Rickettsiales	20	-	-	-	20
Alphaproteobacteria/Others	63	41	60	41	3
Alphaproteobacteria/Rhizobiaceae	5	5	5	5	-
Betaproteobacteria/Bordetella	3	3	3	3	-
Betaproteobacteria/Burkholderiaceae	20	19	20	19	-
Betaproteobacteria/Neisseriaceae	3	1	1	1	2
Betaproteobacteria/Others	19	14	19	14	-
Gammaproteobacteria/Enterobacteriales	25	23	22	22	2
Gammaproteobacteria/Pasteurellaceae	8	4	3	2	3
Gammaproteobacteria/Vibrionaceae	12	6	12	6	-
Gammaproteobacteria/Pseudomonadaceae	8	8	8	8	-
Gammaproteobacteria/Xanthomonadaceae	5	-	4	-	1
Gammaproteobacteria/Others	62	42	51	40	9
Proteobacteria/Others	2	2	2	2	-
Total	540	319	410	287	98

Figure 1.6. Distribution of cobalt (and nickel) utilisation genes in bacteria (from Zhang *et al*., 2009). Phyla in which none of the organisms use cobalt or nickel are shown in blue (if containing at least 3 organisms, shown in bold). Phyla in which all organisms use both Co and Ni are shown in red (if containing at least 3 organisms, shown in bold).

1.1.17 Cobalamin pathway in *Salmonella*

Scavenging cobalt is required for the vitamin B_{12} (cobalamin) production that was confirmed to be synthesized *de novo* but only under anaerobic conditions (Bobik *et al.,* 1999; Jeter *et al.*, 1984*)*. The coenzyme form of vitamin $B_{12,}$ either deoxyadenosylcobalamin or methylcobalamin, is required as a cofactor in a number of enzyme-catalyzed reactions (Jones *et al.*, 2013). All salmonellae dedicate 1% of their genome to the synthesis of cobalamin (vitamin B_{12}) and another 1% of their genome to use this cofactor in support of anaerobic growth on two non-fermentable carbon sources - ethanolamine and 1,2-propanediol. Thus, the functions of these genes must significantly contribute to *Salmonella* fitness in a natural setting. The entire pathway remains unclear, however it is suggested that this ability enhances *S.* Typhimurium proliferation in an inflamed mouse gut (Roth *et al.*, 1993). Cobalamins are complex, non- polymeric molecules biosynthesised in cells and present generally in bacteria. Animals need cobalamins in their diet, unlikely higher plants (Roth *et al.*, 1993a). In humans, vitamin B_{12} deficiency causes the condition pernicious anaemia and also neural tube defects ashumans are unlikely to biosynthesise it.

Bacteria, for complete biosynthesis of adenosyl cobalamin require around 30 different enzymes as a coenzyme (Raux *et al.*, 1996). Most of the genes involved in vitamin B_{12} synthesis are clustered in a single operon (*cob*) mapping to 41 minutes on the chromosome (Ailion *et al.,* 1993; Jeter *et al.*, 1984). The *cob* operon includes 17 *cbi* genes needed for the synthesis of cobinamide, a precursor of vitamin B_{12} (Chen *et al*., 1995).

The structure of B_{12} coenzyme is built from three major parts. The first represents tetrapyrrole-derived corrin ring structure encoded by *cobI* genes. Second part, encoded respectively by *cobII* genes is dimethylbenzimidazole (DBM) nucleotide. And the third part responsible for attachment of the corin ring to the DBM, as well as the addition of the coordinating upper coordinating ligand for the cobalt, adenosyl or methyl group (Raux *et al.*, 1996). The third part is then encoded by *cobIII* genes (Jeter *et al.*, 1984). Among bacteria, some of them have the ability to produce cobalamin de novo and require it for growth. There are also some that only require it to grow but do not possess capacity to synthesise coenzyme *de novo*. *S.* Typhimurium possesses this ability but can only synthesis the coenzyme under anaerobic conditions (Raux *et al.*, 1996).

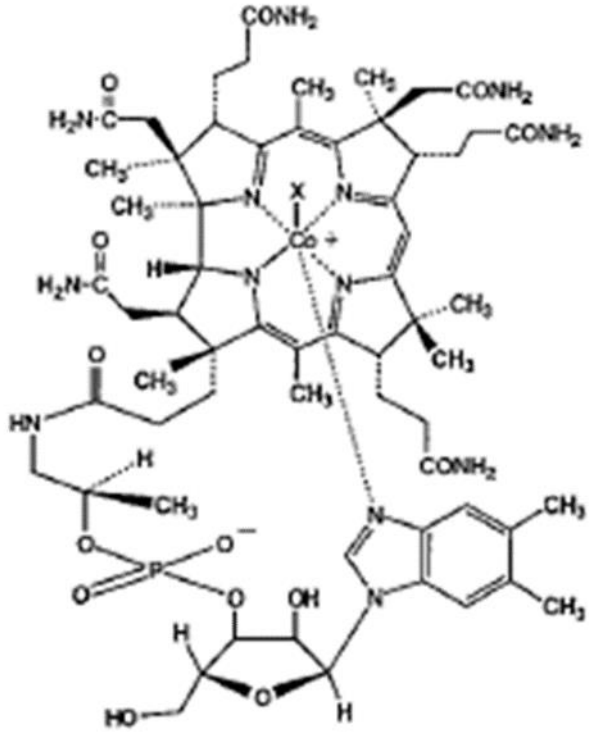


Fig. 1.7. Vitamin B_{12} and its coenzyme forms (Raux *et al.*, 1996).

1.1.18 *cbi, pdu* and *eut* genes

In many bacterial genomes *cbi* (or *cob*) genes are clustered with *pdu* and *eut* genes which are responsible for cobalamin-dependent 1,2-propanediol degradation and utilisation of ethanolamine respectively (Klumpp & Fuchs, 2007).

The products of *pdu* operon encodes enzymes required for 1,2-propanediol utilisation (Bobik *et al*., 1999). Their properties allow *S.* Typhimurium to grow using propanediol a sole carbon source which appears in an adenosylocobalamin-dependent manner (Adkins *et al*., 2006). *Salmonella* catabolises propanediol in the way it involves vitamin B_{12} coenzyme, adenosylcobalamin (Ado-CBL) (Bobik *et al.*, 1997). Propanediol is derived from the breakdown of fucose and rhamnose (Obradors *et al.*, 1988). These sugars are common constituents of intestinal epithelial cells. Therefore, *Salmonella* utilise 1,2-propanediol under anaerobic conditions (Bobik *et al*., 1997). In anaerobic environment 1,2-propanediol is converted into propioaldehyde by an Ado-CBL-dependent propanediol dehydratase. Propionaldehyde is then metabolised into propanol and propionic acid by coenzyme-A (Co-A)-dependent aldehyde dehydrogenase, phosphotransacetylase, propionate kinase and alcohol dehydrogenase (Obradors *et al*., 1988). In the propanediol utilisation process energy (ATP molecule) and carbon are provided only if reaction takes place anaerobilcally (Jeter, 1990). Experiments conducted with mice showed that *pdu* mutations refer to the virulence deficiency of *Salmonella* virulent strains (Bobik *et al*., 1997). Moreover, the *pdu* operon was revealed to be regulated by CsrA and Fis, which are responsible for regulating *Salmonella* pathogenicity (Eriksson *et al*., 2002). In addition, studies showed that Pdu proteins were observed to be 5 times more frequent in significantly more virulent strains – such as strain 14025 (Adkins *et al.*, 2006).

The *S.* Typhimurium *eut* gene cluster encodes 17 genes required for the degradation and utilization of ethanolamine (*eutS*, -P, -Q, -T, -D, -M, -N, -E, -J, -G, -H, -A, -B, -C, -L,-K, -R) (Lawhon *et al.*, 2003). EutBC allows ethanolamine metabolism to acetaldehyde and ammonia within carboxysome microcompartment that prevents the cell from toxicity of acetaldehyde (Penrod *et al*., 2004). From this reaction, ammonia serves as a source of nitrogen. Acetylaldehyde is converted to acetyl coenzyme A by aldehyde oxydoreductase

and then subsequently utilised in many metabolic cycles, such as tricarboxic acid cycle (Roof & Roth, 1989). Under anaerobic conditions ethanolamine utilisation depends on the supply of cobalamin that is synthesised de novo only under anaerobic conditions by *cob* cobalamin biosynthetic genes (Roth *et al.*, 1996)

Both of these operons are transcriptionally activated by *pocR* regulator when *Salmonella* grows on poor carbon sources (Srikumar & Fuchs, 2011). It was confirmed by Heithoff, (1999) that cobalamin synthesis and propanediol degradation are required for the intracellular replication of *S.* Typhimurium. There is evidence that genes encoding proteins responsible for propanediol and ethanolamine degradation are highly expressed in the gut but only in virulent strains (Garsin, 2010). This ability serves as an indirect contribution to *Salmonella* virulence by providing the useful source of carbon and/or nitrogen and also overcoming competition with other microbes that rely entirely on fermentative metabolism (Thiennimitr *et al.*, 2011). Additionally, tetrathionate was found to be essential as the electron acceptor for the anaerobic growth of *Salmonella* on ethanolamine and 1,2-propanediol (Price-Carter *et al.,* 2001). Various studies have linked *eut* and *pdu* gene expression with the global virulence regulators such as CsrA and Fis that are located on SPI1 serving as direct evidence of possible roles in *Salmonella* pathogenesis (Lawhon *et al.*, 2003).

Studies by Bobik *et al.* in 1999 revealed strong correlation and co-induction of propanediol utilisation genes (*pdu*) and cobalamin synthetic genes (*cob*) revealing that propanediol catabolism is the primary reason for de novo synthesis of vitamin B_{12} coenzymes by *Salmonella*. To confirm the hypothesis independently isolated mutant strains lacking with *cobA* and *pduO* genes were unable to grow on a 1,2-propanediol minimal medium supplemented with vitamin B_{12} but were capable of growth on medium supplemented with Ado-CBL (adenosylcobalamin). Thus, the presence of fully functional *pdu* operon is required for conversion of the inactive form of cobalamin, such as vitamin B_{12}. It was described that *pduO* encodes for protein with adenosyltransferase activity which converts vitamin B_{12} into Ado-CBL that allows 1,2-propanediol degradation (Johnson *et al.*, 2001).

1.1.19 *cbi* genes and similarities to *stm3071-5*

The putative operon *stm3071/3072/3073/3074/3075* was also found to share structural similarity to ABC transporters similar to those identified within *cbi* genes (Taylor, unpublished data). Within *stm3071/2/3/4/5* there were domains found similar to these found in CbiQ and CbiO. To date, the precise functions of these genes are unknown but we hypothesise that *stm3072/3/4/5* are involved in cobalt transport while the upstream *stm3071* gene may be responsible for regulation of the downstream genes (Taylor, unpublished data). Interestingly, STM3071 has been shown to be expressed preferentially within hypoxic tumour cells (Arrach *et al*., 2008).

1.8 Anti-tumour concept

1.1.20 Hallmarks of cancer disease and therapeutic approaches

In 2000 Hanahan and Weinberg first coherently defined six common hallmarks of cancer: self-sufficiency in growth signals, avoidance of programmed cell death (apoptosis), insensivity to growth-inhibitory signals, infinite growth potential, sustained blood vessel formation (angiogenesis), and tissue invasion and spread to other sites (metastasis) (Hanahan & Weinberg, 2000). Aside from these major features of cancer cells there are also many others, such as chromosomal abnormalities, inflammation and evasion of the immune system and abnormalities in metabolic pathways. In solid tumours, cancer cells outgrow healthy tissue and form a lump of cells called tumour (Hanahan & Weinberg, 2011). The tumour is known to recruit seemingly normal cells that contribute to the formation of the tumour microenvironment (Hanahan & Weinberg, 2011).

Cancer is one of the most dreaded diseases in humans and the most common cause of death in the XXI century. New, selective anti-cancer therapies are required mainly due to lack of selectivity and high toxicity of the existing strategies. Since the XX century, both radiotherapy and chemotherapy have advanced into two main therapeutic approaches against cancer. Neither of those therapies however eliminate cancer cells and their spread. In addition, both chemo- and radiotherapy are toxic to healthy tissues and it is due to: incomplete tumour targeting, inadequate tissue penetration, and limited toxicity to cancer cells. Hence why such a high rate of morbidity and mortality occurs in cancer patients (Minchinton & Tannock, 2006).

Selective targeting of the tumour tissue has been the ultimate goal of drug delivery research. Over the years controlled drug delivery has advanced significantly, however there are still areas where substantial improvements are to be made in order to minimise anti-tumour therapy-associated toxic effects (Hoffman, 2008). Targeted drug delivery means that the target drug is combined with a carrier that in principle should aid that drug to the intended site. An effective targeted delivery system must have four characteristics: retain, evade, target and release (Mills & Needham, 1999). Different targeting mechanisms are needed to distribute the drug to the different parts of the body. Most therapies are based on the concept of so-called passive targeting which utilises the nature of the tumour. Tumour tissue is characterised by enhanced permeation and retention (EPR) effect which is based on the concept that molecules of certain size tent to accumulate inside the tumour over the normal tissue (Matsumura & Maeda, 1986). The EPR principle is based on exploitation of the abnormalities observed in the tumour tissue. These are hypervascularisation, aberrant vascular architecture, extensive production of vascular permeability factors stimulating extraversion within tumour tissues, and lack of lymphatic drainage (Greish, 2010). Administration of macromolecular drugs uses the specificity of the tumour tissue to its advantage. Those macromolecular drugs, such as lipids, nanoparticles, when injected intravenously, selectively accumulate in tumour tissues and remain there for an extended period (mainly due to the lack of lymphatic drainage) (Matsumura & Maeda, 1986).

Inflammation and tumour tissue nature have a lot in common. Both are characterised by enhanced vascular permeability, which is mediated by number of factors such as bradykinin, prostaglandins, nitric oxide, and peroxynitrate (Hanahan & Weinberg, 2011). The EPR effect as discussed above has been used as a major characteristic in order to selectively deliver macromolecular drugs in tumour targeting.

In addition, necrotic regions of tumours are hypoxic, acidic and often with elevated levels of metals (Ionescu *et al.*, 2006). These metals are not accumulated in the organism and are processed out through kidneys. However, in the case of inflammation this mechanism might be disrupted (Songdej *et al*., 2010). Pathological levels of metals are closely related to free radical

generation, lipid peroxidation, formations of DNA strand breaks and tumour growth in cellular systems (Ionescu *et al.*, 2006). Cobalt exposure itself enhances inflammation and this process may be a key contributor to malignancy, tumour formation and the onset of cancer (Saini & Shoemaker, 2010). Interestingly, studies in various systems have shown that exposure to certain metals, such as cobalt, promote a response similar to the hypoxia observed in tumours by activating hypoxic signalling by stabilising the hypoxia inducible transcription factor 1α (HIF1α) (Vengellur & LaPres, 2004). On the other hand elevated levels of cobalamin in blood may be a sign of life threatening disease, such as leukaemia *(Ermens et al*., 2003).

1.1.21 Targeting tumour with *S.* Typhimurium

S. Typhimurium has a unique ability to overcome limitations of standard anti-cancer therapies. It has the ability to sense and target tumours, to grow in tumour-specific microenvironment and the ability to penetrate the tumour. *Salmonella* also exhibits low cytotoxicity and immunogenicity and can be relatively easily manipulated genetically (Forbes, 2010).

The ability to sense tumours is mainly based on sensing hypoxia (less than 1% of oxygen). Due to continuous cell proliferation and angiogenesis during tumour growth, the newly formed blood vessels are not sufficiently developed and as a result not enough oxygen and nutrients are delivered to the tumour tissue. Low oxygen attracts facultative anaerobes and favours their proliferation. Genetically engineered auxotrophic strains (purine, arginine, and leucine auxothrops) of *S.* Typhimurium were found to colonise tumours better that WT strains (Zhao *et al.*, 2006).

Unlike traditional anti-cancer methods such as chemo- and radiotherapy, bacteria used to target cancer cells are metabolically active. This allows them to penetrate tumour tissue better than synthetic drugs. Another key feature of bacteria is their ability to move. Motility allows them to actively move around, migrate through complex vascularity of the tumour, and reach it throughout (Dang *et al.*, 2001). Highly motile strains such as *E. coli* and *S.* Typhimurium have been used as microrobots where theranostic molecules were combined with motile strains in order to deliver drug directly to the tumour tissue (Park *et al.*, 2013). Bacterial accumulation inside the tumour tissue leads to nutrients

deprivation and at the same time activation of antitumor immunity lead to tumour death (Sznol *et al.*, 2000).

Tumour nature allows escaping immunity by limiting maturation and infiltration of the specialised immune cells. Systemic spread of attenuated *S.* Typhimurium was shown to activate anticancer immunity leading to regression of the tumour. *S*. Typhimurium was shown to activate the inflammasome during early stages of cancer through NOD-like receptor. Conserved bacterial components called PAMPs (pathogen associated molecular patterns) are recognised by PPRs (pattern recognition receptors), also called TLRs (toll-like receptors), which recognise molecules shared by pathogens but not host cells (Phan *et al.*, 2015). Those bacterial components, such as flagella and, LPS activate TLR signalling pathway and induce both innate and adaptive immune responses. NLRP4 (known as IPAF) is a subset of inflammasomes that recognise cytoplasmic bacterial flagellin through bacterial secretion systems *(*Franchi *et al*., 2006) whilst NRLP3 is activated through endogenous danger signals (damage associated molecular pattern molecules), low intracellular potassium concentration and, endotoxins. This leads to activation of caspase-1 by inflammasomes that results in the cleavage of pro-IL-1β, and pro-IL-18. Thus, attenuated *S.* Typhimurium systemic administration leads to production of pro-inflammatory cytokines, such as IL-1β, IL-18, TNF-α, and IFN-γ, which in turn results in recruitment and activation of macrophages, dendritic cells, and T cells – it converts the tumour environment from immunosuppressive to immunocompetent (Phan *et al*., 2015).

Bacteria can be relatively easily engineered so they can express molecules, such as cytotoxic agents, cytokines, RNA interference and prodrug enzymes (Zheng & Min, 2016). These agents can be expressed under the tumour-specific promoters or specifically induced promoters to avoid unwanted expression in the healthy tissue (Jiang *et al.*, 2013; LI *et al*., 2013; Loeffler *et al*., 2007; Loeffler *et al*., 2009; Loessner *et al*., 2007; Ryan *et al*., 2009). Specifically induced promoters come from the external systems, such as L-arabinose induced P_{BAD} promoter. Mice with murine colon cancer were injected with an attenuated *Salmonella* strain harbouring *clyA* (gene encoding for cytotoxic gene cytolysin). Under the control of P_{BAD} it was shown that *clyA* was expressed leading to tumour regression in mice (Hong *et al*., 2014).

Tetracycline regulated promoter and γ-irradiation-inducible P_{RecA} were also investigated as potential promoters for triggering expression of therapeutic agents (Ganai *et al*., 2009; Jiang *et al*., 2013). Amongst tumour-specific induced promoters hypoxia-inducible fumarate and nitrate reduction regulator (Ryan *et al.*, 2009) and quorum sensing systems were detected which turns on gene expression at high bacterial densities that is usually observed in tumours (Swofford *et al*., 2015).

New approaches, such as vaccines and biological therapies are providing hope with the specific premise of killing of the cancer cells. For instance, bacterial therapies exhibited hopeful perspective in targeting cancer more precisely. Specifically selected, live, attenuated or genetically modified bacterial strains with tumour targeting abilities are capable of multiplying selectively inside the tumour cells. Bacteria, as well spores, were used in delivering engineered prodrugs and proteins inside the tumour cells (Zheng & Min, 2016).

It was examined that attenuated *S.* Typhimurium cells can infect cancerous cells in mice. Tumour reduction was observed which was related to the local bacterial expression or expression of immune-stimulating molecules IL-18, Fas ligand. It was also found that IL-2 encoding *Salmonella* strains showed better suppression of the tumour tissue than *Salmonella* wild type strains. Attenuated *S.* Typhimurium introduced with human tumour endothelial marker 8 (TEM8) DNA was reported to generate a CD8 cytotoxic T-cell response in the animal models (Ruan *et al*., 2009).

1.1.22 Hypoxia, cobalt and potential candidates for tumour specific expression

Avirulent *Salmonella* mutants were found to accumulate inside the tumour at ratios that range between 250:1 and 9,000:1 and were found to lead to reduction or even the cure of cancer in animal models (Pawelek *et al*., 2003; M. Zhao *et al*., 2006). Necrotic regions of tumours are hypoxic and relatively acidic which possibly attract *Salmonella* to preferentially colonise tumour tissue over normal tissue. Arrach and colleagues (2008) used a high-throughput method to screen for those *Salmonella* promoters that are preferentially expressed in tumours over healthy spleen tissue. $P_{stm3071}$ was noted to be induced inside tumour over 2-fold more than in normal spleen tissue. Thus, the data suggests that $P_{stm3071}$ can serve as a candidate for a hypoxia/tumour specific induced

promoter and in the future may be used as a tool for constructing anti-tumour recombinant proteins expressed under the specifically induced promoter.

1.9 Hypothesis and research objectives

To date Arrach *et al.*, (2008) have observed the upregulation of $P_{stm3071}$ when *Salmonella* is present within the solid tumours. Gene *stm3071* according to NCBI prediction is thought to encode for a potential DNA-binding protein, thus a potential transcription regulator of downstream genes. Interestingly, those downstream genes are predicted to be involved in cobalt transport. It was therefore important to determine NCBI presumed annotation which was based on similarities with the *cob* operon. Interestingly, the conditions observed inside solid tumours are similar to those that are thought to induce *cob* operon expression (Srikumar & Fuchs, 2011; Thiennimitr *et al.*, 2011).

The conditions required for *stm3071* promoter upregulation are similar to those required for activation of cobalt transport genes along with cobalamin synthesis genes (Jeter, 1990). Thus, it is predicted that if $P_{stm3071}$ is upregulated selectively in conditions mimicking those present within tumour cells, then attenuated *Salmonella* strains may be used as a novel selective therapeutic tool for delivering drugs encoded downstream from the $P_{stm3071}$.

The aim of the study was to determine and characterise the conditions in which $P_{stm3071}$ is expressed. It was intended to identify whether STM3071 acts as a DNA-binding protein and as a consequence, if it is involved in transcription regulation of downstream *stm3072/3073/7374/3075*. Additionally, it was important to find out whether the absence of *stm3071* would affect *Salmonella* anaerobic growth and exposure to elevated levels of cobalt anaerobically in order to find out whether the predicted operon is involved in metal transport. It was intended to determine whether *stm3071* loss would be essential for anaerobic growth in the presence of elevated levels of cobalt. Further, it was interesting to determine whether the *stm3071* loss would be important in $P_{stm3071}$ activation

Investigating functions of putative *stm3071/3072/3073/3074/3075* operon as well as conditions that determine expression of these genes is essential to establish possible roles in *S.* Typhimurium virulence. Understanding of all aspects of expression of *stm3071/3072/3073/3074/3075* genes could be

exploited in terms of seeking for novel antimicrobial and or/anti-tumour strategies.

2. Materials and methods

2.1 Bacterial strains

All bacterial strains used in this study are listed and described in Table 2.1. Additionally strains provided as a control for phenotype determination experiments are described in Table 2.2.

S. Typhimurium SL1344 was the wild type strain used throughout this study and was obtained from the Taylor Laboratory Culture Collection (TLCC). *S.* Typhimurium LT2 (TR10000) wild type strain and derivatives were kindly provided by Professor John Roth (UC Davis, USA) and were used as described in appropriate experiments. *S.* Typhimurium LB5010a strain was used in site directed λ-red mutagenesis. Mutant *Δstm3071* was constructed using site-directed mutagenesis in LB5010a and transduced using P22ϕ into SL1344 and TR10000. *S*. Typhimurium LB5010a *galE+* was used as a host strain for P22ϕ propagation. *Escherichia coli* α-Select strain was purchased from Bioline, UK and used for all plasmid propagations; except for plasmid pKD3 which requires *E. coli* DH5α λpir (Bioline, UK). *E. coli* BL21 (DE3) (Bioline, UK) was used for STM3071 protein overexpression.

Table 2.1 Bacterial strains used in this study.

Strain	Strain description	Reference
TR10000	Wild type *S.* Typhimurium LT2	(Ailion & Roth, 1997)
TR6583	*metE205 ara-9*	(Ailion & Roth, 1997)
TT11202	*metE205 ara-9 metH2355::MudJ $COM blue Mud*	(Ailion & Roth, 1997)
TT10327	*metE205 ara-9 cob-24::MudA (CobI)*	(Ailion & Roth, 1997)
TT17131	*metE205ara-9pdu-12::MudJ*	(Ailion & Roth, 1997)
TT17104	*metE205ara-9pocR12::MudJ*	(Ailion & Roth, 1997)
SL1344	Wild type *S.* Typhimurium	TLCC
LB5010a	*S.* Typhimurium R^-/M^+; *galE*$^+$	TLCC
Δstm3071::cm LB5010a	Intermediated deletion mutant resulting with *stm3071::cm* LB5010a	This study
Δstm3071 LB5010a	Deletion mutant of *stm3071* resulting with complete removal of *stm3071*	This study
Δstm3071 SL1344	P22ϕ was used for transduction of *stm3071* deletion into the WT S1344 strain	This study
Δstm3071 LT2	P22ϕ was used for transduction of *stm3071* deletion into the WT LT2	This study
E. coli α-Select	F- deo*R end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(rk-, mk+) *sup*E44 thi-1 *pho*A Δ(*lac*ZYA-argF) U169 Φ80*lac*ZΔM15 λ-	Bioline, UK
E. coli DH5α λpir	*sup E44, ΔlacU169 (ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir* phage lysogen	TLCC
E. coli BL21 (DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	TLCC

Table 2.2 Description of strains provided by Roth's laboratory.

Strain	Strain description
TR10000	Wild type - have the ability to produce B12, to grow on 1,2-propanediol, and to grow on minimal glucose (met+).
TR6583	A *metE* mutant - requires methionine on minimal glucose, however has the ability to grow when B12 is provided. B12 allows the synthesis of methinione using the alternative B12 dependent *metH* enzyme. Used as control strain for testing the medium. If anything is contaminated with B12 -- this strain will grow without methionine. If B12 is not produced no growth should occur on minimal medium.
TT11202	This strain lacks both methionine syntheses. Requires methionine even when B12 is provided.
TT10327	This strain is not able to synthesise B12. If growth on 1,2-propanediol media occurs, medium must be contaminated with B12, or with some alternative carbon source.
TT17131	This strain is defective for use of 1,2-propanediol. If the growth occurs on propanediol, then some alternative carbon source had contaminated the media.
TT17104	This strain is *pocR* defective - it should not grow on 1,2-propanediol and it is not able to make B12.

Table 2.3 List of primers used in this study.

Primer name	Sequence	Target
PAL*3071*R	5'CGGAGTAAAGCTTTGATGACTGTATCTCAC 3'	*stm3071* cloning into pPAL7
PAL*3071*F	5'GAGTGAATTCTTAACGGGAAGAAATATGC3'	
STM*3071*F	5'TGGCCGCTTACGCTGCAATTT3'	*stm3071* flanking regions to confirm deletion
STM*3071*R	5'GATGACGGCCCTAACAGCA3'	
*epd*F2	5'CGCGTTTCCCGGCCAGATCCAGATCGGTC ATCTTAATT3'	*epd* 5'end flanking region
*3071*F	5'TCGCTATGCGTACGGTCATAGGTC3'	*stm3071* flanking region
*3072*F	5'TTATGGCGCGTCGCCCGTTT 3'	*stm3072* flanking region
*3071*R	5'CGCGCTGTAGAGGCATTGTGGT3'	*stm3071* flanking region
*3071*R2	5'ACGCGCCATAAAGTACCTCTTAAAGAGTCG TCAA3'	*stm3071* flanking region
*3072*R	5'ATTTAGCGTACGGCTGACATCGC3'	*stm3072* flanking region
3073R	5'CGTGTCGCATCGGACGGAAGA3'	*stm3073* flanking region
*3073cat*F (formly named *3073kan*F)	5'CATTTTCCTGCGATGTCAGCCGTACGCTAA ATGCAT3'	*stm3073 knock-out*
*3073cat*R (formly named *3073kan*F)	5'TATCCATACTCCCGCTTCCGCCAGTATTAG CAGTAC3'	
*2021cat*F	5'ACCACAGGTCTGAAGTGGCAAGCATAATA GTCCTTA3'	*stm2021 knock-out*
*2021cat*R	5'AAGGTAAACAACGCCGTGATGACCGGGCT TGATCGA3'	

*3073*_UP	5'ATTTATTCGTCGCCTGGATG3'	*stm3073 knock-out confirmation*
*3073*_DOWN	5'ATGGTGGGCGACTGTTCTAC3'	
*2021*_UP	5'CTCATTCTGATAACGAAACCAC3'	*stm2021 knock-out confirmation*
*2021*_DOWN	5'CAAGTGATTACCGTACAAGGAC3'	
P*stm3071*F	5'- GCTATGAATCCGGTCATAGG -3'	*stm3071 promoter cloning site, lacZ* fusion
P*stm3071*R	5'- TGGGATCCAGTCATCGTTAC -3'	
P*cbiA*_F	5'-ACCCgaaTCCCTACCACAAATG3'	*cbiA* promoter site cloning primers; *lacZ* fusion
P*cbiA*_R	5'-TCATTTCGGATCCAAAATTTATC3'	
P_Arrrach_*stm3071*_F	ATACAAAGCACGAACCACGTTACG	*stm3071* cloning as in Arrach *et al*, 2009 promoter site cloning primers, *lacZ* fusion
P_Arrach_*stm3071*_R	GGATGGATCCGTGATTCTTAAATA	

2.2 Bacterial growth media

2.1.1 Luria-Bertani growth medium

Luria-Bertani (LB) broth containing 10% (w/v) tryptone (Oxoid, UK), 5% (w/v) yeast extract (Oxoid, UK) and 5% (w/v) NaCl (Prolabo, UK) was used for aerobic growth at 37ºC or 30ºC in liquid culture at 200 rpm (unless stated otherwise) and for STM3071 protein expression. For growth on solid medium, LB broth was solidified by addition of 1.5% (w/v), agar or for soft agar at 0.7 % (w/v) bacteriological no. 3 agar (Oxoid, UK) was added. All LB media were prepared using distilled H_2O and sterilised by autoclaving at 121ºC for 20 min.

2.1.2 NCE (No-carbon-E) minimal medium

NCE minimal medium containing 29 mM KH_2PO_4 (Sigma-Aldrich, UK), 28 mM K_2HPO_4 (Sigma-Aldrich, UK) and 16 mM $NaNH_4HPO_4{\cdot}4H_2O$ (Sigma-Aldrich, UK) was prepared in distilled H_2O and sterilised by autoclaving at 121ºC for 20 min. The medium was supplemented with trace metals (0.3 µM $CaCl_2$, 0.1 µM $ZnSO_4$, 0.045 µM $FeSO_4$, 0.2 µM $Na_2Se_2O_3$, 0.2 µM Na_2MoO_4, 2 µM $MnSO_4$, 0.1 µM $CuSO_4$, 3 µM $CoCl_2$, and 0.1 µM $NiSO_4$; Sigma-Aldrich, UK). Unless otherwise indicated carbon sources were provided at the following concentrations: 11mM glucose (Sigma-Aldrich, UK); 43 mM glycerol (Sigma-Aldrich. UK); and 50 mM 1,2-propanediol, 50 mM potassium acetate (Sigma-Aldrich, UK), and 40 mM sodium tetrathionane (Sigma-Aldrich, UK) as a final electron acceptor for the anaerobic growth. Regardless to carbon source used NCE minimal medium was supplemented with 20 µg ml^{-1} histidine (Sigma-Aldrich, UK). All medium components were prepared in acid washed glassware (washed in 4% nitric acid, Sigma-Aldrich, UK) or plastic ware and using metal-free reagents. Media supplements were sterilised by autoclaving or filter sterilised using 0.2 µm sterile filters (Whatman, UK).

2.1.3 M9 minimal medium

M9 minimal medium containing 50 mM Na2HPO4* 7H2O (Sigma-Aldrich, UK), 22 mM KH_2PO_4 (Sigma-Aldrich, UK), 8 mM NaCl (Sigma-Aldrich, UK), 18 mM NH_4Cl (Sigma-Aldrich, UK); salts were sterilised by autoclaving at 121ºC for 20 min. Medium was supplemented with 2 mM $MgSO_4$ (Sigma-Aldrich, UK), 0.1 mM $CaCl_2$ Sigma-Aldrich, UK), 0.4 % (w/v) glucose and 20 µg ml-1 histidine.

2.1.4 Media supplements

Antibiotics ampicillin (Sigma-Aldrich, UK) and chloramphenicol (Sigma-Aldrich, UK) were prepared at 100 mg ml^{-1} and 25 mg ml^{-1}, and used at 100 µg ml^{-1} and 25 µg ml^{-1}concentrations respectively, unless stated otherwise. Antibiotics were prepared in distilled H_2O and filter sterilised using 0.2 µm sterile filters.

2.3 Plasmid purification

2.1.5 Plasmid purification using Qiagen Qiaprep Miniprep Kit

Bacterial plasmids used in this study were purified using Qiaprep Miniprep Kit (Qiagen, UK), where high quality DNA was required for downstream reactions, according to manufacturer's instructions. A volume of 1-5 ml of overnight culture was transferred to a sterile microfuge tube. Bacterial cells were harvested by centrifugation at 10,000 x *g* for 1 min at room temperature (all centrifugation was conducted in Mikro 200 R Benchtop Centrifuge, Hettich, UK). Plasmid DNA was extracted using Qiaprep spin columns provided with the kit. DNA was eluted from the column in 30-50 µl of PCR water (Bioline, UK). Purified plasmid DNA was verified on 1 % (w/v) agarose (agarose molecular grade, Bioline, UK) gel electrophoresis and restriction digest and stored at -20°C.

2.1.6 Plasmid purification using TELT miniprep

TELT solution containing 50 mM Tris base (Thermo Fisher Scientific, UK), pH 7.5, 62.5 mM EDTA (Sigma-Aldrich, UK), 2.5 mM LiCl (Sigma-Aldrich, UK), and 0.4 % (v/v) Triton 100x (Biorad, UK) was prepared in distilled H_2O and sterilised by autoclaving at 121ºC for 20 min. A volume of 2-5 ml of overnight culture was centrifuged at 10,000 x *g* for 1 min at room temperature. The resulting pellet was resuspended in 800 µl of TELT solution and 80 µl of 100 mg ml^{-1} lysozyme (Sigma-Aldrich, UK); prepared in TELT solution. Samples were boiled for 120 s at 95ºC and then incubated on ice for 15 min. Following the incubation, precipitated protein content was collected by centrifugation for 15 min at 10,000 x *g.* Supernatant was transferred to a fresh tube containing 600 µl of isopropanol. Samples were mixed by inversion and incubated for 5 min on ice to allow DNA precipitation. DNA was pelleted by centrifugation for 30 min at 10,000 x *g* followed by subsequent washing with 750 µl of 70 % ethanol (Sigma-Aldrich, UK). DNA pellet was air dried for approximately 15 min at room temperature and then suspended in 50 µl of PCR water (Bioline, UK). In order

to allow more efficient DNA resuspension, samples were incubated at 65ºC for approximately 1h. Purified plasmid DNA was verified on 1 % (w/v) agarose gel electrophoresis and restriction digest and stored at -20°C.

2.4 Restriction digest

Analytical restriction digests of vectors or constructs were prepared in a sterile microfuge tube in a final volume of 20 µl. A volume of up to 10 µl of plasmid DNA, 2 µl of appropriate 10 x concentrated buffer (Roche, UK) was added. Depending on the number of restriction enzymes (Roche, UK) used for the reaction PCR water was added to a final volume of 20 µl. Restriction enzyme was added last at the volume of 1 µl. Reaction tube was incubated in a water bath at 37°C (unless stated otherwise) for 1h. The outcome of the reaction was verified using 1% (w/v) agarose gel electrophoresis and stained with Gel Red stain (Biotium, UK) or Safe View (NBS Biologicals, UK)

2.5 Genomic DNA isolation

2.1.7 Qiagen – Puregene Yeast/Bact. Kit B method

Genomic DNA was isolated using QIAGEN – Puregene Yeast/Bact. Kit B. A volume of 1 ml of bacterial overnight culture was harvested and pellet collected by centrifugation at 10,000 *x g* for 5 min at room temperature. Overnight cultures were grown in a shaking incubator at 37°C or 30°C (depending on the antibiotic sensitivity temperature) at 200 rpm.

2.1.8 CTAB method

CTAB/NaCl mixture containing 4.1 % (w/v) NaCl and 10 % (w/v) CTAB (hexadodecyltrimethyl ammonium bromide), Thermo Fisher Scientific, UK) was prepared in distilled H_2O and heated to 65ºC prior the procedure. A volume of 1-5 ml of overnight culture was harvested by centrifugation at 10,000 x *g* for 5 min at room temperature. Bacterial pellet was resuspended in 567 µl of TE buffer (10 mM Tris, 1mM EDTA) followed by addition of 3 µl of 20 mg ml^{-1} proteinase K (Sigma-Aldrich, UK) and 30 µl 10 % (w/v) SDS (Sigma-Aldrich, UK). Samples were incubated at 37ºC for 1h until the solution became clear and viscous. A volume of 100 µl of 5 M NaCl was added and samples were mixed. Previously prepared CTAB/NaCl mixture was preheated to 65ºC. A volume of 80 µl CTAB/NaCl was added, mixed well and incubated at 65ºC for 10 min. A

volume of 750 µl Phenol:chloroform:isoamyl alcohol (25:24:1) (Thermo Fisher Scientific, UK) was added, mixed well and followed by centrifugation at 10,000 x *g* for 5 min. Top layer was carefully transferred to a fresh microfuge tube containing 450 µl of isopropanol. The samples were mixed by inverting 5-7 times until the DNA was visible. DNA was centrifuged down for 5 min at 10,000 x *g* followed by subsequent wash with 750 µl of 70 % ethanol. DNA pellet was air dried for approximately 15 min at room temperature and then suspended in 200 µl of PCR water (Bioline, UK). In order to allow more efficient DNA resuspension samples were incubated at 65ºC for approximately 1h or overnight at room temperature to allow slow dissolving. Genomic DNA samples were verified using 1 % (w/v) agarose gel electrophoreses in order to confirm quality. DNA was stored at 4ºC.

2.6 **Agarose gel electrophoresis**

Tris-acetate-EDTA (TAE) 10 x concentrated buffer (Sigma-Aldrich, UK) was diluted to the final concentration of 1x concentrated solution with distilled H_2O. TAE 1 x buffer is a solution containing a mixture of 0.4 M Tris acetate and 0.01 M EDTA with pH approximately 8.3. The appropriate amount of agarose was weighed out to obtain desired gel concentration. Agarose was dissolved in 1x concentrated TAE buffer by heating in the microwave oven on defrost setting for approximately 2 min until agarose was dissolved completely. The mixture was then cooled down to approximately 50ºC and SafeView (NBS Biologicals, UK) nucleic acid stain (where stated) was added to the final concentration of 5µl per 100ml agarose. Gel was then poured into tray and comb was inserted. Once gel was set it was placed in the tank filled with 1x TAE buffer. Where stated, agarose gel was post-stained with Gel Red stain according to manufacturer's instructions.

Samples were prepared by mixing 5x DNA loading buffer (Bioline, UK) and were applied onto the gel. A concentration of about 400 ng (varies from strain to strain, where needed gDNA was diluted 1:10 or 1:100) of DNA was applied onto the wells. The gel was run until the bromophenol blue tracking dye has run to the front of the electrophoresis (approximately 40 min at 100 V). DNA was visualised using Bio-Rad-Mol Imager ChemiDoc XRS+ imaging system with ImageLab™ software at ethidium bromide setting.

2.7 Preparation of competent cells

2.1.9 Chemically competent cell preparation

Solution 1 containing 10 mM $MgCl_2$ (Thermo Fisher Scientific, UK), 50 mM $CaCl_2$, and 10 mM MES Sodium salt (2-(N-Morpholino) ethanesulfonic acid sodium salt, Sigma-Aldrich, UK) was prepared. Solution 2 containing 10 mM $MgCl_2$, 50 mM $CaCl_2$, 10 mM MES Sodium salt, and 15 % (v/v) glycerol was prepared. Both solutions were prepared in distilled H_2O and sterilised by autoclaving at 121ºC for 20 min. Both solutions were cooled down to 4ºC prior use.

E. coli strains α-select; BL21 (DE3); DH5α λpir and *S.* Typhimurium strains SL1344; LB5010a and TR10000 were incubated overnight in 10 ml LB broth at 37ºC at 200 rpm. A volume of 10 ml LB broth was supplemented with 15 mM $MgCl_2$ and inoculated 1:50 with the overnight culture. Cells were grown until mid-log growth phase (OD_{600} approximately 0.4-0.6). Optical density was measured using spectrophotometer Spec 6300 Jenway. Cells were harvested by centrifugation for 10 min at 4000 x *g*, at 4ºC (Multifuge 3LR). Pellet was washed in 3 ml ice cold Solution 1 and incubated on ice for 20 min. Cells were harvested by centrifugation for 5 min at 4000 x *g* at 4ºC. Supernatant was discarded and bacterial pellet was resuspended in 600 µl of ice cold Solution 2. Competent cells were prepared in aliquots of 100 µl and stored at -80ºC.

2.1.10 Electrocompetent cell preparation

S. Typhimurium strains SL1344, LB5010a and TR10000 were grown overnight in 10 ml LB broth at 37ºC at 200 rpm. A volume of 1 ml of fresh LB broth was inoculated 1:50 with the appropriate overnight culture. Bacterial cells were grown at 37ºC at 200 rpm until mid-log growth phase (OD_{600} approximately 0.4-0.6). Cells were harvested by centrifugation for 5 min at 4000 x *g* at 4ºC. Subsequently cells were washed twice with sterile ice cold distilled H_2O followed by resuspension in 250 µl of 10 % (v/v) sterile, ice cold glycerol. Prepared electrocompetent bacterial cells were aliquoted at 100 µl and stored at -80ºC until further use.

2.8 Bacterial transformation

2.1.11 Chemical method with $CaCl_2$

A volume of 50 µl of previously prepared or purchased (Bioline, UK) chemically competent cells was mixed with 5 µl of plasmid DNA. Cells and DNA mix was incubated on ice for 15 min. Cells were heat-shocked at 42ºC in water bath for 90 s followed by 2 min incubation on ice. A volume of 1 ml was added to the mixture followed by 30-60 min recovery incubation at 37ºC (or at 30ºC for pKD45 and pCP20) at 200 rpm. Recovered cells were spread out on a selective LB agar and incubated overnight at 37ºC (or 30ºC where appropriate).

2.1.1 Electroporation method

Sterile electroporation cuvettes, 2mm (Geneflow, UK) were cooled on ice. A volume of 50 µl electrocompetent cells were mixed with 5 µl of plasmid DNA (DNA concentration: 30-50 µg ml^{-1}) unless stated otherwise. Followed by 10 min incubation on ice the mixture of cells and DNA was transferred to a pre-cooled electroporation cuvette. Electroporation was performed using Gene Pulser, Biorad, UK using pre-set bacterial protocol - 2mm gap, 2.5 kV setting. A volume of 1 ml of fresh LB broth was added immediately to shocked cells followed by 30-60 min incubation at 37ºC (or at 30ºC for pKD45 and pCP20) at 200 rpm. Recovered cells were spread out on a selective LB agar and incubated overnight at 37ºC (or 30ºC where appropriate).

2.9 2.9 Gene disruption

2.1.2 λ-Red based homologous recombination

λ-Red mediated mutagenesis was used to introduce non-polar mutation in *stm3071*, *stm3073* and *stm2021* genes in *S*. Typhimurium LB5010a. The procedure was based on the protocol of Datsenko and Wanner (2000).

LB5010a transformants carrying Red helper pKD46 plasmid were grown in 10 ml LB broth with ampicillin and 1 mM L-arabinose overnight at 30ºC. Fresh 10 ml of LB broth, supplemented with ampicillin and L-arabinose was inoculated 1:50 and grown at 30ºC until OD_{600} reached 0.4-0.6. Then the cells were made electrocompetent by concentrating 100-fold and washing twice with 10 % (v/v) ice cold glycerol. PCR products were generated using primers with homology to the disrupted genes. pKD3 plasmid carrying *cat* cassette (chloramphenicol

resistance gene) was used as a template – table 2.3. PCR products were verified using agarose gel electrophoresis and purified using QIAquick PCR purification kit (Qiagen, UK) according to manufacturer's instructions. Electroporation with PCR product at the concentration 10-100 ng was performed as stated in 2.8.2. Recovery step was prolonged to maximum of 2h, 37ºC at 200 rpm. Cells were spread out on selective agar with chloramphenicol and incubated at 37ºC. Obtained colonies were tested for ampicillin sensitivity for loss of the Red-helper pKD46 plasmid. If some still carried resistance, bacteria were grown at 43ºC and then tested for resistance loss again.

2.10 **PCR verification**

A *cat* gene encoding chloramphenicol resistance was previously constructed in place of *stm3071* using λ-Red site directed mutagenesis (Datsenko & Wanner, 2000) and was confirmed by PCR with *stm3071*F and *stm3071*R primers (this study).

2.11 **Antibiotic resistance gene elimination**

Temperature-sensitive, chloramphenicol and ampicillin resistant plasmid pCP20 that shows thermal induction of flippase (FLP) synthesis was transformed into the strain carrying mutation *Δstm3071::cm*. Transformants were selected at 30ºC and then cultured at 43°C to eliminate pCP20 plasmid; and then tested for loss of all antibiotic resistances. The loss of *cat* cassette from the bacterial chromosome was confirmed by PCR with *stm3071*F and *stm3071*R primers.

2.12 **P22 transduction**

2.1.3 Donor phage propagation and quantification

S. Typhimurium LB5010a *galE*+ host strain was grown overnight in LB broth at 37ºC, 200 rpm. Bacteriophage P22 HT101 was diluted serially in 0.9 % (w/v) NaCl. A volume of 100 µl of each phage dilution and 100 µl of LB broth was mixed with 2.5 ml of melted LB soft agar. The mixture was poured onto the fresh LB agar plate and incubated overnight at 37ºC. Bacteriophage titre was determined. The plate with confluent plaques was soaked overnight with 0.9 % NaCl. The soak was aliquoted and stored at 4ºC with few drops of chloroform. This step was performed to propagate WT P22 HT strain. For donor strain propagation *Δstm3071::cm* bacterial strain was used as a host strain.

2.1.4 Transduction with bacteriophage P22

Recipient strain cells (*S.* Typhimurium SL1344 or TR10000) were grown overnight in 10 ml of LB broth at 37ºC, 200 rpm. Donor bacteriophage was diluted to 10^8 pfu ml^{-1}. A volume of 100 µl (10^8 bacterial cells) and 100 µl of the phage dilution was mixed to allow multiplicity of infection of 0.1 phage per cell. The mixture was incubated at 37ºC for 60 min for chloramphenicol resistance at 200 rpm. Following the incubation period the cells and phage mix was plated directly on selective medium and incubated overnight at 37ºC. The transductants were picked early in order to avoid excessive contamination. Colonies were picked using a small, sterile tip, from the top of the colony to avoid contact with the surrounding medium. Colonies were streaked out on green indicator plates and incubated overnight at 37ºC. Bacteriophage P22 strain H5 was diluted in T2 buffer and 20 µl was struck on a green indicator plate in a line down the centre of the plate. Large light green colony from the streak plates were selected and tested for correct phenotype and incubated overnight. Light green colonies are phage-free (Smith & Levine, 1967). Dark green colonies are phage-infected lysed cells. Phage free transductants were streak out on LB plates and growth overnight. Strains were grown and frozen in 50 % (v/v) glycerol at -80ºC.

2.1.5 Green indicator plates

Green plates used for differentiation between phage infected and phage free cells are poorly buffered rich plates with an excess of glucose and two pH indicator dyes Alizarin yellow and Aniline blue. Plate mix containing 0.8 % (w/v) triptone, 0.1 % (w/v) yeast extract, 0.5 % (w/v) NaCl, 1.5 % (w/v) agar, 6.5 mg ml^{-1} Aniline blue (Sigma-Aldrich, UK) and 62.2 mg ml^{-1} Alizarin yellow (Sigma-Aldrich, UK) was prepared in 1000 ml of distilled H2O and sterilised by autoclaving at 121 ºC for 20 min. The mixture was cooled down to 50 ºC and prior pouring medium was supplemented with 16.8 ml of 50 % (w/v) glucose.

2.13 **Metal tolerance assays**

Cobalt (II) sulphate hepta-hydrate (Sigma-Aldrich, UK) at a concentration of 1M was prepared in metal free distilled H_2O and filter sterilised with 0.2 µm filter. Further concentrations of cobalt were prepared aseptically in plastic ware with metal free sterile distilled H_2O. *S.* Typhimurium SL1344 and mutant strain

Δstm3071 were exposed aerobically and anaerobically to cobalt concentration ranging from 0 – 500 µM in M9 or NCE glucose metal depleted minimal media. Media conditions as described in media section (2.2.2 and 2.2.3). Cells were incubated (inoculum 1:50) anaerobically in minimal media at 37°C. To compare the growth of both strains the OD_{595} readings were obtained using Dynex Tech spectrophotometer, MX02 model. Data was generated using Revalation 4.25 software and analysed using Microsoft Excel 2007.

Overnight culture for M9 experiments was M9 medium and for NCE glucose, NCE glycerol was used for overnight aerobic culture growth. For anaerobically grown cells, medium was pre-incubated 24h prior inoculation to ensure medium anaerobicity.

2.14 **Viable count – Miles and Misra method**

A dilution series of an aliquot of the growing cultures exposed to different conditions as described were performed in PBS (phosphate buffered saline, Sigma-Aldrich, UK). Three spots of 10µl of each dilution were pipetted onto the LB agar plate and incubated overnight at 37ºC. The viable count was determined by counting the number of colonies present in each 10 µl zone at a particular dilution, obtaining the mean and calculating by multiplying by the appropriate dilution factor. Growth was described as colony forming units per ml in original culture (cfu ml^{-1}).

2.15 **Protein expression and purification**

2.16 **Overexpression on STM3071**

E. coli BL21 (DE3) cells bearing recombinant plasmids were grown overnight in 10 ml of LB broth supplemented with ampicillin. A volume of 50 ml LB medium was pre-warmed and supplemented with ampicillin prior the inoculation (1:50) with of overnight culture. Cells were grown until mid-log growth phase (OD_{600} approximately 0.4-0.6) was obtained. A volume of 1 ml of pre-induced samples was taken prior the induction. Bacterial cells were harvested by centrifugation at 10,000 x *g* for 1 minute at room temperature and stored at -20ºC for further protein analysis of pre-induced sample. Induction of expression was performed by addition of Isopropyl β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich, UK). IPTG was added to the final concentration of 1mM to remaining culture and

incubated either at 37ºC with vigorous shaking (200 rpm) for 4h or 20ºC with shaking overnight (14-16h). Following incubation period 1 ml of the culture was harvested by centrifugation at 10,000 x *g* for 1 minute at room temperature, labelled as post-induced sample and stored at -20ºC for further analysis. The remaining cells from post-induced culture were harvested by centrifugation, stored at -20ºC and used further in protein purification experiments.

2.17 Determination of protein concentration – Bicinchoninic acid assay (BCA assay)

Proteins concentration was determined by the technique developed by Bradford, 1976 using the QantiPro BCA protein assay kit (Sigma-Aldrich, UK). BCA working reagent was prepared by mixing bicinchoninic acid (prepared according to manufacturer's instructions) and copper (II) sulphate, pentahydrate 4% solution in 50:1 ratio until the correct colour was obtained.

Protein Standard Solution containing 1.0 ml of solution consisting of 1.0 mg ml^{-1} bovine serum albumin in 0.15 M NaCl with 0.05% sodium azide (Sigma-Aldrich, UK) as a preservative. Protein standards were prepared to concentrations ranging from 0.0 mg ml^{-1} to 1.0 mg ml^{-1} using 10mM Tris/HCl (Thermo Fisher, UK) pH 7.4 as a diluent.

Protein samples were prepared by diluting the obtained bacterial pellet in 50µl of 10mM Tris/HCl pH 7.4 for pre-induced samples and 100µl for post-induced protein samples.

The assay was conducted according to the manufacturer's protocol for microtitre plate experiment. A volume of 10 µl of each sample was loaded onto the 96-well microtitre plate in duplicates. Protein standards were applied onto the microtitre plate in duplicates in order to generate standard curve. A volume of 200 µl of BCA working regent prepared prior the experiment was applied into all wells. The plate was incubated at 37ºC for 30 minutes until the colour developed. The absorbance was measured at 550 nm using Dynex Tech spectrophotometer, MX02 model. Data was generated using Revalation 4.25 software and analysed using Microsoft Excel 2007.

2.18 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.1.6 Preparation of samples

Bacterial pellet from pre-induced samples was suspended in 50 µl 2x SDS sample buffer (Sigma-Aldrich, UK), and pellet from post-induced samples in 100 µl 2x SDS sample buffer. Samples were denatured by boiling at 95ºC for 5 min using heating block (Stuart SBH13OD) and loaded onto the SDS gel.

Samples used for BCA assay were prepared with 10x BugBuster protein extraction reagent (Novagen, UK) and 1mM Tris pH 7.4, 10x SDS sample buffer (20% (w/v) SDS, 500 mM Tris (v/v), pH 7.6, 1% bromophenol blue (Sigma), 50% (w/v) glycerol, and 1 µl 1 M dithiothreitol (DTT, Sigma-Aldrich, UK). Samples were denatured by boiling at 95ºC for 5 min using heating block. The appropriate volume of samples loaded onto SDS gel was estimated using BCA assay.

2.1.7 Monitoring expression levels by SDS-PAGE analysis

Electrophoresis was performed using Bio-Rad Mini-Protean Tetra System tank. Gels (15% (v/v) acrylamide) were prepared accordingly; resolving gel: 1.2 ml H_2O, 2.5 ml 30% acrylamide (Sigma-Aldrich, UK), 1.3 ml 1.5M Tris, pH 8.8, 0.05 ml 10% (w/v) sodium dodecyl sulphate, 0.05 ml 10% (w/v) ammonium persulphate (APS, Sigma-Aldrich, UK), 0.002 ml tetramethylethylenediamine (TEMED, Sigma-Aldrich, UK); stacking gel: 0.68 ml H_2O, 0.17 ml 30% acrylamide, 0.13 ml, 0.01 10% SDS (w/v), 0.01 ml 10% APS (w/v), 0.001 ml TEMED.

Casting apparatus was assembled according to the manufacturer's instructions (Biorad, UK). Resolving gel was prepared and poured between the glass slides at appropriate volume, covered by layer of isopropanol (Sigma-Aldrich, UK) and left to set (approximately 20 min). Isopropanol layer was poured off, and stacking gel was applied. The comb was placed immediately after the stacking gel was poured between the glass slides. The comb was left to set and for the further use.

SDS electrophoresis 10x concentrated running buffer was prepared prior the electrophoresis, containing 3.03% (w/v) Tris base, 14.42% (w/v) Glycine (Sigma-Aldrich, UK) and 1% (w/v) SDS.

Gel was placed in the tank containing 1x running buffer. Samples were applied into wells at a volume ranging 5-30 µl depending on the concentration of samples. Protein marker was applied at a volume of 5 µl (ColorBurst, Sigma-Aldrich, UK or SeeBlue Plus standard, Invitrogen, UK). The gel was run at 140 V for approximately 60 min (Biorad, UK Power Pac 3000).

Gels were stained using SimplyBlue (Invitrogen, UK) according to the manufacturer's instructions. Where appropriate, the recommended staining time was increased from 1h to overnight to improve quality of protein bands. The intensity of protein bands was analysed using Bio-Rad-Mol Imager ChemiDoc XRS+ imaging system with ImageLab^{TM} software.

2.1.8 Monitoring expression levels by Silver Staining SDS-PAGE analysis

Silver staining procedure was conducted using reagents from ProteoSilver^{TM} Plus Silver Stain Kit (Sigma-Aldrich, UK). The following solutions were prepared prior to the procedure: 30% (v/v) ethanol; Fixation Solution containing 30% (v/v) ethanol, 10% (w/v) acetic acid (Sigma-Aldrich, UK); Sensitizer solution containing sodium dithionite (Sigma-Aldrich, UK) 25 mg ml^{-1} (freshly prepared); Staining solution containing 0.2% $AgNO_3$ (Sigma-Aldrich, UK) with 37% Formaldehyde solution (Sigma-Aldrich, UK); Developer solution containing 6% sodium carbonate (Sigma-Aldrich, UK), 4 µg ml^{-1} sodium thiosulfate (Sigma-Aldrich, UK).

Gels were placed in a clean dish with sufficient volume of fixation solution to cover and incubated for 2h with gentle shaking at room temperature using Stuart mini orbital shaker SSMI at 50 rpm. Gels were washed 3 times for 10 min with 30% ethanol and 2 times for 10 min with ultrapure deionised water at room temperature with gentle shaking. After washing steps, gels were incubated with sensitizer solution for 1 min and then washed immediately 2 times with ultrapure deionised water for 1 min. Staining solution was applied to the gel and incubated for 25 min with gentle shaking at room temperature followed by further washing with ultrapure deionised water for 1 min. Developer solution

was then applied and incubated with gentle mixing. A volume of 5 ml of Stop solution (EDTA, Sigma-Aldrich, UK) was added immediately after bands were developed. Gels were then stored in the dark at 4 ºC. The intensity of protein bands was analysed using Bio-Rad-Mol Imager ChemiDoc XRS+ imaging system with ImageLab^{TM} software.

2.1.9 Protein purification

Profinity exact Mini purification starter kit (Biorad) was used for STM3071 protein purification.

Previously expressed protein in *E. coli* BL21 (DE3) was extracted from the cells with 10x BugBuster protein extraction reagent (Novagen, UK) in lysis buffer containing 100 mM sodium phosphate, pH 7.2 (Sigma-Aldrich, UK). A volume of 10 ml of bacterial culture was centrifuged and the resulting pellet treated with 700 µl of 1x BugBuster in 100 mM lysis buffer, and vortexed at high speed for 1 min to aid cell lysis. Lysed cell extract was centrifuged for 15 min at 10,000 *x g*. The supernatant was transferred to a fresh microfuge tube and labelled as the soluble fraction. The remaining pellet was resuspended in 700 µl of 1x BugBuster in 100 mM sodium phosphate and labelled as the insoluble fraction. Solubility of the protein was verified by SDS PAGE analysis. Proteins were stored at 4°C until purification.

Bind/wash buffer 100 mM sodium phosphate, pH 7.2 was pre-.chilled to 4°C. The storage buffer 100 mM sodium phosphate, 0.02 % sodium azide, pH 7.2 was removed by centrifugation 30s at 1,000 *x g.* The resin was equilibrated by washing twice with bind/wash buffer to remove any residual NaN_3 from the resin. A volume of 500 µl pre-chilled to 4°C protein lysate was applied on a resin and incubated for 30 min on ice on a rocking platform to allow the tagged protein with β-subtilisin subunit to bind to the resin. The column was centrifuged for 30s at 1,000 *x g* at room temperature. The unbound material was collected and labelled as a flowthrough and stored at 4°C. The resin was washed twice with bind/wash buffer. Washed flowthrough material was collected, labelled as a wash material and stored at 4°C. Elution buffer containing 100 mM sodium phosphate and 100 mM sodium fluoride (Sigma-Aldrich, UK) was applied at a volume of 500 µl onto the resin and was incubated at room temperature for 30 min on a rocking platform. Following the incubation period the supernatant

containing purified protein was collected by centrifugation at room temperature for 30s at 1,000 *x g*. Purified protein was stored at 4°C and analysed by SDS PAGE. After analysis protein was kept in 20% (v/v) glycerol and stored at -20°C.

2.19 **Modified β-galactosidase assay**

S. Typhimurium SL1344 strains carrying *lacZ* fusion plasmids were used in this assay to test conditions inducing gene expression. All *lacZ* fusions were based on a promoter-less pRS415 (control plasmid, encoding *lacZ* reporter gene). Strains were exposed to various conditions under anaerobic and aerobic conditions until OD_{600} reached 0.4-0.6. Cells were grown in M9 or NCE minimal medium with or without the addition of metal at MPC (minimal permissive concentration). Overnight cultures were always grown aerobically for M9 in M9 glucose and for NCE in NCE glycerol. Cultures were inoculated 1:50 with an appropriately prepared overnight culture.

Z buffer containing 0.06 M $Na_2HPO_4*7H_2O$ (Sigma-Aldrich, UK), 0.04M NaH_2PO_4 (Sigma-Aldrich, UK), 0.01 M KCl (Sigma-Aldrich, UK), 0.001M $MgSO_4$ $7H_2O$ (Sigma-Aldrich, UK), 0.5M β-mercaptoethanol, always added fresh (2-ME) (Sigma-Aldrich, UK) was prepared in distilled H_2O. Phosphate buffer, pH 7 and Stop buffer 1M Na_2CO_3 and 4 mg ml^{-1} ONPG in phosphate buffer were prepared in distilled H_2O immediately prior the procedure.

Cultures were diluted 1:100 or 1:10000 in Z buffer and treated with 0.1% (w/v) SDS (1 drop) and chloroform (2 drops) in order to permeabilise cell membranes. The content were vortex at high speed and left to settle for 30 min at room temperature. A volume of 176 µl of each sample was added to 96-wel plate in duplicates. Test samples were treated with 35 µl of ONPG to test for colorimetric reaction. As soon as the yellow colour was developed the reaction was stopped by addition of 88 µl of Stop buffer; added to all test and blank wells. Then a volume of 35 µl of ONPG was also added to all remaining blank wells. ONPG mimics lactose but it does not activate the *lac-Z* operon (Lapage & Jayaraman, 1964). After ~20 min when the yellow colour was developed, the Stop buffer was added to each row. A concentration of 1M Na_2CO_3 inhibits the reaction. Addition to each row prevents odd rows from *lac-Z* operon activation – negative controls. After Stop buffer was added, a volume of 35 µl of ONPG solution was added to odd rows. Then using microtitre reader the absorbance

was measured at 405 nm. The result was analysed using MS Office excel software.

2.20 **Electromobility shift assay (EMSA)**

EMSA protocol was adapted from Campbell *et al*., 2007. Plasmid DNA pUC19 was re-circularised by restriction digest with EcoRI and used as a competitor DNA. Predicted operator-promoter regions (probes) were amplified with primers listed in table 2.3. For each construct 5 parallel PCR reactions were combines and purified using purified using QIAquick PCR purification kit according to manufacturer's instructions. Equal quantities of competitor DNA and probes were mixed together with the binding buffer containing 20 mM Tris ph 7.8, 1 mM DTT, 1mM EDTA, 2.5% (v/v) glycerol, 50 µM spermidine (Sigma - Aldrich, UK). Pre-equilibrated, purified STM3071 was added to binding reactions at different concentrations. Binding reactions were then incubated at room temperature for 30 min. Following incubation, the entire reaction volume was applied onto the 5% non-denaturing Tris-borate-EDTA (TBE. 446 mM Tris, 445 mM boroc acid, 10 mM EDTA, ph 8) polyacrylamide gels prepared using Bio-Rad Mini Protean 3 system. Polyacrylamide gels underwent preliminary electrophoresis in 1 x TBE at 90 V for 90 min prior sample loading. Samples were then applied and run at 250 V for 3 min before being separated at 120 V for 60 min. One lane containing DNA loading buffer was used as an indicator for migration of DNA fragments through the gel. Gel was stained using Gel Red stain and visualised using Bio-Rad-Mol Imager ChemiDoc XRS+ imaging system with ImageLab^{TM} software at ethidium bromide setting.

2.21 **B12 assay**

S. Typhimurium cultures were grown overnight from a single colony in M9 medium with or without 1.2 µg $CoSO_4$*6H2O for 24h under anaerobic conditions. The medium was pre-equilibrated in anaerobic chamber for 24h prior the inoculation.

MacConkey base agar plates containing 2 % (w/v) peptone (Oxoid, UK), 0.5 % (w/v) bile salts (Sigma-Aldrich, UK), 0.5 % (w/v) sodium chloride, 0.0075 % neutral red (Sigma-Aldrich, UK), 0.0001 % cristal violet (Sigma-Aldrich, UK) and 1.2 % agar. MacConkey agar base plates were supplemented with supplemented with 1% 1,2-propanediol and 1 mg ml-1 $CoCl_2$*$6H_2O$. Negative control plates were not supplemented with $CoCl_2$*$6H_2O$ (Paiva *et al*., 2009).

Medium was inoculated 1:50 from the aerobic overnight culture and incubated anaerobically overnight. Then, bacteria were cultured on MacConkey base agar plated and incubated at 37°C for 24h.

2.22 **Molecular cloning**

In order to clone operator-promoter sites of *stm3071* , *stm3071* Arrach *et al*., 2008 version and *cbiA* into pRS415 appropriate primers were designed and are listed in table 2.3. All regions were amplified from *S.* Typhimurium SL1344 genomic DNA (Materials and Methods; 2.5.2) using appropriate primers. Polymerase Chain Reactions (PCR) were set up on ice according to the table below:

	Single reaction (µl)
2x MyTaq HS Mix (Bioline, UK)	25.0
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
Template – genomic DNA	1.0
PCR grade water (Bioline, UK)	23.0

PCR cycling was carried out under the following conditions:

5 min @ 95°C - 1 cycle

30 s @ 95°C
30 s @ X°C - 25 cycles
X min @ 72°C

10 min @ 72°C - 1 cycle

Following the amplifications procedure PCR products were purified using Qiagen purification kit (Qiagen, UK) according to manufacturer's instructions. PCR products and donor vector promoterless pRS415 were subjected to

restriction enzyme procedure using EcoRI and BamHI restriction enzymes (Roche, UK) accordingly following as in Materials and Methods section 2.4. Following restriction inserts and recirculised vector were subjected to incubation at 65°C for 1h in order to allow restriction enzymes heat-inactivation. Ligation PCR product : pRS415 vector ratio was adjusted to 3:1. Ligation was performed using T4 ligase and the reaction was set up as follows: DNA, 10x ATP (Roche, UK), T4 ligase and 10x ligation buffer (Roche, UK) and incubated at 4°C overnight. Ligation reaction was transformed into E. coli α-select Bioline, UK, Gold efficiency cells according to 2.8.1 procedure (Materials and Methods). Constructs were confirmed by DNA sequencing and introduced into the restriction minus *S.* Typhimurium strain LB5010a. Isolated constructs extracted from LB5010a were electrotransformed into SL1344 as described in (2.7.2 Materials and Methods). B-galactosidase assays were performed with obtained strains as described in 2.13.

2.23 **Bioinfomatics analysis**

All nucleotide and amino acid sequences were obtained from the NCBI database and subjected to BLAST analyses using either the BLASTp or BLASTx algorithm as appropriate. For multiple alignments of sequences, the ClustalW alignment tool and Multalin were applied.

2.24 **Statistical analysis**

Bacterial numbers (growth) and changes in β-galactosidase activity were transformed logarithmically before statistical analysis using a Student t-test.

3. Chapter 3 – Bioinformatics analysis

3.1 Predicting the function of stm3071

In the Introduction section, it was described that transition metals are essential for multiple biological functions (Lemire *et al*., 2013). There were given examples of such metals in the context of their biological role and transcriptional regulation of genes responsible for metal sensing and expression regulation of their acquisition mechanisms. This section focuses on seeking the evidence as to whether *stm3071* is a part of the putative operon involved in cobalt transport and whether STM3071 is important in expression regulation of this potential *S.* Typhimurium cobalt transport system.

3.1.1 Nutrient acquisition – classification of transporters in bacteria

The ability to transport nutrients across biological membranes is fundamental in all three kingdoms. Transporters are grouped with regards to energy used by transporting proteins. Primary transporters are proton-pumping ATPases or ATP-binding cassettes (ABCs) that use the energy obtained from ATP hydrolysis. Secondary transporters on the other hand couple transport to electrochemical gradients across the membrane (Eitinger *et al*., 2011).

ABC transporters form one of the largest protein superfamilies of paralogous sequences across species (Davidson & Maloney, 2007). With regards to genome size, the highest number of ABC systems is found in bacteria (Davidson *et al.*, 2008). Canonical ABC transport systems share a common structural feature, which is two transmembrane domains (TMDs) which form a translocation channel and two nucleotide-binding domains (NBDs) which hydrolyse ATP. In the ABC superfamily, there have been distinguished exporters, importers and non-transporters which do not to possess transmembrane domains. In 2008 Davidson proposed classification method of the ABC transporters. Based on sequence similarities there have been described III classes of ABC systems have been described; class I with fused TMDs and NBDs, class II including nontransport ABCs, and class III with TMDs and NBDs formed by separate polypeptide chains. In ABC importers substrate binding proteins (SBPs) are required for function (Eitinger *et al.*, 2011).

ECF transporters represent a newly discovered category of ABC transporters (Eitinger *et al*., 2011; Rodionov *et al*., 2006; Slotboom, 2013; Swier *et al*., 2015).

This type of transporters consists of ABC ATPase domains (A components, described as EcfA and EcfA'), a conserved transmembrane protein (T component EcfT) and a transmembrane substrate-capture protein (S component, also known as S-unit) (Fig 3.1 (a)). ECF components lack SBP domain, which differs them the most from ABC transporters (Rodionov *et al*., 2009).

For years, it was a subject of discussion to understand the mechanism fully as well as to explain the machinery of the S-unit. It was recently proposed that the S-unit serves as a substrate binding and substrate translocation unit (Slotboom, 2013; Swier *et al*., 2015), presented in fig 3.1 (a). They are usually involved in transport of transition metals such as Co^{2+} and water soluble vitamins (Eitinger *et al*., 2011; Slotboom, 2013). EcfA, EcfA' and T-component together with ATP facilitate translocation of the S-unit (described below in fig. 3.1 (b) (Slotboom, 2013; Xu *et al*., 2013).

As reviewed by Swier *et al.,* (2015), presented in fig. 3.1 (b), it was observed based on crystallography studies that S-unit translocates to an almost perpendicular position to the T-component. Thus, when S-unit was observed in that position and did not contain the substrate, the state of the transporter has been defined as resting state (step 1, fig 3.1 b). Presented in fig. 3.1 (b), S-unit undergoes almost 90° rotation (step 2), which allows it to reach the substrate binding site on the outer surface of the membrane. This mode of action is still considered speculative due to lacking evidence on a biochemical level and the crystal structure does not provide information on dynamic rearrangements (Finkenwirth *et al*., 2015).

(a) (b)

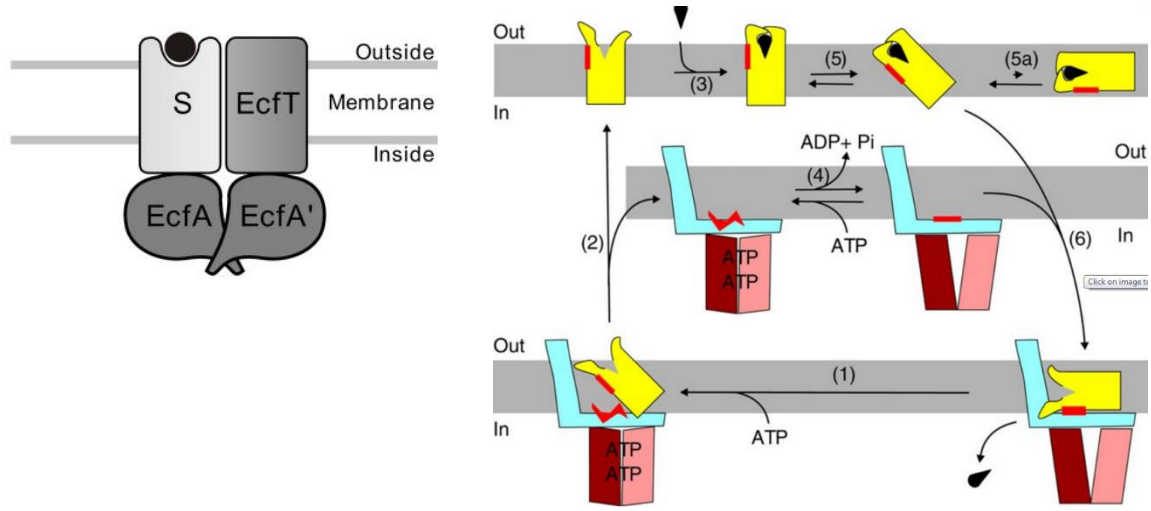


Fig. 3.1 (a) Schematic representation of the ECF transporter. EcfA and EcfA' represent cytoplasmic, highly similar ATPases. EcfT represent the integral membrane protein and S subunit represents substrate specific domain. EcfAA' and EcfT together form a module the binds and translocates the S-unit. Black dot embedded in the S subunit represents substrate (adapted from ter Beek *et al.*, 2011). **(b) Schematic representation of the ECF transporter mechanism of action.** Step 1 – ECF complex in post-translocation state binds ATP, which releases empty S-unit (yellow) by disruption of the hydrophobic interface (red). This results in reorientation of the S-unit into perpendicular position towards the outer membrane – step 2. In this position S-unit is able to bind the specific substrate (3) from the extracellular side of the membrane. Step 4 – ATP hydrolysis aids S-unit to the starting position (5) while regenerating the hydrophobic interface (6) and binds it back to the EcfT component (cyan). This results in release of the substrate to the cytoplasm (adapted from Swier *et al.*, 2015).

Diverse nutrients, such as sugars, amino acids, peptides, polypeptides, xenobiotics, vitamins and ions are transported with ABC transporters. These transporters have specific high affinity substrate-binding proteins (SBPs) that mediate the uptake of nutrients (Lubelski *et al*., 2007).

Transporter classification system includes 33 subfamilies of ABC importers that take onto account both functional and phylogenetic information (Saier *et al.*, 2016). Those subfamilies of canonical ABC transporters include importers such as: carbohydrate uptake transporters, amino acid uptake transporters, sulphate transporters (OppABCDF in *S.* Typhimurium and *Lactobacillus lactis*), ferric iron uptake transporter (SfuABC in *Serratia marcescens*), vitamin B12 uptake transporter (BtuCDF in *E. coli*), manganese/iron/zinc chelate uptake transporter (MntABC in *Neisseria meningitides*), and methionine uptake transporter (MetNIQ in *E. coli*) (Eitinger *et al.*, 2011). The transporter classification system does not include ECF transporters, however it classifies members of ECF transporters as ABC systems or secondary active transporters.

ECF are described as an abundant class of importers for micronutrients in bacteria and archaea. Substrates described as specific for ECF type transporters are water soluble vitamins, cofactors and metabolic precursors, tryptophan and queuosine and its metabolic precursors, and the metals nickel and cobalt. Similarily to canonical ABC importers, they contain ABC ATPase subunit and therefore couple ATP hydrolysis to substrate uptake. The fundamental difference between those two systems is the absence of the extracytoplasmic SBP in ECF uptake systems. ECF systems have been classified into two classes. I class is encoded by operons that consist of two genes for A units, T unit and an S-unit gene. Class II on the other hand consist of conserved A and T units and has a highly diverse S unit that can function for different transporters and can be scattered around the genome and might be expressed constitutively, or under control of metabolite-responsive riboswitches or transcription factors (Rodionov *et al*., 2009).

3.1.2 Back-up plan – *cbi* operon

It is clear that bacteria sense metals and those metals take part in regulation of expression (Lemire *et al*., 2013). It is also clear that the regulation of metal acquisition needs to be strictly controlled as the excess of those metals may be lethal to the cell (Eriksson *et al.*, 2002). In nature, most organisms have at least

one alternative to a gene that plays an essential role (Zhang & Lin, 2009). In the event of mutation which might result in loss of the function of an essential gene of some sort, the cell either prokaryotic or as well as eukaryotic has a ‘back up plan’. There are usually genes that are likely to be expressed under different conditions and regulated by different mechanisms (Koonin, 2003).

As previously described in Chapter 1, *S.* Typhimurium has a demand for cobalt as it requires this metal for cobalamin biosynthesis. The *cbiMNQO* genes, presented below in fig. 3.2, share structural similarities with other ABC-type transporters and are located within close proximity to cobalamin biosynthetic genes (Raux *et al.*, 1996; Rodionov *et al.*, 2006). Genomic context of *cbiM* (*stm2023*) presented in fig 3.2 below annotates it as encoding for cobalamin biosynthesis protein CbiM (protein coding). Subsequent genes of the putative operon *cbiN* and *cbiQ* are described as cobalt ABC-transporter substrate-binding protein CbiN and cobalt ECF transporter T component CbiQ, respectively. Downstream *cbiO* encodes for energy coupling factor (ECF) ABC transporter ATP-binding protein. Roth and colleagues, (1993) identified *cbiMNQO* gene cluster and predicted that it functions without periplasmic substrate-binding protein. Their hypothesis was confirmed a few years later which suggests that functionality of transporter (*cbiMNQO*) can be corrected by an increase in cobalt concentration in the medium (Rodionov *et al.*, 2006). This represents a prime example of ECF type of transporter which this cluster is classified as (Eitinger *et al.*, 2011). T component of the transporter, encoded by *cbiQ*, was identified to be abundant across the other species and is usually flanked by ATPase genes (*cbiO*) but not the periplasmic substrate binding protein. According to Eitinger *et al.*, (2011) many annotations were done by mistake – the presence of the CbiO determines that those were annotated correctly. They usually appear independent or within proximity of an adjacent S-unit gene (encoding substrate-binding subunit) (Eitinger *et al.*, 2011).

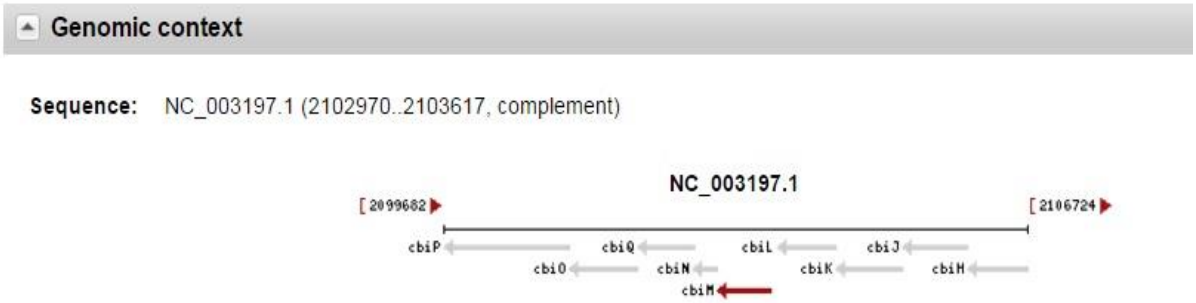


Fig. 3.2 Genomic context of *cbiM*; source NCBI. Prediction result of genomic context of *cbiM* – indicated in red, showing as protein coding, counterclockwise transcribed. Downstream *cbiNQO* are indicated with the same direction of transcription and are predicted to encode ECF ABC-type cobalt transporter (Rodionov *et al.*, 2003) .

Rodionov and colleagues (2006) confirmed that CbiO (A unit) as a nucleotide-binding unit needs to dimerise to function (as presented in fig 3.1 – A and A' dimerise). The function of other components of the system was predicted to form amino acid similarities (CbiMNQ). CbiM was predicted to have seven transmembrane domains, CbiN to have two transmembraine domains, and CbiQ belonging to a family of T proteins. It was confirmed that in the absence of CbiO in recombinant *E. coli*, basal transport activity of Co^{2+} was observed. This suggest the alternative cobalt transporter activity (Rodionov *et al.*, 2006). It was also observed that Co^{2+} and Ni^{2+} transporters are an exception among ECF systems as their S unit (CbiMN) appears heterooligomeric. Cobalt and nickels transporters have been reported to share similarities (CbiMNQO and NikMNQO). However, the Cbi system was found to have a strong preference towards cobalt (Rodionov *et al*., 2006).

Genomic location of genes encoding ECF transporter units determines transporter group classification. There are two subcategories of the ECF transporters. In I group S-unit is specific to the particular ECF module whilst in the II group ECF module interacts with various S-units of different substrate specificities. Additionally, in II group S-unit can be released from ECF module and compete with other units to bind to the module (Karpowich *et al*., 2015). S-units are a very substrate-specific and therefore were difficult to classify as they represent very diverse group and share relatively very little sequence identity, approximately ~15% (Karpowich & Wang, 2013). With accordance to Rodionov and co-workers (2006) CbiMN ECF transporter was classified to I group. They were also described to require additional small integral membrane proteins which are predicted to facilitate binding of the substrate – this mechanism however remains unclear (Rodionov *et al*., 2006; Zhang *et al*., 2010).

3.1.3 *stm3071/3072/3073/3074/3075* identification

NCBI database annotates *cboQ* (*stm2021*) as cobalt ECF transporter T component CbiQ. Search result for *cbiQ* domain in *S.* Typhimurium also identifies STM3073 cobalt ABC transporter permease (*stm3073*) (fig. 3.3), which to date has not been described in the literature and its biological function has not been confirmed. Amino acid sequence alignment has been provided and presented in fug 3.3.

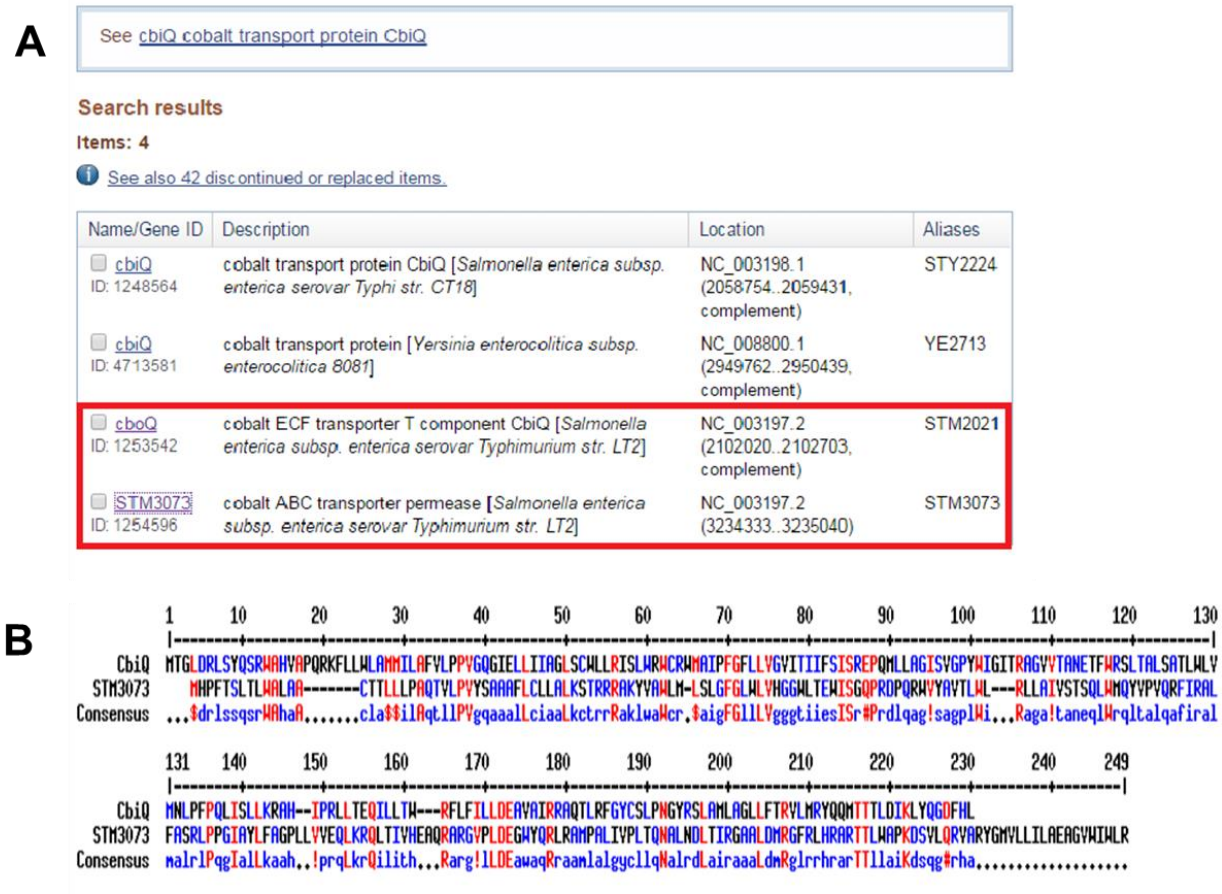


Fig. 3.3. CbiQ and STM3073 – function prediction investigation. A. NCBI gene search result for CbiQ annotation. Search result identifies CbiQ in *S.* Typhi and *Yersinia entercolitica* subsp. Entercolitica 8081. Red box indicates for CbiQ annotations in *S.* Typhimurium; *cboQ* and *stm3073*. B. Amino acid sequence alignment of CbiQ and STM3071.

Fig. 3.4. Schematic visualisation of genomic context of *stm3073* and *stm3071* queried from NCBI. A Represents genomic context of *stm3073* (in red) and its neighbouring genes location in the genome; indicated as protein coding and clockwise transcribed. B Represents genomic context of *stm3071* (in red) and its neighbouring genes. Gene type described as protein coding and described as putative DNA-binding protein; and clockwise transcribed. Transcription direction of upstream *epd* indicated in the opposite, counter clockwise direction.

Presented on the previous page, in fig 3.4 A genomic context of *stm3073* shows localisation, orientation and neighbouring genes. Upstream *stm3071* and *stm3072* as well as downstream *stm3074* and *stm3075* are transcribed in the same direction, which is the main criterion for operon prediction (Brouwer *et al.*, 2008; Rogozin *et al.*, 2004).

Domains located downstream from *stm3073* (*stm3074* and *stm3075,* fig. 3.4 A) are annotated as ATP-binding proteins (table 3.1). This, as described by Roth *et al*. (1993) and Rodionov *et al*. (2006), suggests correct classification to ECF transporters – S-unit should be located within close proximity to ATP-binding units. Additionally, according with ECF classification it is likely that *stm3072/3073/3074/3075* module belongs to the II group of the ECF transporters – similarly to *cbiNQO.* ECF transporters as described earlier, are defined as a subgroup of ABC transporters involved in the uptake of micronutrients in prokaryotes (Erkens *et al*., 2012). Described in fig 3.2 are ECF transporters that have been characterised to consist of two conserved peripherial ATPases (here *stm3074* and *stm3075*), the integral membrane protein EcfT (*stm3073*) and a non-conserved integral membrane protein that determines substrate specificity which here represents cobalt-specific transporter (Rodionov *et al*., 2006; ter Beek *et al*., 2011). All predicted functions were listed in the table 3.1 and predicted annotations compared to corresponding genes of the *cbiMNQO* cluster.

Sequence encoding *stm3071* opens a putative operon. According to NCBI, *stm3071* is annotated as a putative DNA-binding protein (fig. 3.4 B). Summarised and compared in table 1, *stm3071* does not have an equivalent in *cbiMNQO* cluster.

Genes located upstream from *cbiM* encode for cobalamin biosynthesis pathway and are regulated by end-product repression and also co-regulated by PocR regulator (Roth *et al*., 1993a). Gene encoding putative DNA-binding protein STM3071 opens the tandem (presented in fig 3.4 B). The sequence located upstream from *stm3071*, *epd* is transcribed in the opposite direction (fig. 3.4 B). Therefore, it is likely that *stm3071* opens the predicted operon and due to sequence similarities with other DNA-binding proteins it is possible that acts as a transcriptional regulator of downstream genes potentially involved in cobalt transport.

Predicted role – NCBI annotation	*stm3071/3072/3073/3074/3075*	Predicted role – NCBI annotation	*cbi* operon
DNA-binding protein	*stm3071*	cobalamin biosynthesis protein CbiM, transmembrane protein	*cbiM*
ECF transporter S-unit	***stm3072***	**cobalt ABC transporter substrate-binding protein CbiN, transmembrane protein, (ECF transporter S-unit)**	***cbiN***
cobalt ABC transporter permease (cobalt ECF transporter T component CbiQ)	***stm3073***	**cobalt ECF transporter T component CbiQ**	***cbiQ***
cobalt ABC transporter ATP-binding protein	***stm3074***	**energy-coupling factor ABC transporter ATP-binding protein**	***cbiO***
ABC transporter ATP-binding protein	*stm3075*		

Table 3.1. Summary of the predicted function of identified predicted operon *stm3071/3072/3073/3074/3075* in comparison to predicted functions of previously described *cbiMNQO* genes. Indicated in bold genes names are predicted to have analogous function. Summary based in accordance with NCBI predicted annotations.

3.2 **Operon prediction**

Operon prediction is described in literature as one of the biggest challenges in microbial functional genomics (Brouwer *et al.*, 2008; Rogozin *et al.*, 2004). Generally, genes that are classified as operons usually are arranged in tandem in a genomic sequence, delimited by promoter at the beginning of the tandem (Chen *et al.*, 2004). In literature an operon is described as a cluster of consecutive genes arranged in tandem that share the same orientation of the transcription (Rogozin *et al*., 2004). Intergenic distances between should be shorter than the inter-operon distances and operon is usually delimited by a promoter and terminator. Additionally, genes should have similar predicted functions and functional category. Ideally, those genes should be well conserved amongst phylogenetically related species – comparative genomics are one of the most reliable approaches in protein prediction. Rogazin and colleagues, (2004) described co-regulation of expression and summarised the co-existence of the regulator genes with functional genes. In some cases the regulator is present within close proximity to the operon, however this is relatively uncommon and the classic and most described example is the *lacZYA* operon in *E. coli* (Lawrence & Roth, 1996).

Analysis of *cbiMNQO* shows that they are co-localised with genes involved in cobalamin biosynthesis and are regulated by coeznzyme B12 riboswitch elements, suggesting implication in Co^{2+} uptake (Rodionov *et al*., 2006; Roth *et al*., 1993b). Experimental analysis confirmed the bioinformatics predictions, thus it *cbiMNQO* cluster is characterised as an operon.

Presented in fig 3.3, *stm3071/3072/3073/3074/3075* share the same transcription orientation on the strand and according to the genome map are adjacent in the cluster. Predicted functions of those genes suggest they are likely to form a cobalt transporter. First of the identified genes in tandem is predicted to encode a putative DNA-binding protein, which in literature suggests that it acts a regulator of downstream genes (Aravind & Landsman, 1998; Galperin, 2006).

Operon prediction software was used to analyse *stm3071/3072/3073/3074/3075* cluster (fig. 3.5). Accessed on microbesoline.com website tool provides operon prediction analysis based on proximity of analysed genes and on how conserved they are in other genomes.

The tool also takes into account whether analysed genes are predicted to encode proteins with related functions as well as expression data when available (Price *et al*., 2005). Prediction indicated transcription orientation as well as that described above identifies conserved domains found across genes. Identified COG1661 domain within *stm3071*, based on the functional predictions, is listed as a putative DNA-binding protein (fig. 3.5). Downstream genes are predicted accordingly as described above to encode putative ECF ABC-type transporter involved in cobalt transport.

VIMSS150368: **STM3071 putative DNA-binding protein (NCBI ptt file), 141 a.a.** [Salmonella typhimurium LT2]

VIMSS Predicted Operon

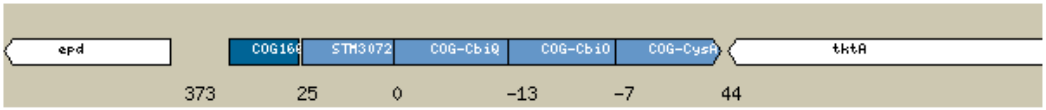
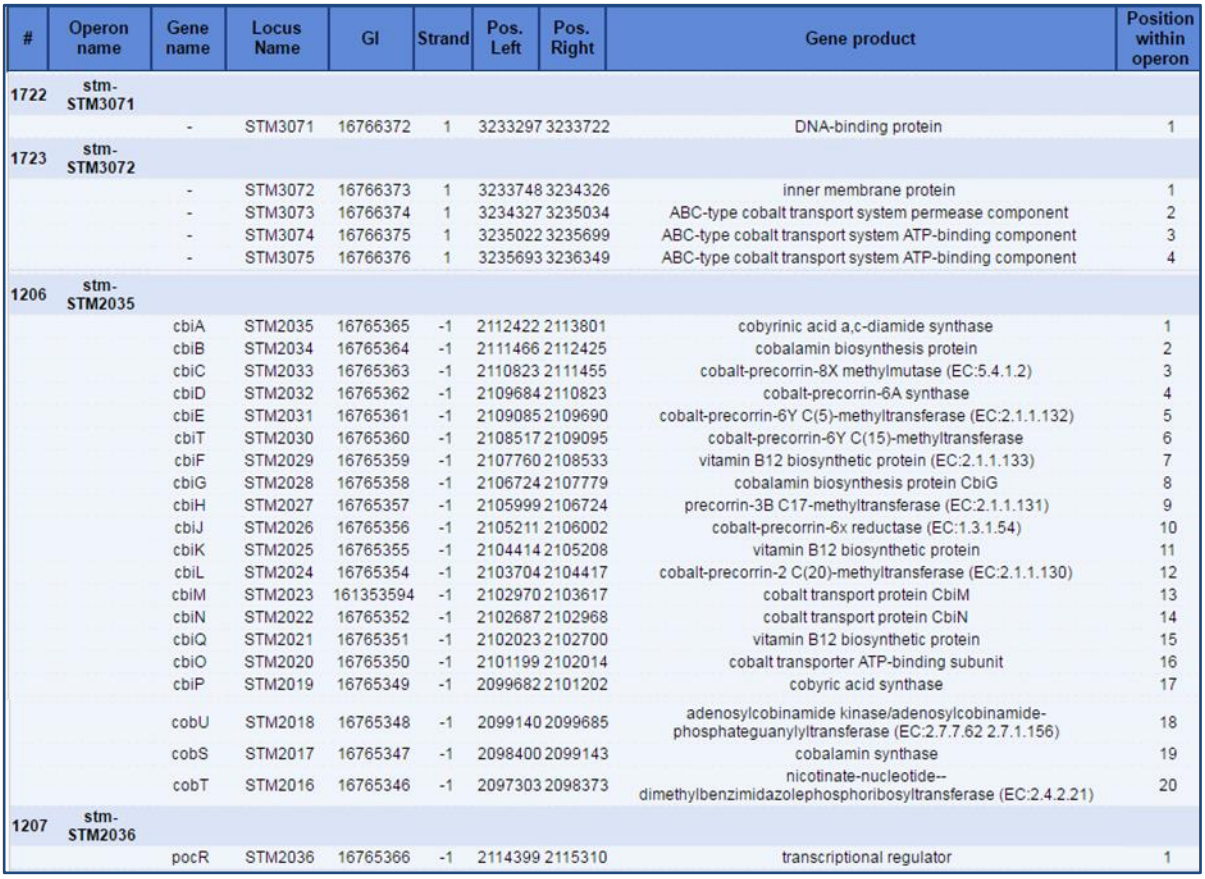


Fig 3.5. Operon prediction analysis. In the operon viewer query gene *stm3071* is shown in dark blue and downstream genes that are predicted to be part of the putative operon are presented in light blue. Arrowhead indicates the transcription direction. Numbers below the genes indicate the intergenic distance between two adjacent genes. Negative numbers indicate overlapping genes. Genes are named either by the gene name or synonym that is linked to its predicted function. Here, *stm3071*, the query gene is labelled with the predicted gene function – DNA-binding protein COG1661. Genes *stm3073* and *stm3074* are indicated as COG-CbiQ and COG-CbiO respectively. CbiQ indicates for the similarities with ABC-type cobalt transport systems, permease component CbiQ, whereas CbiO for ATPase component. (source: http://www.microbesonline.org/cgi-bin/fetchLocus.cgi?disp=1&locus=150368) (Dehal *et al*., 2010).

Additionally, the Procaryotic Operon DataBase was used to compare the above prediction. This software uses an operon prediction algorithm, which provides operon data prediction across 1200 procaryotic genomes and collectively aims to list all operons not only based on prediction results but also to include experimentally confirmed data, if available (Taboada *et al.*, 2012). Both, *cbiMNQO* as well as *stm3071/3073/3074/3075* were analysed using Operon DataBase prediction tool (fig. 3.5). The algorithm used considers intergenic spaces between consecutive genes as well as functional relationship scores of the STRING database between different groups of orthologous proteins accordingly with COG (Clusters of Orthologues) database (Taboada *et al.*, 2012).



#	Operon name	Gene name	Locus Name	GI	Strand	Pos. Left	Pos. Right	Gene product	Position within operon
1722	stm-STM3071								
		-	STM3071	16766372	1	3233297	3233722	DNA-binding protein	1
1723	stm-STM3072								
		-	STM3072	16766373	1	3233748	3234326	inner membrane protein	1
		-	STM3073	16766374	1	3234327	3235034	ABC-type cobalt transport system permease component	2
		-	STM3074	16766375	1	3235022	3235699	ABC-type cobalt transport system ATP-binding component	3
		-	STM3075	16766376	1	3235693	3236349	ABC-type cobalt transport system ATP-binding component	4
1206	stm-STM2035								
		cbiA	STM2035	16765365	-1	2112422	2113801	cobyrinic acid a,c-diamide synthase	1
		cbiB	STM2034	16765364	-1	2111466	2112425	cobalamin biosynthesis protein	2
		cbiC	STM2033	16765363	-1	2110823	2111455	cobalt-precorrin-8X methylmutase (EC:5.4.1.2)	3
		cbiD	STM2032	16765362	-1	2109684	2110823	cobalt-precorrin-6A synthase	4
		cbiE	STM2031	16765361	-1	2109085	2109690	cobalt-precorrin-6Y C(5)-methyltransferase (EC:2.1.1.132)	5
		cbiT	STM2030	16765360	-1	2108517	2109095	cobalt-precorrin-6Y C(15)-methyltransferase	6
		cbiF	STM2029	16765359	-1	2107760	2108533	vitamin B12 biosynthetic protein (EC:2.1.1.133)	7
		cbiG	STM2028	16765358	-1	2106724	2107779	cobalamin biosynthesis protein CbiG	8
		cbiH	STM2027	16765357	-1	2105999	2106724	precorrin-3B C17-methyltransferase (EC:2.1.1.131)	9
		cbiJ	STM2026	16765356	-1	2105211	2106002	cobalt-precorrin-6x reductase (EC:1.3.1.54)	10
		cbiK	STM2025	16765355	-1	2104414	2105208	vitamin B12 biosynthetic protein	11
		cbiL	STM2024	16765354	-1	2103704	2104417	cobalt-precorrin-2 C(20)-methyltransferase (EC:2.1.1.130)	12
		cbiM	STM2023	161353594	-1	2102970	2103617	cobalt transport protein CbiM	13
		cbiN	STM2022	16765352	-1	2102687	2102968	cobalt transport protein CbiN	14
		cbiQ	STM2021	16765351	-1	2102023	2102700	vitamin B12 biosynthetic protein	15
		cbiO	STM2020	16765350	-1	2101199	2102014	cobalt transporter ATP-binding subunit	16
		cbiP	STM2019	16765349	-1	2099682	2101202	cobyric acid synthase	17
		cobU	STM2018	16765348	-1	2099140	2099685	adenosylcobinamide kinase/adenosylcobinamide-phosphateguanylyltransferase (EC:2.7.7.62 2.7.1.156)	18
		cobS	STM2017	16765347	-1	2098400	2099143	cobalamin synthase	19
		cobT	STM2016	16765346	-1	2097303	2098373	nicotinate-nucleotide--dimethylbenzimidazolephosphoribosyltransferase (EC:2.4.2.21)	20
1207	stm-STM2036								
		pocR	STM2036	16765366	-1	2114399	2115310	transcriptional regulator	1

Fig. 3.6. Operon predictions analysis for *stm3071/3072/3073/3074/3075* and *cbiABCDEFGHJKLMNQOPUST* operons and *pocR*. Result obtained from Procaryotic Operon DataBase (Taboada *et al*., 2012). Selected STM3071 in the analysis appears independently to predicted operon *stm3072/3073/3074/3075*. Analysis provides information about gene name (if characterised), gene location, left and right position, gene orientation, gene product, and position in the predicted operon. The result was selectively chosen out of the entire list of operons predicted for *S.* Typhimurium.

In terms of *cbi* operon, it was clear from previous research that *cbiMNQO* are part of *cbi* genes that encode for cobalamin biosynthesis. It was also previously described that the operon is regulated by the end-product (Roth *et al.*, 1993b). Located in the nearest neighbourhood of *cbi* genes, PocR acts upon as a transcriptional regulator (Rondon & Escalante-Semerena, 1992, 1996). It is possible that STM3071 is expressed under specific conditions, as it requires activation of cobalt transport of some sort.

Further to speculations as to whether investigated genes function as an operon, it was analysed whether the orthologous cluster appears within other species. Microbesonline software provides a phylogenetic approach to comparative genomics of queried genes with other organisms. Result obtained provide information about identified domain (COG) name, and neighbouring clusters (COGs) as well as and tree orthologues (Dehal *et al*., 2010).

To further investigate similarities and conservancy of the described genes, nucleotide sequence of *stm3071* was analysed using gene tree finder (microbesonline.org). Similarly, to the previous analysis most conservancy was detected amongst other *Salmonella* strains. There were also identified in *Citrobacter koseri, Laribacter hongkongensis* and *Providentia rettgeri. Citrobacter* is a non-pathogenic bacterium but is also associated with the gut. Importantly, it can be observed that query genes are flanked in all genomes with the same genes suggesting their similar function and importance in these organisms. This predicted operon is also likely to have conserved function in other organisms due to similarities in adjacent genes also suggesting analogous location on their genomes. Flanking upstream *epd* encoding for erythrose 4-phosphate dehydrogenase located upstream from *stm3071* and is marked as transcribed anticlockwise and at the same time in the opposite direction from the *stm3071*. Located downstream from *stm3075, tktA* and *trpA* encoding for transketolase and transposase respectively, are transcribed clockwise similarly to the predicted operon.

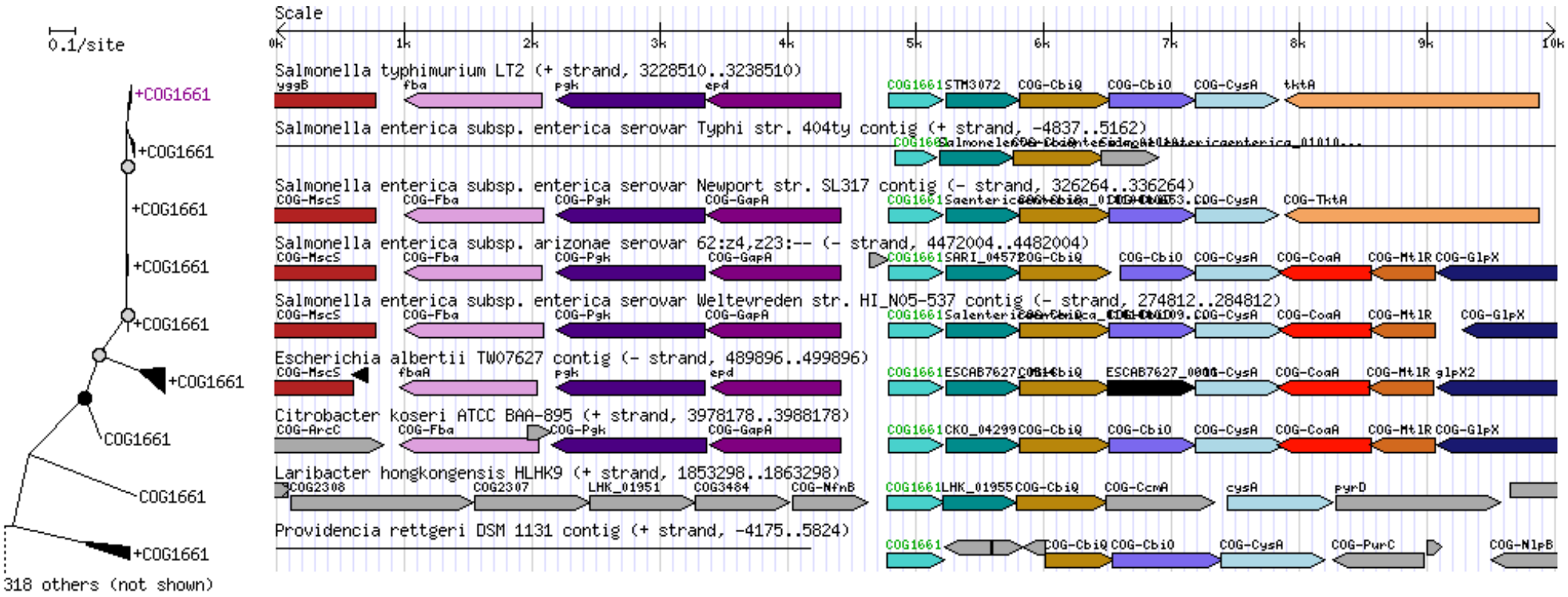

0.1/site
+COG1661
+COG1661
+COG1661
+COG1661
+COG1661
+COG1661
COG1661
COG1661
+COG1661
318 others (not shown)
Scale
0k
1k
2k
3k
4k
5k
6k
7k
8k
9k
10k
Salmonella typhimurium LT2 (+ strand, 3228510..3238510)
yggB
fba
pgk
epd
COG1661
STM3072
COG-CbiQ
COG-CbiO
COG-CysA
tktA
Salmonella enterica subsp. enterica serovar Typhi str. 404ty contig (+ strand, -4837..5162)
Salmonella enterica subsp. enterica serovar Newport str. SL317 contig (- strand, 326264..336264)
COG-MscS
COG-Fba
COG-Pgk
COG-GapA
COG-TktA
Salmonella enterica subsp. arizonae serovar 62:z4,z23:-- (- strand, 4472004..4482004)
SARI_04572
COG-CoaA
COG-MtlR
COG-GlpX
Salmonella enterica subsp. enterica serovar Weltevreden str. HI_N05-537 contig (- strand, 274812..284812)
Escherichia albertii TW07627 contig (- strand, 489896..499896)
fbaA
ESCAB7627
glpX2
Citrobacter koseri ATCC BAA-895 (+ strand, 3978178..3988178)
COG-ArcC
CKO_04299
Laribacter hongkongensis HLHK9 (+ strand, 1853298..1863298)
COG2308
COG2307
LHK_01951
COG3484
COG-NfnB
LHK_01955
COG-CcmA
cysA
pyrD
Providencia rettgeri DSM 1131 contig (+ strand, -4175..5824)
COG-PurC
COG-NlpB

Fig. 3.7. Tree Browser gene centric view for *S.* Typhimurium gene *stm3071*, accessed at Microbesonline (Dehal *et al*., 2010). COG1661 domain identified within *stm3071* encodes for putative DNA-binding domain (indicated in cyan). Downstream *stm3072* is described as hypothetical protein and/or across listed organisms as putative inner membrane protein (indicated in turquoise). COG-CbiQ is described as ABC-type cobalt or multidrug transport system (indicated in gold). COG-CysA with predicted ATPase component, conserved across other species (indicated in light blue).

Tree browser analyser was used for gene context analysis of the *cbi* genes. This shows conservative domains across the putative operon and predictions of the gene functions. The view determined for *stm3071* shows that all genes in the predicted operon are protein coding (fig. 3.7).

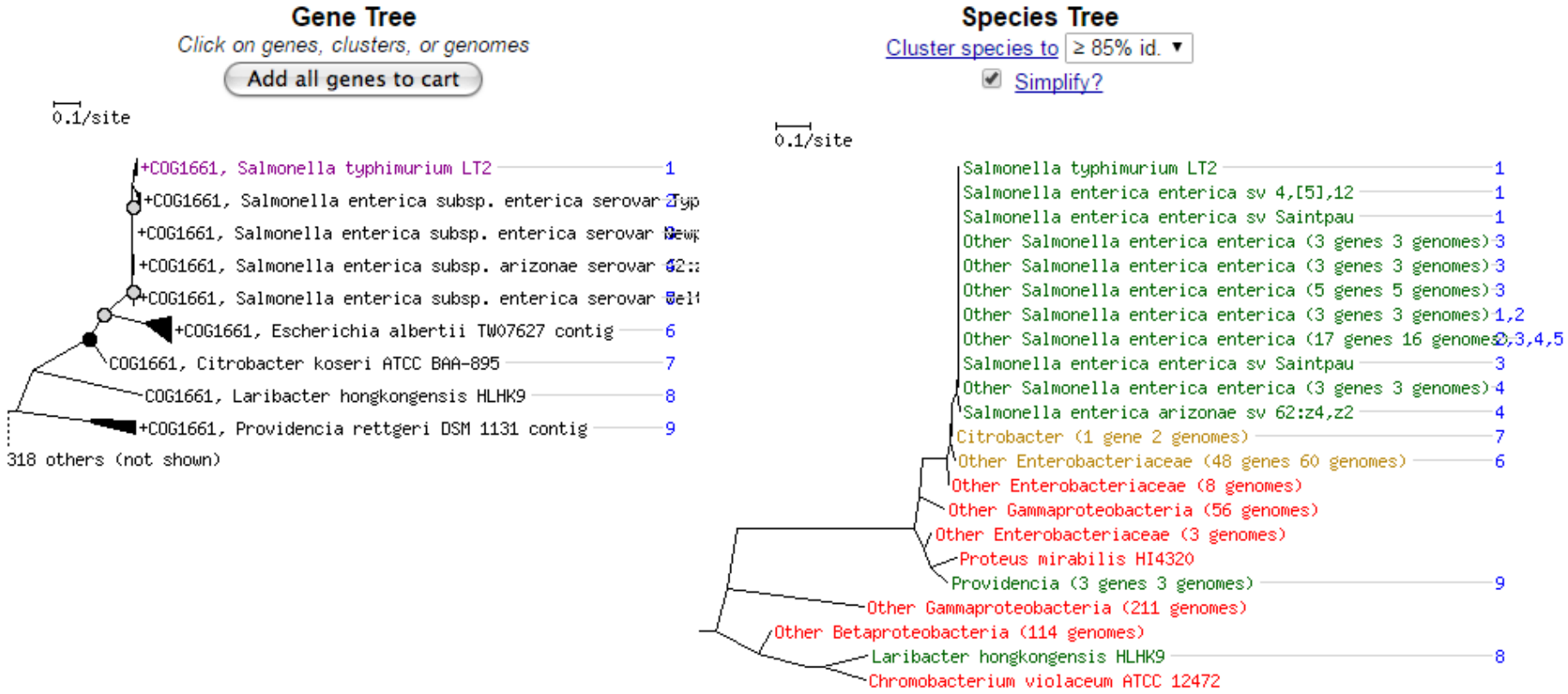

Gene Tree
Click on genes, clusters, or genomes
Add all genes to cart
0.1/site
+COG1661, Salmonella typhimurium LT2 1
+COG1661, Salmonella enterica subsp. enterica serovar 2
+COG1661, Salmonella enterica subsp. enterica serovar
+COG1661, Salmonella enterica subsp. arizonae serovar
+COG1661, Salmonella enterica subsp. enterica serovar
+COG1661, Escherichia albertii TW07627 contig 6
COG1661, Citrobacter koseri ATCC BAA-895 7
COG1661, Laribacter hongkongensis HLHK9 8
+COG1661, Providencia rettgeri DSM 1131 contig 9
318 others (not shown)
Species Tree
Cluster species to ≥ 85% id.
Simplify?
0.1/site
Salmonella typhimurium LT2 1
Salmonella enterica enterica sv 4,[5],12 1
Salmonella enterica enterica sv Saintpau 1
Other Salmonella enterica enterica (3 genes 3 genomes) 3
Other Salmonella enterica enterica (3 genes 3 genomes) 3
Other Salmonella enterica enterica (5 genes 5 genomes) 3
Other Salmonella enterica enterica (3 genes 3 genomes) 1,2
Other Salmonella enterica enterica (17 genes 16 genomes) 2,3,4,5
Salmonella enterica enterica sv Saintpau 3
Other Salmonella enterica enterica (3 genes 3 genomes) 4
Salmonella enterica arizonae sv 62:z4,z2 4
Citrobacter (1 gene 2 genomes) 7
Other Enterobacteriaceae (48 genes 60 genomes) 6
Other Enterobacteriaceae (8 genomes)
Other Gammaproteobacteria (56 genomes)
Other Enterobacteriaceae (3 genomes)
Proteus mirabilis HI4320
Providencia (3 genes 3 genomes) 9
Other Gammaproteobacteria (211 genomes)
Other Betaproteobacteria (114 genomes)
Laribacter hongkongensis HLHK9 8
Chromobacterium violaceum ATCC 12472

Fig. 3.8. The species view for *stm3071* (Dehal *et al*., 2010). The gene tree is presented on the left and species tree on the right. Gene tree leaves were labelled numerically and correspond with species tree. In species tree green colour indicates that every genome in this group contains a gene from the shown part of the tree. *Citrobacter* appears to have only one gene in 2 genomes so it might be likely that COG1661 was acquired in the event of horizontal gene transfer. Species tree also indicates that is very common in *Salmonella* genus.

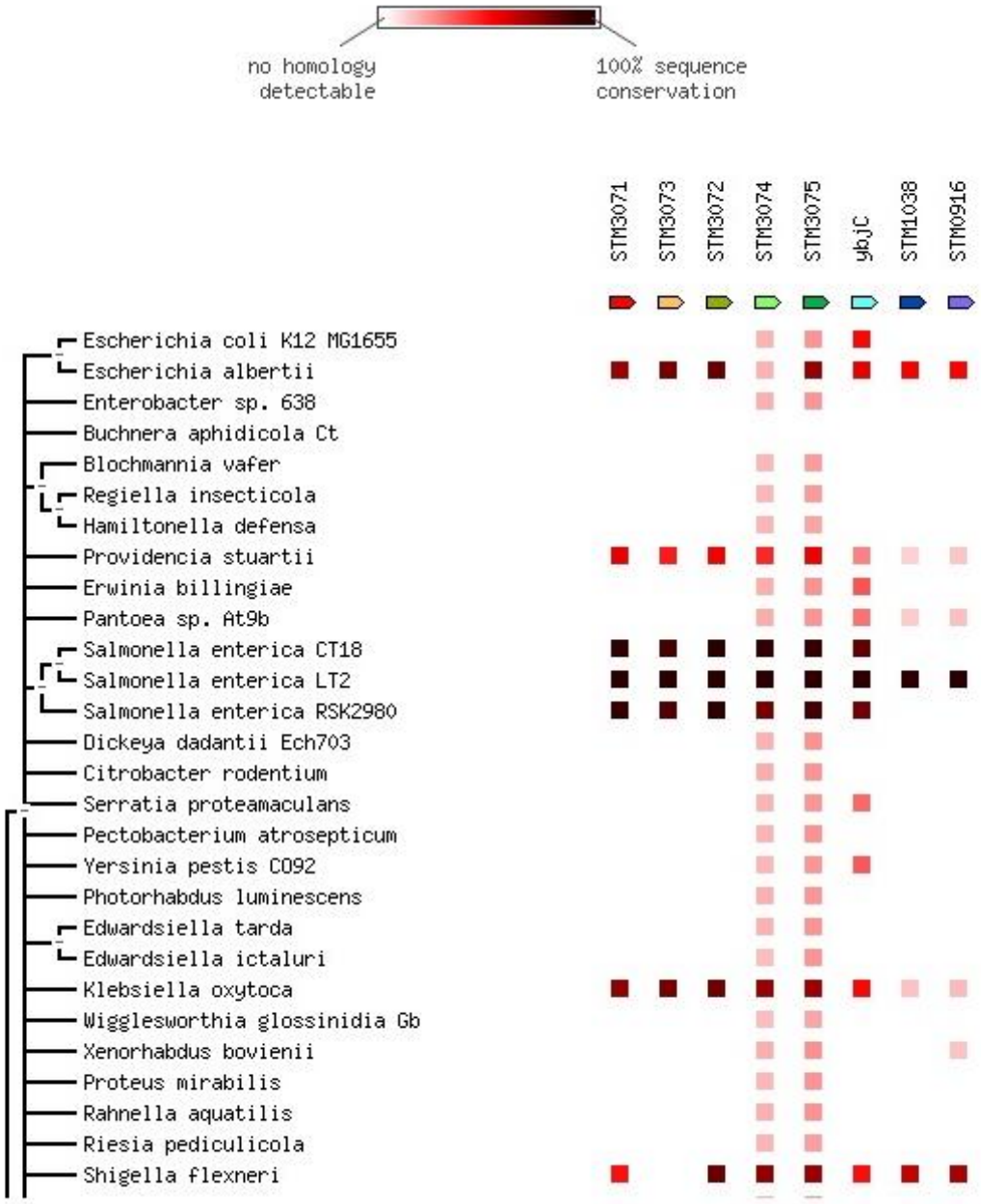


Fig. 3.9 The evidence view of the similarity conservancy patterns amongst other organisms. This view represents a phylogenetic display of the organisms. This allows viewing clusters of orthologous groups of genes in other organisms, here referring to proteins. Colours represent quantitative similarities of the protein in other species, where dark brown stands for 100% identity. Required medium confidence score was standardised at p≤0.4.

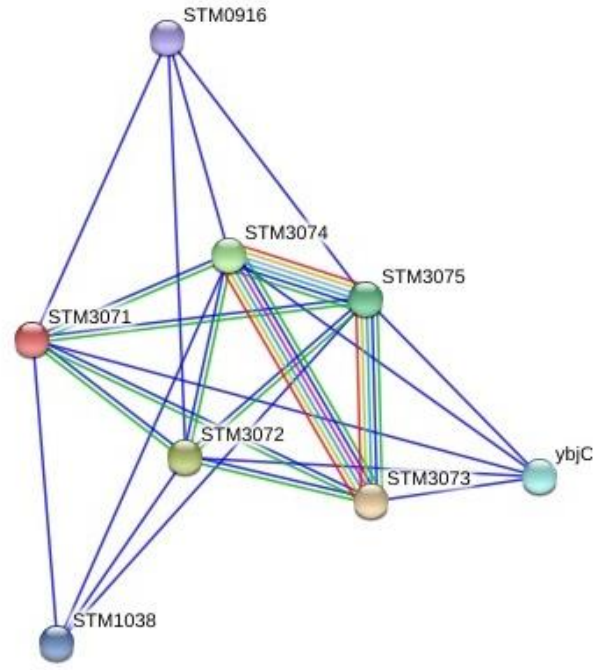
The picture on previous page (fig. 3.9) represents visual illustration of the phylogenetic occurrence of the queried genes. It shows that the putative operon occurs with highest sequence identity in *Salmonella enterica*.

Investigated further from the evidence view (fig 3.9), proteins that shared most conservancy amongst other organisms were analysed based on the nucleotide sequence identities. The most similar organisation of the putative operon was found in *Salmonella* species. Similar patterns were also detected in other pathogens, such as *Escherichia albertii*, *Providencia stuarti*, *Klebsiella oxitoca*, and also *Shigella flexneri*. Interestingly, most commonly occurring genes found in other species were *stm3074* and *stm3075.* Both of these encode for putative cobalt ABC transporter ATP-binding proteins.

The phylogenetic approach is another important criterion in operon prediction approach serving as another piece of evidence that *stm3071/3072/3073/3074/3075* might function as an operon.

3.3 **STM3071**

Using String 10 software, a correlation view of *stm3071* was generated. Correlation evidence of *stm3071*, presented over the page, represents interaction evidence between *stm3071* and other genes. Gene *stm3071* is described to encode for putative DNA-binding protein STM3071. Fig. 3.9 over the page further shows the evidence of possible interaction with downstream genes. Most interactions in the evidence view of *stm3071* are linked to downstream genes predicting for co-occurrence and neighbourhood. Genes *stm0916* and *stm1038* described as predicted major tail proteins and hypothetical *ybjC* were linked to *stm3071* based on co-occurrence evidence. NCBI results describe major tail proteins as containing Ig-like domain which are members of the family that are found in bacterial and phage surface proteins such as intimins. Most interactions were detected between *stm3072*, *stm3073*, *stm3074* and *stm3075*.



Your Input:

STM3071 DNA-binding protein (141 aa)
(Salmonella enterica LT2)

Predicted Functional Partners:

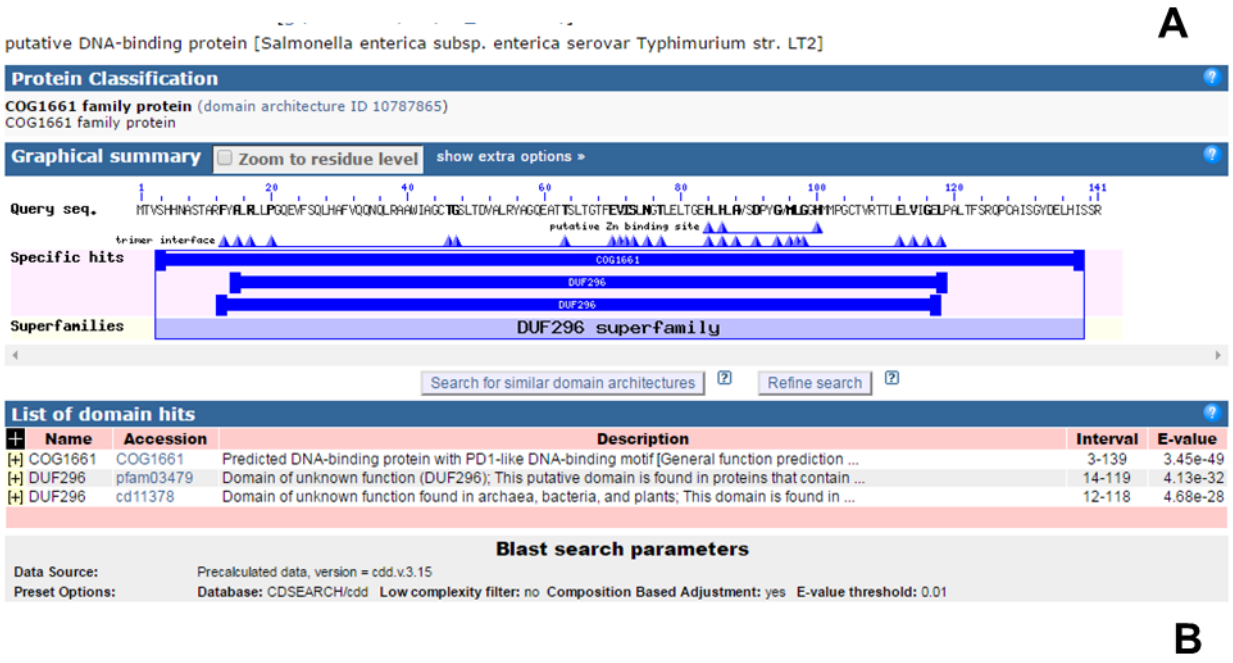
		Neighborhood	Gene Fusion	Cooccurrence	Coexpression	Experiments	Databases	Textmining	[Homology]	Score
STM3073	ABC-type cobalt transport system permease component (235 aa)	•		•						0.949
STM3072	inner membrane protein (192 aa)	•		•						0.945
STM3074	ABC-type cobalt transport system ATP-binding component (225 aa)	•		•						0.919
STM3075	ABC-type cobalt transport system ATP-binding component (218 aa)	•		•						0.908
ybjC	hypothetical protein (96 aa)			•						0.500
STM1038	major tail protein (249 aa)			•						0.450
STM0916	major tail protein (247 aa)			•						0.427

Fig. 3.10 The String 10 network view for *stm3071*. Combined screenshots from String 10 software analysis which has been queried with *stm3071 of S. Typhimurium*. Coloured lines between proteins indicate various types of interaction evidence. Network view shows correlations between genes in the predicted operon. Blue indicates co-occurrence amongst other organisms, green stands for neighbourhood of analysed genes, red indicates for gene fusion, and light blue for homology. String 10 software (source: http://string-db.org/).

3.2.1 Analysis of proteomic context

NCBI conserved domains finder was used to analyse STM3071 amino acid sequence (source: http://www.ncbi.nlm.nih.gov/protein/). Amino acid sequence of STM3071 was obtained from NCBI and presented in fig. 3.11 B over that page.

The first of the genes *stm3071* was analysed using NCBI conserved domains finder and DUF296 was identified (Fig. 3.11 A). This represents a domain of unknown function. According to NCBI this protein is largely found in archea, bacteria and plants and contains an AT-hook motif that suggests a role in DNA-binding. Three conserved histidine residues appear to form a zinc-binding site and that domain has been observed to form homotrimers. It occurs together with a thioredoxin-like domain. Proteins possessing zinc fingers typically function as molecules that bind RNA, DNA proteins or other small molecules (Ali *et al*., 2012). There are three types of AT-hook proteins and these were classified according to their sequence similarities (Aravind and Landsman, 1998). AT-hook motifs are associated with known functional domains as seen in a chromatin proteins and DNA-binding proteins (histone folds, homeodomains, zinc fingers). They usually support changes in a DNA structure and function as a polypeptide of multidomain. The number of experiments conducted on AT-hook containing proteins demonstrated that they play an important role in chromatin structure and act as transcription cofactors (Kim *et al*., 1997; Slama-Shwok *et al.*, 2000).



B

MTVSHHNASTARFYALRLLPGQEVFSQLHAFVQQNQLRAAWIAGCTGSLTDVALRYAGQE
ATTSLTGTFEVISLNGTLELTGEHLHLAVSDPYGVMLGGHMMPGCTVRTTLELVIGELPA
LTFSRQPCAISGYDELHISSR

Fig.3.11 NCBI conserved domains finder. A. This putative domain is found in protein containing AT-hook motif that strongly suggests DNA-binding function of this protein, zinc-binding site (Aravind and Landsman, 1998). B. Amino acid sequence of STM3071.

3.4 **Summary**

Summarising the above findings, it is likely that *stm3071/3072/3073/3074/3075* represent a putative operon. It may be that they exist as a copy for *cbiMNQO* genes and are important for the organism in case of loss of function. It is also possible that they are expressed under completely different conditions and their occurrence is separate to *cbi* genes. These genes are present across 91 other species associated with the gut, which might suggest that they are expressed and are needed for intracellular survival. Thus it is also likely that they were not acquired by horizontal transfer. This also suggests that they are likely to be functional. There is a question, however whether *stm3071* plays a separate role or is a part of the putative operon.

STM3071 is predicted to act as a DNA-binding protein and it is not clear whether it plays a role as a transcriptional regulator of downstream genes or whether it is not related to the downstream cluster. There is also potential that STM3071 might act as a metal regulatory protein due to the presence of predicted zinc binding site. Investigation of the role of STM3071 is described in the next chapter.

4. Chapter 4 – Investigation of STM3071

4.1 Introduction - DNA-binding and zinc-binding proteins in bacteria.

In chapter 3 it was discussed that based on the sequence identity STM3071 might represent DNA-binding protein due to the presence of an AT-hook motif and a putative zinc-binding site. Thus, it is possible that it may function as a transcriptional regulator of downstream genes putatively involved in cobalt transport.Cellular regulation of transcription occurs at the level of initiation. In order for the process to happen recognition of a specific DNA sequence needs to occur and is mostly performed by proteins under strictly controlled conditions (Alberts *et al.*, 2002). Regulation of transcription is the most critical control point in gene expression (Seshasayee *et al*., 2011). Transcriptional factors are usually identified based on the presence of a DNA-binding motif using sequence searches against protein family databases (Finn *et al.*, 2014). DNA-binding proteins in bacteria represent a varied group of proteins. The first identified DNA-binding protein in prokaryotic organisms was helix-turn-helix (HTH), and that type of motif was initially considered to be exclusive for bacteria. It was characterised by an X-ray crystallography and it was the cyclic AMP receptor protein of *E. coli* (McKay & Steitz, 1981). Now, HTH is a well-known motif that was identified in many DNA-binding proteins and across many species both prokaryotic and eukaryotic. Another commonly identified DNA-binding motif is Zn-binding site (Alberts *et al*., 2002). Whilst the HTH motif consists of amino acids, zinc fingers coordinate one or more zinc atoms as structural components. DNA-binding proteins that contain a zinc-binding site are common in eukaryotic organisms, however not so frequently identified in bacterial organisms. Those found in bacteria are termed histone-like proteins and they are involved in DNA modulation in processes such as replication, recombination, repair, and transcription (Dillon & Dorman, 2010; Drlica & Rouviere-Yaniv, 1987).

Bacterial proteins that contain zinc-binding sites are usually involved in repression of transcription in response to heavy metal toxicity but the Zn-finger DNA-binding domain is mostly found in eukaryotic organisms. Zinc acts as a conformational inducer to structural rearrangements leading to repression

(Ebert & Altman, 2008; Petrarca *et al*., 2010). Zinc-binding transcriptional regulators represent a rather diverse group and in some cases binding is specific not only to zinc but can also involve other divalent metal cations (Hantke, 2001).

There is a rather small set of non-HTH bacterial DNA-binding proteins amongst those that were identified. Accordingly, with as mentioned above the zinc-finger DNA-binding domain is prevalent across eukaryotic transcription factors (TFs) but is rare in bacteria. C2H2 Zn-finger (cysteine and histidine coordinated zinc ion) was identified in Ros/MucR of *Agrobacterium tumefanciens* (Chou *et al*., 1998; Esposito *et al*., 2006) and also MucR of *Rhizobium meliloti* (Keller *et al.*, 1995), however it differs to the eukaryotic ones. They have less residues and only 2 pairs of metal-chelating ligands (Esposito *et al*., 2006). Zinc ribbon is also found in bacterial TFs, however it is not a primary DNA-binding domain but rather interacts with RNA polymerases, such as NrdR found in *E. coli* that acts as a regulator of the ribonucleotide reductase operons (Torrents *et al*., 2007). There has also been identified AT-hook motif to occur in bacterial TFs. AT-hook proteins in eukaryotic organisms belong to the high mobility non-histone chromosomal group (HMG-I(Y)) which bind to minor grooves of DNA via conserved 9 amino acid domain called AT-hook. This motif is also prevalent in eukaryotic organisms and not so frequent in prokaryotic (Aravind & Landsman, 1998). Thus, bacterial proteins containing AT-hook have not been very well characterised.

4.2 Protein function investigation

4.1.1 Prediction analysis

Based on sequence characteristics and predicted function STM3071 was proposed as DNA-binding protein acting as potential regulator of downstream genes.

Initially, STM3071 was annotated with DNA-bind prediction. In-depth investigation of the sequence revealed that the amino acid sequence of STM3071 does not contain the PRGRP sequence, which represents the functional AT-hook domain responsible for minor groove binding (Aravind & Landsman, 1998). However, as it was previously annotated, the domain

resembles similarities with proteins that contain AT-hook. CnD3 software was used to visualise 3D protein structure – presented in figure 4.1 over the page.

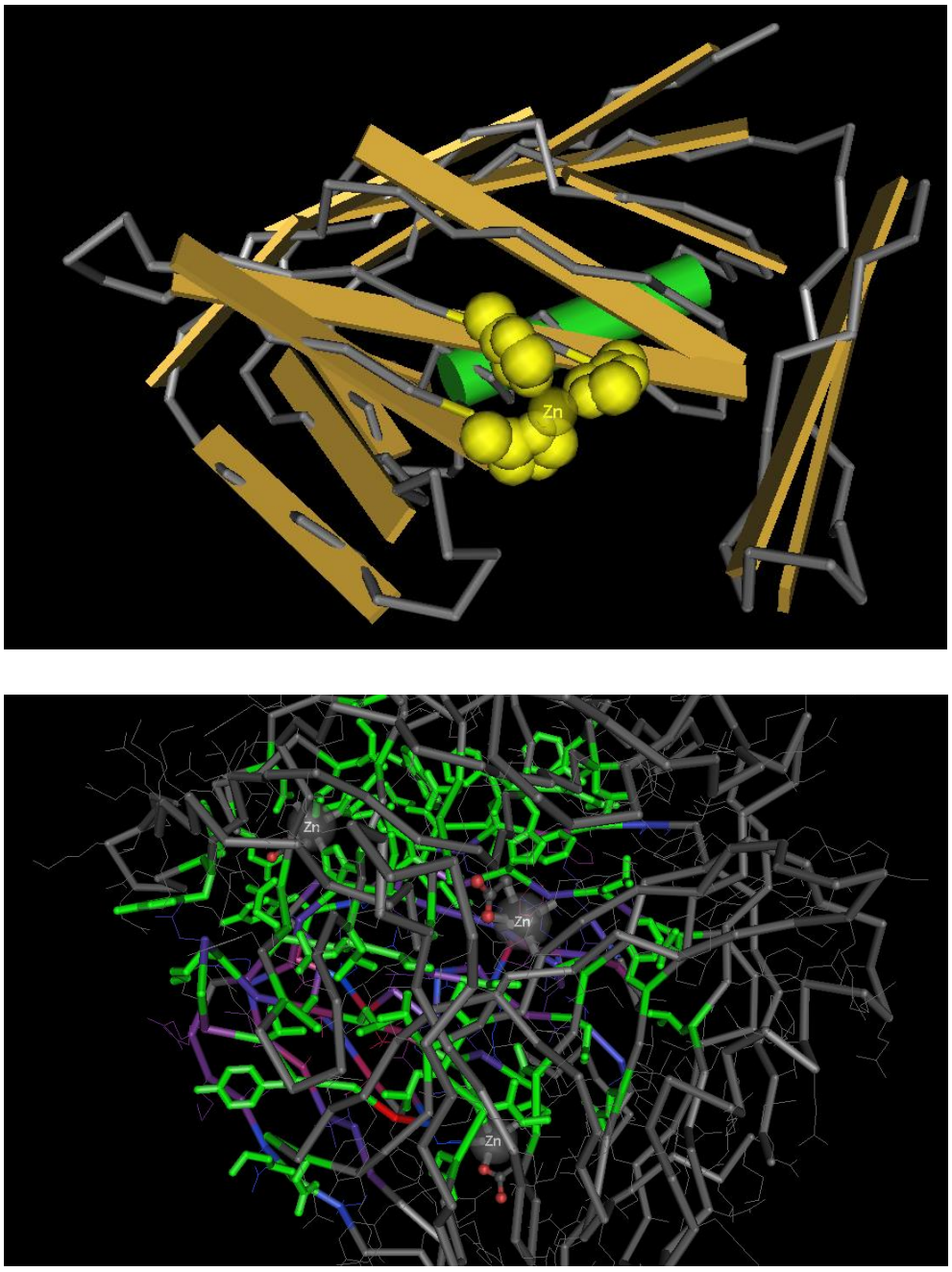


Fig. 4.1 Cn3D software prediction of conserved domains identified within STM3071. (a) Visual representation of DUF296 (domain of unknown function). Usually associated with proteins that also contain AT-hook motifs based on which protein is predicted to play role in DNA-binding as a whole. Three conserved His residues indicated in yellow, appear to form zinc-binding site – the domain was observed to form homotrimers and was associated with thioredoxin-like in uncharacterised cyanobacterial proteins. (b) Visual

presentation of 3 domains (in green), homotrimer with coordinated 3 zinc atoms. (combination of screen shots; generated using Cn3D 4.3 software) (Marchler-Bauer *et al.*, 2015).

Annotation summary accessed on NCBI predicts zinc-binding site evidence based of *Vibrio cholerae* Q9KM02 protein that also represents putative zinc-binding site. Based on sequence similarities with *Archaeglobus fulgidus* AF0104 protein homotrimer formation prediction was annotated (NCBI).

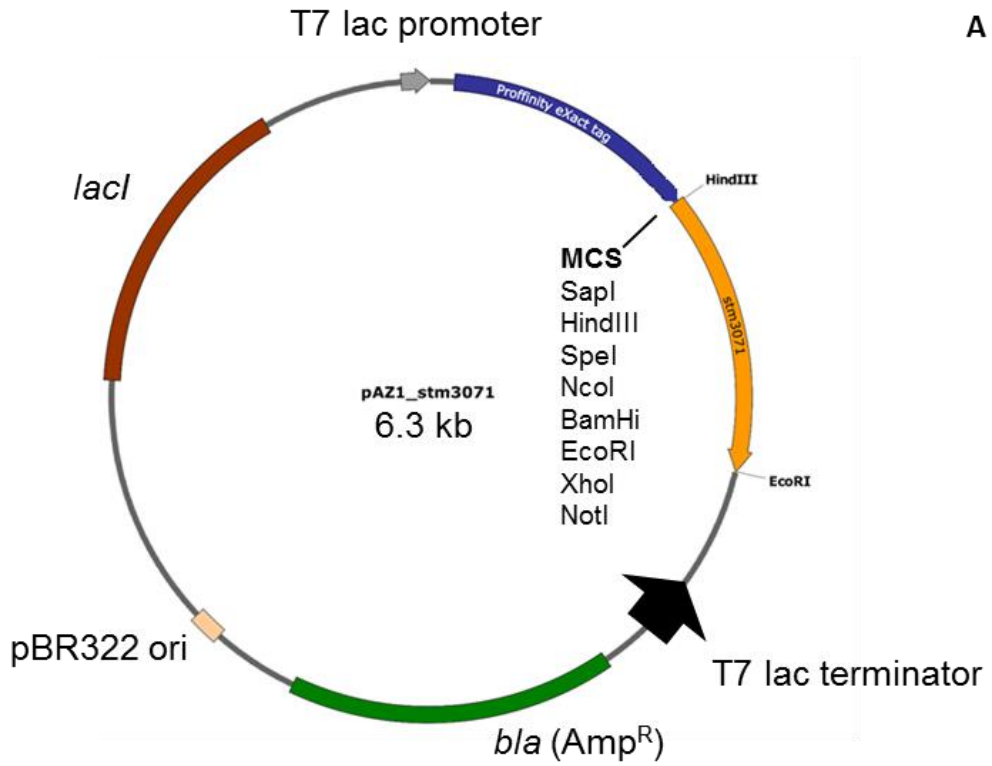
4.1.2 Construction of recombinant protein

To investigate the function of STM3071, nucleotide sequence encoding predicted protein was cloned into pPAL7 expression vector in order to obtain recombinant protein for further analysis.

The ability to express and purify large quantities of recombinant proteins in combination with whole genome sequencing has revolutionised many fields of biology including drug discovery. The most commonly used expression system is *E. coli* due to relatively high yields of protein, quick doubling time and high growth rates (Miroux & Walker, 1996). The ability to express and purify recombinant protein in large quantity allows to determination of its biochemical properties, which are crucial for complete protein characterisation (Rosano & Ceccarelli, 2014).

Engineered subtilisin pro-domain was fused to the N-terminus of the STM3071. The pro-domain acts as a high affinity tag that is automatically removed by an auto-cleavage reaction during the elution from subtilisin affinity chromatography resin. Encoding STM3071 nucleotide sequence was cloned in frame into pPAL7 expression vector under T7 strong promoter in order to obtain heterologous protein construct. The 8 kDa Profinity eXact tag containing EEDKLFKAL amino acid sequence is specifically recognised by genetically engineered proteolytic enzyme subtilisin active site that was immobilised on the resin (Biorad, UK). The tag is a form of a wild-type subtilisin BPN prodomain that contains cleavage recognition sequence of the subtilisin mutant. Exo-recognition sequence for the subtilisin increases the binding activity to the enzyme active site which enhances cleavage specificity to the tag (Ruan *et al*., 2004).

The nucleotide sequence was amplified and ligated into HindIII and EcoRI sites in pPAL7 (fig. 4.2).



HindIII

start

5'-AAGCTTTGATGACTGTATCTCACCACAATGCCTCTACAGCG
CGCTTTTACGCGCTTCGTCTGCTTCCCGGCCAGGAAGTGTTTTCCCAATTACACGCCTTT
GTGCAACAGAACCAATTACGTGCCGCCTGGATAGCAGGGTGTACCGGCAGTCTGACCGAT
GTTGCGCTGCGTTATGCCGGGCAAGAGGCGACCACATCGCTCACCGGCACGTTTGAAGTT
ATCTCGCTAAACGGCACGCTTGAATTGACCGGCGAGCATCTGCATCTCGCGGTATCGGAT
CCCTACGGGGTTATGCTCGGCGGCCATATGATGCCAGGGTGTACCGTGCGTACCACGCTG
GAACTGGTTATCGGCGAGCTGCCAGCGCTGACGTTCAGCCGTCAACCGTGCGCCATTTCC
GGTTATGACGAACTGCATATTTCTTCCCGTTAAGAATTC-3'

stop

EcoRI

Fig. 4.2 Schematic drawing of pAZ1/*stm3071* expression vector construct. A. Sequence encoding *stm3071* was cloned into HindIII and EcoRI restriction site (indicated in orange); inserted in frame with Profinity eXact tag (in blue), under the control of T7 lac promoter (light blue). Indicated in green *bla* encodes for ampicillin resistance. Indicated in maroon *lacI* repressor binds to DNA and prevents RNA polymerase from escaping promoter region. In the presence of IPTG repressor changes allosteric coordination allowing RNA polymerase to elongate – expression of the recombinant protein is controlled upon IPTG induction. B. Sequence of Profinity eXact and cloning region. Cloning into HindII and BamHI allows precise fusion. Purification results in a tag-free target protein without amino acids after cleavage Profinity eXact of the tag.

Nucleotide sequence of *stm3071* (**NC_003197.2)** was amplified from *S.* Typhimurium SL1344 with PAL3071R and PAL3071F primers (table 2.3) and cloned into pPAL7 expression vector (Taylor, unpublished data).

Selected clones were recovered and transformed into *E. coli* α-select cells (Bioline, UK). Plasmid DNA was isolated from 26 selected clones. Each clone was tested for insert presence by PCR with primers used for cloning (PAL3071R and PAL3071R). PCR products were analysed using gel electrophoresis and cloning was confirmed successful in most selected constructs (fig. 4.3).

A number of 4 successful clones were selected and sequenced. Sequencing was carried out with provided pPAL7 sequencing primer. Obtained sequences were aligned against the original *stm3071* sequence (**NC_003197.2)** to test for any potential errors (fig. 4.4).

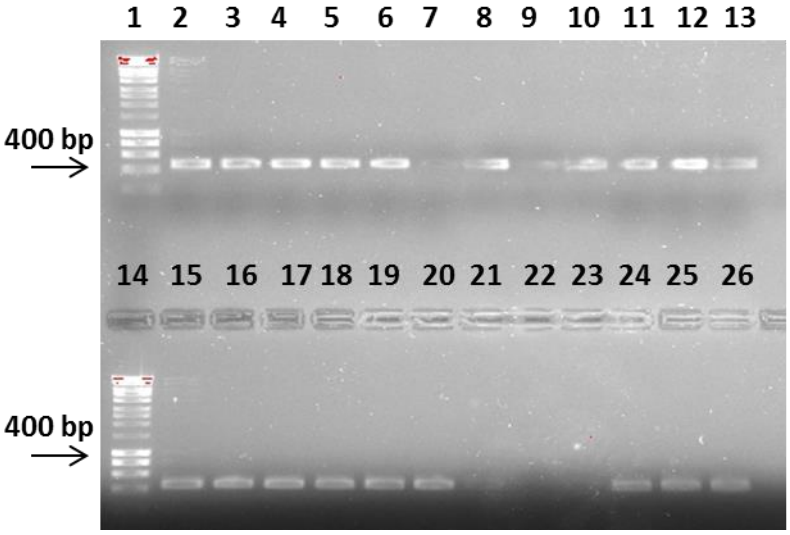


Fig. 4.3 Agarose gel electrophoresis analysis of amplified *stm3071* inserts from cloning vector pPAL7. Lines 1 and 14: DNA size marker (Hyperladder 1). Lines 2-13 and 15-26 PCR products. Successful cloning was confirmed by PCR with PAL3071F and PAL3071R cloning primers. PCR product ~400 bp. Gel electrophoresis (1% agarose gel) analysis indicated cloning was successful in most selected clones.

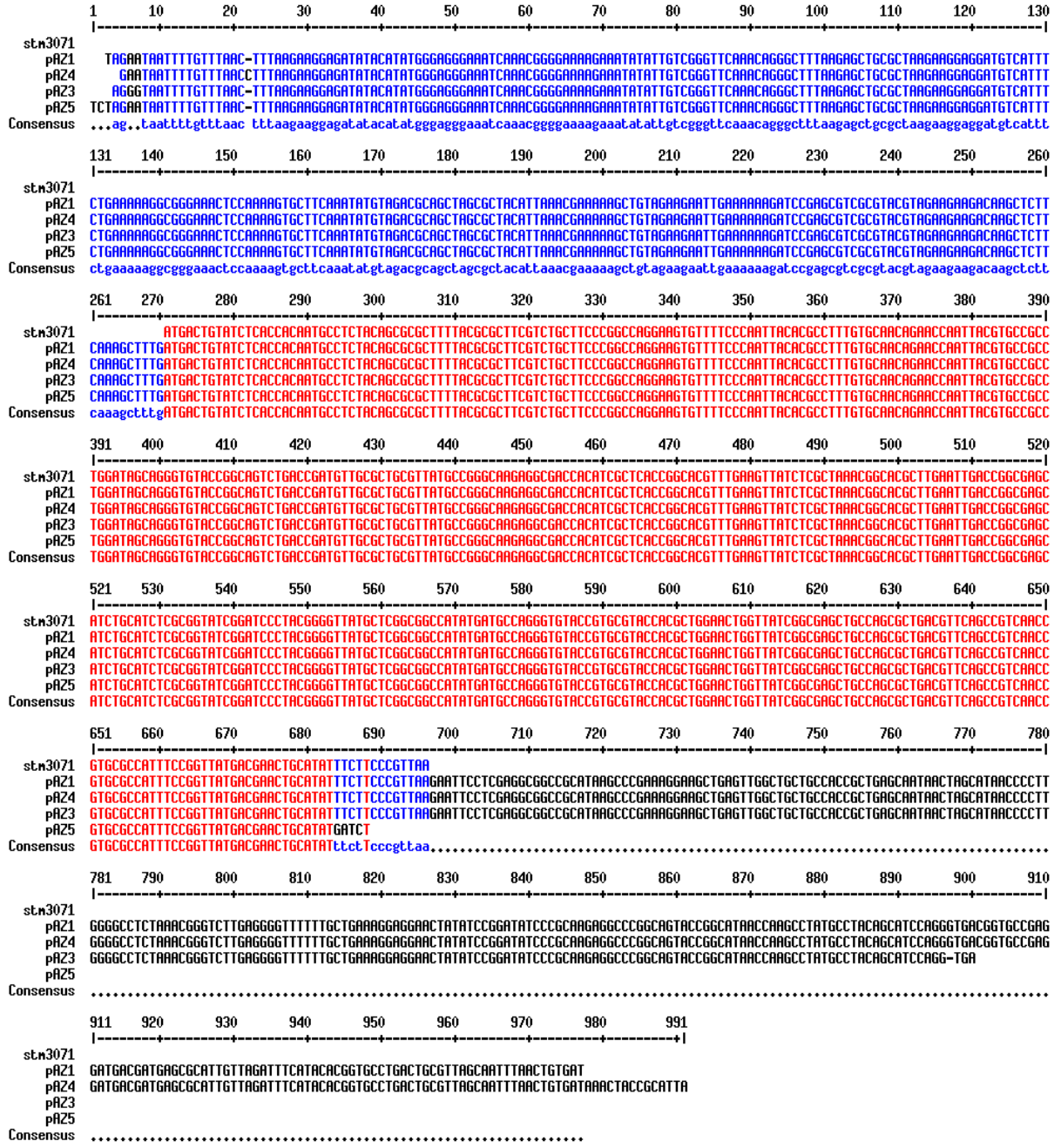


Fig.4.4 Alignment of 5 selected constructs against to the original query sequence representing *stm3071* from *S.* Typhimurium (NC_003197.2**).** Analysis was conducted using Multalin software (http://multalin.toulouse.inra.fr/multalin/). pAZ5 was found to have incomplete gene sequence and was not used further in this study. Remaining constructs were correct and pAZ1 was renamed pAZ1/*stm3071* and used further in this study (Corpet, 1988).

4.1.3 Recombinant protein expression and purification

Selected pAZ1/*stm3071* construct was transformed into *E. coli* BL21 (DE3) cells for expression. The purification procedure was conducted according to the manufacturer's instructions (Biorad, UK) as described in chapter 2.

Expression of STM3071 was induced by addition of IPTG to a final concentration of 1 mM in LB medium when cells were in mid-log phase (OD_{600} at approximately 0.5). Induction at 37°C was carried for 4 h and O/N at 20°C. Expression experiments were conducted at 20°C and 37°C in order to optimise the procedure and obtain high level of expression with the highest possible ratio of recombinant protein in soluble form. Total protein content was separated into soluble and isoluble fractions using BugBuster protein extraction reagent and analysed by SDS-PAGE electrophoresis (fig. 4.5).

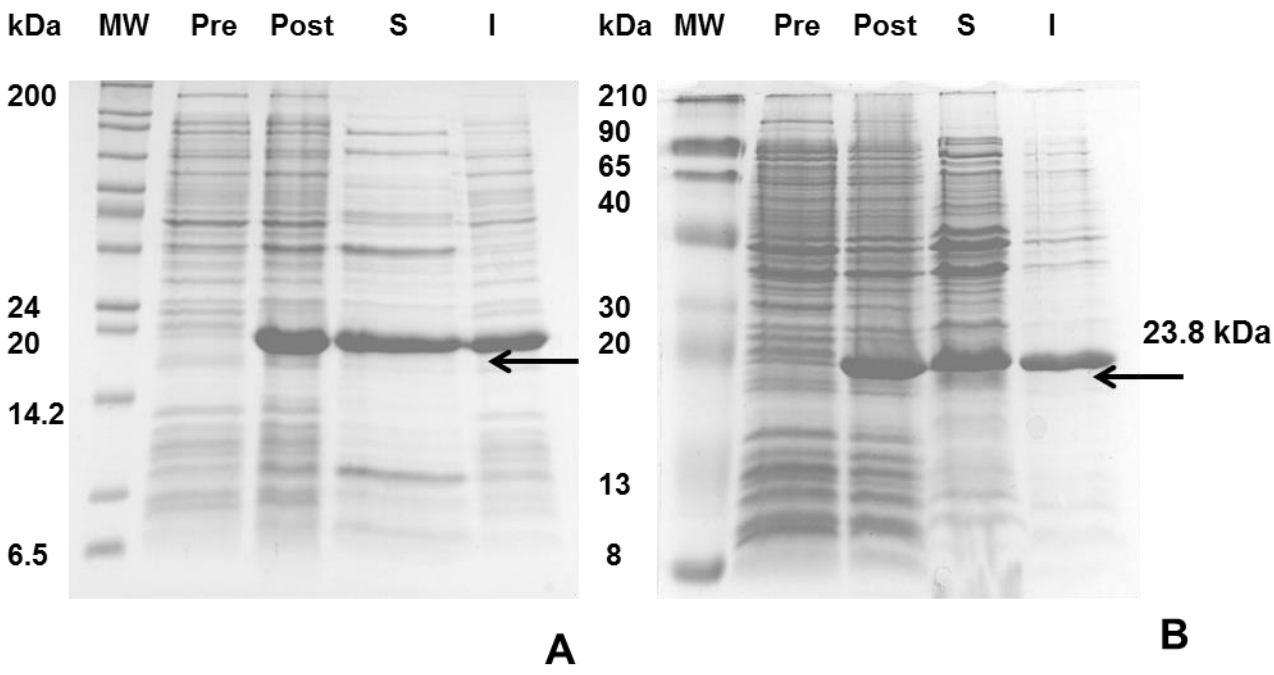


Fig 4.5 Solubility determination of STM3071 overexpressed at different temperatures in LB rich media. Expression was analysed by SDS-PAGE. A volume of 10 µl of insoluble and soluble fractions extracted from *E. coli* BL21 (DE3) using 1x BugBuster in 0.1 M sodium phosphate, pH 7.2. Pre-induced (Pre) and post-induced (Post) with IPTG samples were treated with 2x SDS sample buffer. A volume of 8 µl of pre-induced samples and 4 µl of post-induced samples was loaded onto 15% polyacrylamide gel and SimplyBlue (Invitrogen, UK) stained after separation. Protein size markers were used as a molecular weight reference to confirm the correct size of the protein. A shows solubility analysis of STM3071 expressed at 37°C and B at 20°C. Protein was equally expressed both at 20°C (B) and 37°C (A). Solubility studies shown more soluble STM3071 was obtained from cells cultured at 20°C. Overexpressed recombinant proteins of the correct size are indicated with arrows.

As presented in fig. 4.5, it was determined that STM3071 can be expressed in *E. coli* in soluble form. Solubility determination showed that certain fractions of overexpressed STM3071 remained in insoluble form. SDS-PAGE analysis of overexpressed, recombinant STM3071 showed it was soluble both at 20°C and 37°C, with relatively higher proportion of soluble STM3071 obtained at 20°C (fig. 4.5 B).

According to Sorensen and Mortensen, (2005) medium composition and growth temperature are important factors in optimisation of heterologous protein expression (Sørensen & Mortensen, 2005). It was difficult to observe whether soluble subset of STM3071 was achieved at 20°C or 37°C (Fig. 4.5). Thus, purification procedures were conducted with proteins expressed at both temperatures.

4.1.4 Purification of STM3071

Prior purification procedure molecular weight of STM3071 was determined based on the information accessed on NCBI. The protein consists of 141 amino acids and its molecular weight was calculated at 15.6 kDa. The tag attached to the STM3071 is 8.2 kDa. Molecular weight of recombinant protein is 23.8 kDa (already indicated in fig. 4.5).

Purification of STM3071 was performed using Profinity exact Mini purification starter kit (Biorad, UK). Purity was analysed by SDS PAGE (fig. 4.6). Purification procedure was carried out under exactly the same conditions for both protein extracts obtained at 20°C and 37°C. Cleavage was obtained by subtilisin activation using sodium fluoride elution buffer (according to manufacturer's instructions). This allowed C-terminus cleavage of the recognition sequence, resulting in purified and tag-free native form of STM3071. SDS PAGE analysis shows that there was observably higher levels of purified STM3071 obtained from cells induced at 20°C (fig. 4.6).

Purification of STM3071 overexpressed at 20°C indicated higher ratio of soluble subset (fig. 4.6) and following temperature was selected as more efficient in obtaining higher levels of soluble STM3071.

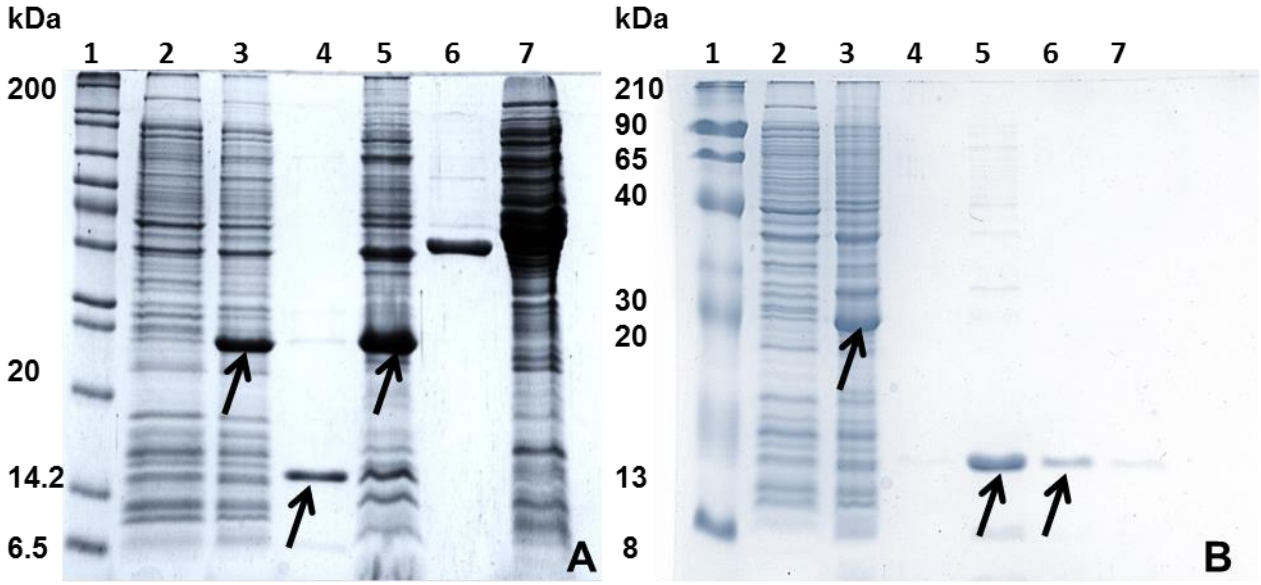


Fig. 4.6 Profinity eXact affinity-tagged STM3071 purified Profinity eXact purification system (Biorad, UK). Samples were analysed on a 15% polyacrylamide gel and stained with Simply Blue (invitrogen, UK). Lanes 1 (fig. A and B) indicate protein markers used as a molecular weight reference (Sigma-Aldrich, UK). Lanes 2 (fig. A and B) a volume of 8 µl of pre-induced; lanes 3 (A & B) a volume of 4 µl of post-induced samples were treated with 2x SDS sample buffer. Lane 4 in fig A represents eluted tag free STM3071 of overexpressed at 37°C. Lane 5 in fig B represents eluted tag free STM3071 overexpressed at 20°C.Lanes 5 in fig A and lanes 6 and 7 in fig B are host protein contaminants washed from the resin by 0.1 M sodium phosphate, pH 7.2 bind/wash buffer. Line 6 represents eluted tag-free maltose binding protein (MBP) control of the purification procedure. In fig B STM3071 purified protein was run twice the column therefore not as many contaminants in the flowthrough are present in comparison with purification at 37°C (A). Protein of the correct size indicated with arrows.

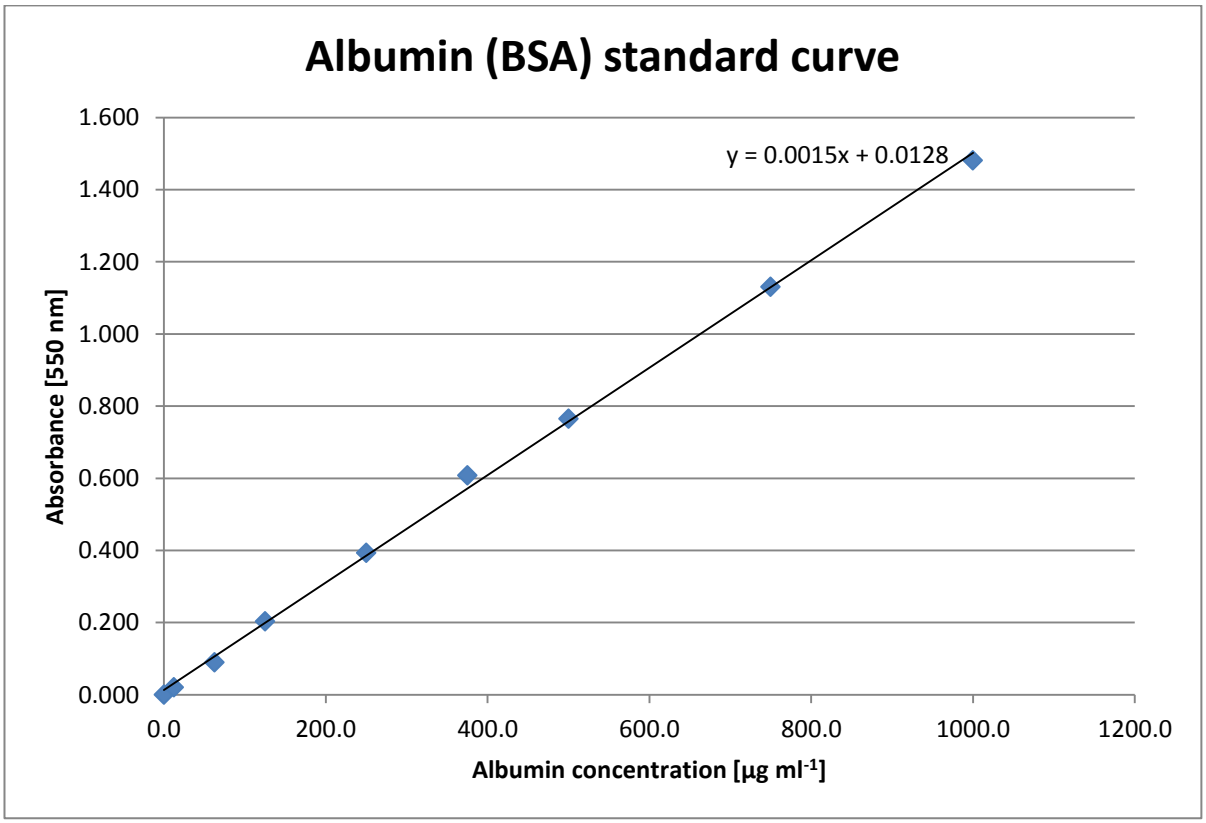


Fig. 4.7. Bovine serum albumin (BSA) standard curve. Absorbance was determined using plate reader at 550 nm wavelength absorbance. Based on linear trend line standard curve equation, concentration of STM3071 was calculated.

Following purification Bicinchoninic assay (BCA) was carried out to determine the concentration of STM3071 (fig. 4.7). BCA assay is a biochemical method that allows protein concentration determination in the solution based on colorimetric reaction (Smith *et al.*, 1985). Prepared set of standards at different concentration and two samples of STM3071 of unknown concertation were measured at 550 nm wavelength with plate reader. The concentration of STM3071 was calculated based on linear trend standard curve equation presented in fig 4.7.

STM3071 protein concentration obtained from 2 cultures (sample 1 (20°C): 489 µg ml^{-1}, sample 2 (20°C): 546 µg ml^{-1}). Molecular weight of the purified protein was determined based on its amino acid sequence (MW of STM3071 = 15230.396 g mol^{-1}). Protein was stabilized in 20% glycerol, aliquoted and stored at -20°C until further analysis.

4.1.5 Investigation of STM3071 DNA-binding properties

Electrophoretic mobility shift assay (EMSA) also known as retardation shift assay is well described and a sensitive method for DNA-protein interactions (Garner & Revzin, 1981). EMSA was carried out according to Campbell and colleagues, (2007). Prior to the procedure, an aliquot of STM3071 was analysed using SDS-PAGE to confirm the presence of protein in the sample and rule out potential protein degradation (fig. 4.8).

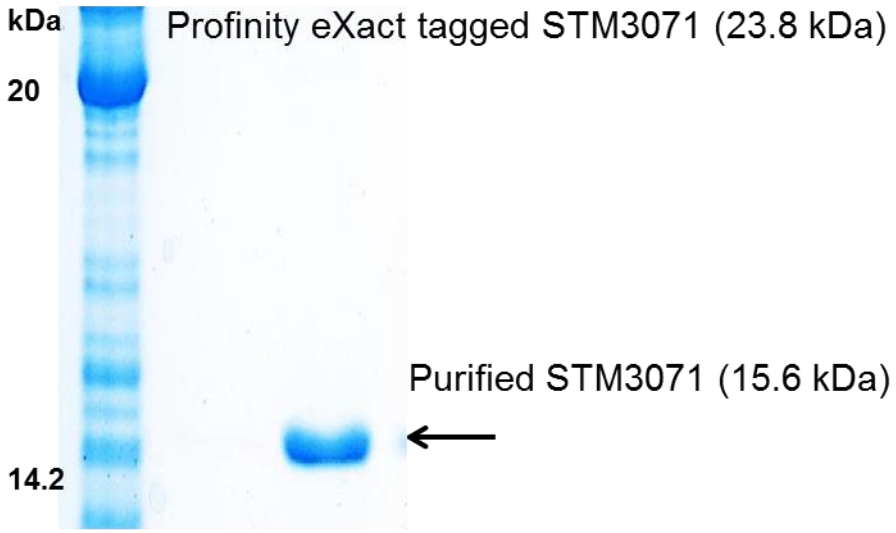


Fig. 4.8 Profinity eXact affinity-tagged STM3071 and purified STM3071 SDS-PAGE analysis. Samples were analysed (a volume of 5 µl of each, prepared in 2x sample buffer) on a 15% polyacrylamide gel and stained with Simply Blue (Invitrogen, UK). Figure represents cropped image that visualises proteins of interest. Tagged STM3071 (Profinity eXact tagged STM3071) was obtaned from total lysate of the corresponding culture that STM3071 was purified from and saved for further analysis. Thawed purified STM3071 was confirmed present and not degraded.

Gel retardation assya used to detect DNA-protein complexes is a powerful method for charaterisation of the interactive systems between nucleid acids and proteins (Hellman & Fried, 2007). In a classical way, cell lysate is combined with the probe cognate nucleotide binding. Protein interaction with DNA usually occurs within the promoter-operator region. In prokaryotic organisms the promoter region is usually located at -10 and -35 upstream from the transcription start site of the first gene in the operon (Apostolaki & Kalosakas, 2011).

Promoter prediction analysis was conducted with software that estimates the potential consensus sequence. The upstream region from *stm3071* was accessed at NCBI and analysed using PePPER webserver for prediction of prokaryote promoter elements and regulons (de Jong *et al*., 2012). Analysis upstream from *stm3071* region from of *S.* Typhimurium detected two potential promoter sites (fig. 4.9 a).

As described in chapter 3 and earlier in this chapter it remains unclear whether STM3071 should be considered DNA-binding. In chapter 3 it was discussed that it remains ambiguous whether *stm3071* functions as a part of *stm3071/3072/3073/3074/3075* operon or whether it functions as an independent regulator. Therefore, for DNA-binding investigation study upstream and downstream region of *stm3071* was investigated. In order to perform this, as schematically presented in fig. 4.9 B, regions located from the nearest proximity from the stm3071 were amplified and used in DNA-binding investigations.

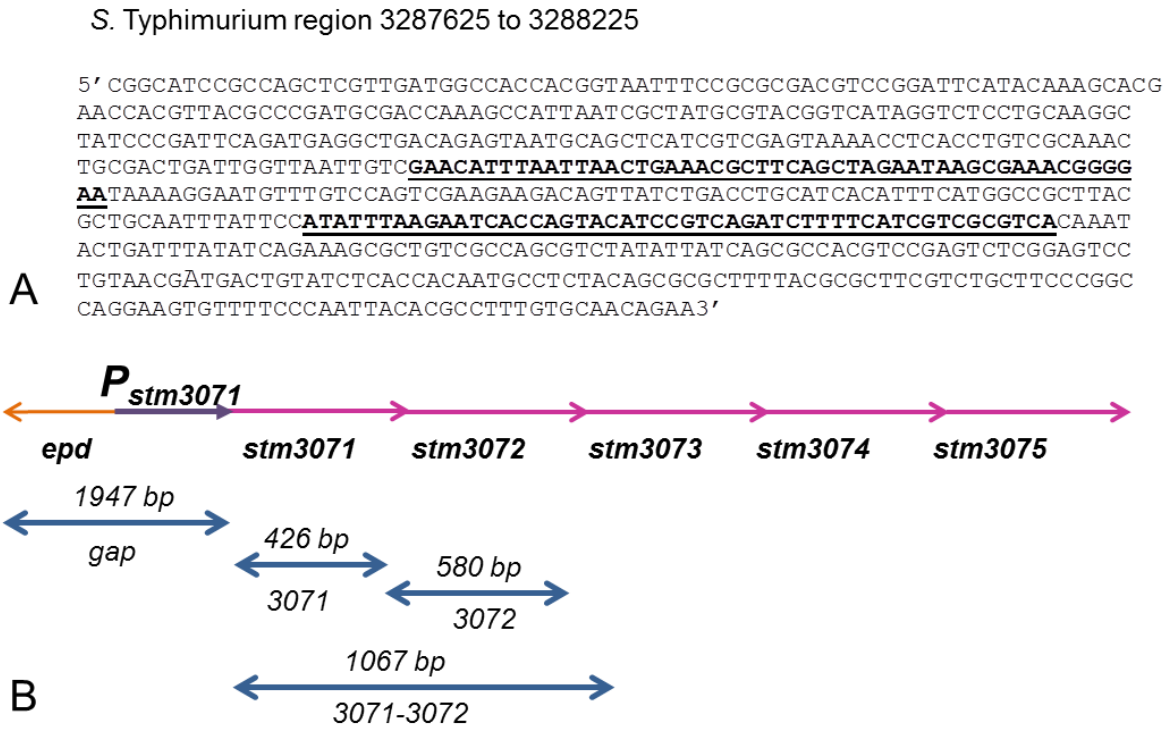


Fig. 4.9. DNA-binding sequence predictions. (A) Represents sequence of region located upstream from *stm3071*; this region represents most probable binding DNA-binding sites for STM3071 with underlined predicted consensus sequences, underlined in bold. (de Jong *et al.*, 2012). Sequence represents 601 bp region from base 3287625 to 3288225 of *S.* Typhimurium. This sequence was also used further in expression experiments. (B) Schematic visualisation of predicted regions. Blue arrows indicate regions testes against STM3071. Regions were amplified with primers as listed in table 2.3 and used in DNA-binding study. Amplicons are presented in fig. 4.10.

To test potential binding activity four regions were chosen due to nearest proximity to the *stm3071* were amplified using PCR. The specific primers used for amplification are listed in chapter 2, table 2.3. PCR products were analysed using agarose gel electrophoresis. Following amplification, PCR products were purified using Qiagen purification kit (Qiagen, UK) according to manufacturer's instructions PCR purification method. DNA concentrations of amplicons were determined spectroscopically. Samples were stored at -20°C until further use in EMSA binding assay.

Primers were designed for amplification of regions as indicated in fig. 4.9 B. PCR was performed and results are presented in fig. 4.10 A and B. Indicated amplified sequences were used in electrophoretic mobility shift assay (EMSA) studies (Campbell *et al.*, 2007) to test for interaction with purified STM3071. Located upstream from *stm3071* region was predicted with two possible consensus sequences. However, considering controversy as to whether *stm3071* is part of the predicted operon, downstream regions were also amplified. It is possible that is STM3071 acts as a regulator then it could theoretically bind within *stm3071* or *stm3072* region. Thus, EMSA were carried out with 'gap', '*3071*', '*3072*', and '*3071-3072*' probes. Probes indicated in fig. 4.10 as 'not used for analysis' also represent regions within putative operon and can be applied in future analysis. Primers for these regions were design for controls in case of successful binding activity of STM3071.

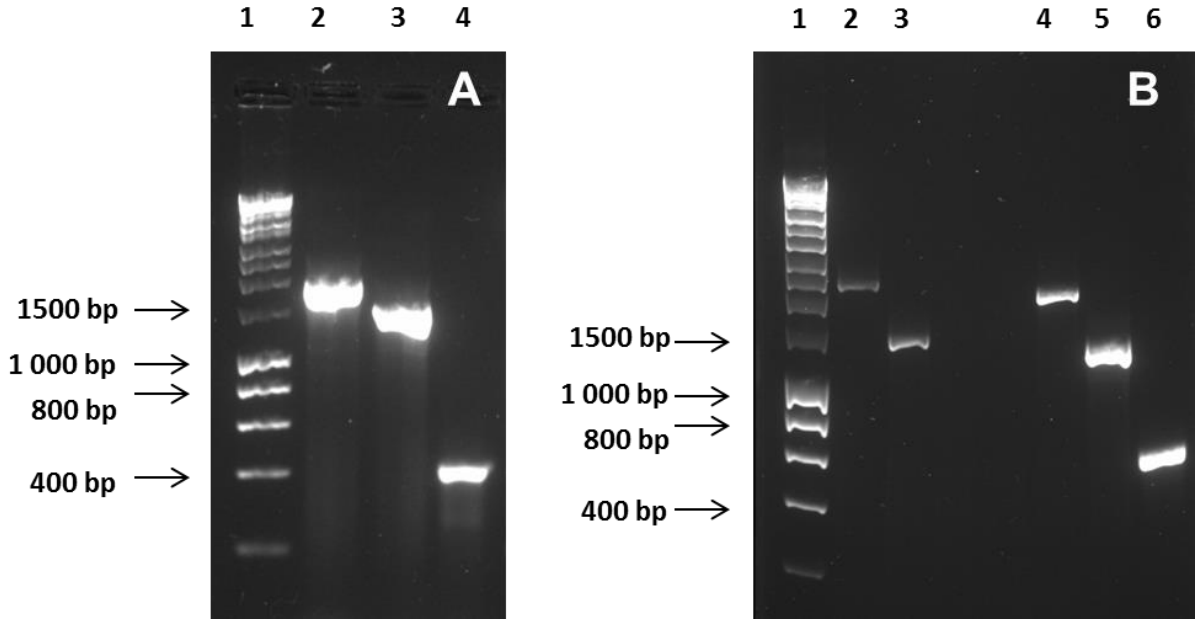


Fig. 4.10. **Agarose gel electrophoresis analysis of PCR products prepared for EMSA binding assay**. Purified genomic DNA of SL1344 was used as PCR template. **A**.1. DNA size marker (Hyperladder 1); 2. Region amplified with *epd*F2 and *3071*R2 ('GAP' probe - 1947bp;); 3.*epd*F2 and *3071*R (not used for analysis); 4. *3071*F & *3071*R ('*3071*' probe - 426 bp). B. 1. DNA size marker (Hyperladder 1); 2. Region amplified with *3071*F and *3073*R (not used for analysis) 2489bp. 3. *3071*F and *3072*R ('*3071-3072*' probe - 1067bp). 4. *3072*F and *3073*R (not used – 1320 bp); 5. *3072*F and *3073*R (not used- 1321bp); 6. *3072*F and *3072*R ('*3072*' probe - 580bp). Samples analysed on 1% agarose gel. Probe names correspond to scheme in fig. 4.9 (b)

Mobility shift assay was used to test the ability of STM3071 to bind double-stranded amplified regions. Probes named ‘gap’, ‘*3071*’, ‘*3072*’ and ‘*3071-3072*’ were tested against STM3071. All probes were adjusted to the same concentration at approximately 100 ng of target and competitor DNA. Competitor used for reactions was re-circularised with restriction enzyme digest vector pUC19 (2.6 kb). Binding reactions were set up with varying concentrations of purified STM3071. Retardiation of the DNA probe’s extent of migration indicated that the specific DNA-protein interaction occurred. No retardation was observed in any of tested conditions presented in figure 4.11 over the page.

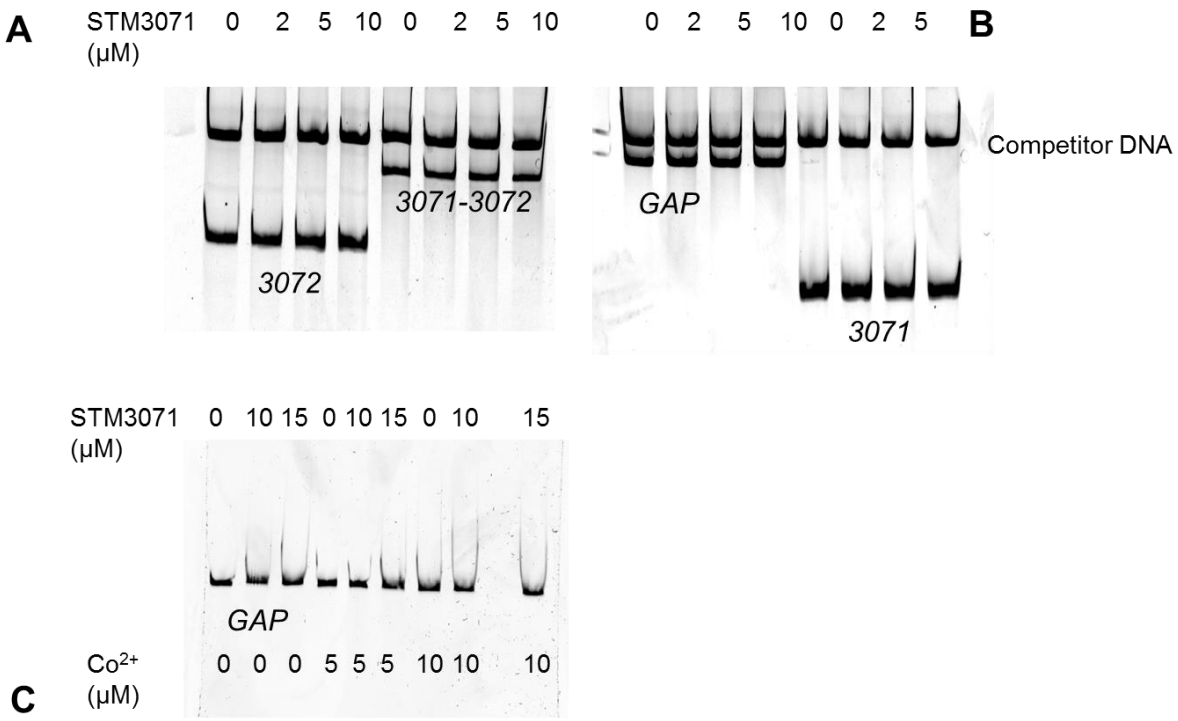


Fig. 4.11. STM3071 binding analysis. A, B, C represent pictures of gel retardation assay. A and B from left as labelled 0,2,5 or 10 µM of purified STM3071 was tested against probes '*3072*', '*3071-3072*', 'gap' and '*3071*'. Equal amounts of specific target DNAs as well as competitor DNA were added. Re-circularised pUC20 (2.6 kb) plasmid was used as unspecific DNA competitor. No retardation was observed with tested probes. C. Probes were prepared consequently as in A and B. Additionally cobalt was added at concentrations as indicated in the picture. Competitor DNA was not added. No retardation was observed.

4.3 **Summary**

Taking into account that based on predictions the structural nature of STM3071 as a potential DNA-protein are not exaclty known, it is difficult to predict and determine the conditions to test its biological activity.

The average *S.* Typhimurium genome GC content is at about 52.2 % (Papanikolaou *et al*., 2009). In most organisms genes that were acquired by horizontal transfer are usually AT rich. GC content of the putative operon was determined and it does not appear to exhibit GC content lower that *S.* Typhimurium genome average (GC content of region from 5' end of *epd* to 3' end of *stm3075* is 56%). Thus, it can be predicted that the analysed region was not acquired by horizontal gene transfer, which in any case is a parallel assumption with that discussed in chapter 3, the co-occurrence of analysed genes in other organisms and prevalence across *Salmonella* genus (chapter 3, fig. 3.7 and 3.8).

Papanikolaou and colleagues, (2009) demonstrated that genes with a lower AT ratio are usually targeted by H-NS rather than conserved genes within genome. During the evolution process bacteria acquire genes through horizontal transfer. Those foreign sequences are usually energy cost effective thus they likely to decrease the fitness of the microbe. Histone-like proteins were found to act as silencers of horizontally acquired genes due to their ability to bind AT-rich regions and thus regulate their transcription. MvaT heterogeneous silencer was found to act upon AT-rich region. However, even though it possessed some functional similarities with PPC-like domain and H-NS proteins it was not found to share any structural similarities with AT-hook motif (Ding *et al.*, 2015). This example shows that this group of regulators may vary in terms of structural features as well as functional properties. It is also possible that the AT-hook motif was selectively chosen for eukaryotic chromatin in the process of evolution. Why it is present in some prokaryotic organisms however, remains unclear.

STM3071 was found to contain a predicted metal-binding site and conservative domain found in other AT-hook motif domains, but not contain the AT-hook motif itself. It is predicted however that it exists as a homotrimer that coordinates 3 atoms of zinc together. Three conservative histidines do not resemble similarities with zinc-finger like proteins but based on predictions are

likely to act as metal binding protein or metal acquisition genes regulator. In chapter 3 it was discussed that it is not entirely obvious if *stm3071* is a part of the operon with *stm3072/3073/3074/3075* genes. It might be that it is not involved in their regulation. It is also possible that its expression is induced by another factor which might in turn regulate downstream genes.

NCBI prediction suggests similarities with thioredoxin-like proteins. They represent uncharacterised cyanobacterial proteins and the conserved domain in their structure has a linkage to Plants and Prokaryotes Conserved (PPC) domain. This domain consists of hydrophobic C-terminal region and is found in proteins with the AT-hook motif – which explains the annotation (Lin *et al.*, 2007). Three conserved histidine residues possibly form a zinc-binding site and as presented in fig. 4.12 (B) it is likely to form homotrimers. Thus, it might be the case that STM3071 cooperated with another protein which has an AT-hook motif in its structure.

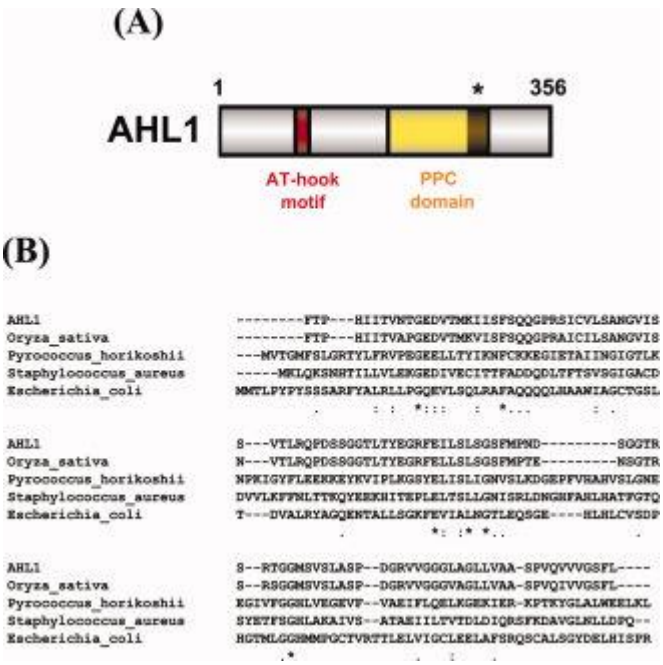


Fig. 4.12 (A) Schematic visualisation of AT-hook motif (in red), PPC domain (in yellow), and hydrophobic site (asterisk) of *Pyrococcus hirikoshii* PPC protein – based on crystallography performed by Lin *et al*., (2007). (B) Sequence alignments of PPC-homologs found in in other organisms. Asterisks indicate highly conserved regions, colons and periods indicate for strong and weak conservancy, respectively. (Lin *et al*., 2007)

According to Aravind and Landsman, (1998) AT-hook motifs are common in eukaryotic organisms but not so much in bacteria. CarD protein of *Myxococcus xantus* was identified in 1994 by Nicolás and co-workers (Nicolás *et al*., 1994). The same group confirmed DNA-binding properties a couple of years later. It was demonstrated that CarD was binding AT-rich sequences. The protein was however identified as slightly different to corresponding eukaryotic motif; CarD was missing the first proline in the motif (Nicolas *et al*., 1996). The protein was annotated as transcriptional regulator (NCBI; fig. 4.13 below). The record was discontinued in the light of relatively recent results. CarD contains C-terminal DNA-binding domain with four repeats of RGRP, AT-hook that resembles high mobility group A (HMGA) (Nicolas *et al*., 1996). HMGA in eukaryotic organisms are described as DNA architectural factors that interact with other proteins and take part in various processes from transcription regulation, RNA processing and, DNA-repair to chromatin remodelling (Sgarra *et al*., 2010). It was described, however, that CarD mirrors HMGA in terms of its DNA-interaction properties but CarD itself was also found to mediate interactions with itself and with RNA polymerase (Garcia-Moreno *et al*., 2010). CarG that forms complex with CarD, which contains a zinc-binding site and is required for stabilisation of the whole complex with DNA. CarD/CarG complex (CarG always co-exists with CarD) has been reannotated recently and it is therefore possible that STM3071 (possibly in similar way to CarG) coordinates with another protein that possibly anchors specific DNA-binding motif. Due to the three conserved His residues and the putative zinc-binding site it is possible that it takes part in regulation of the transcription and facilitates the DNA-binding process.

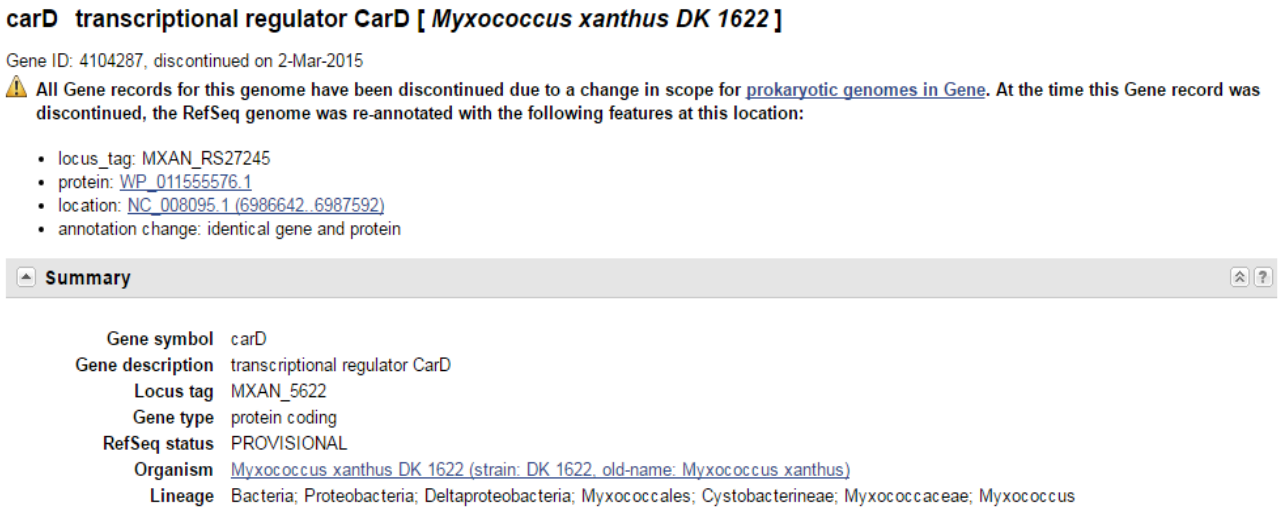


Fig. 4.13. NCBI search for CarD of *Myxococcus xanthus* gene ID. Record shows that gene annotation was discontinued in May 2015.

Additionally considering the predicted metal-binding site and possible regulation of metal acquisition process, the addition of metal in excess might be necessary to consider for further analysis. Reportedly, EDTA as a strong chelator has exhibited inference with the DNA-binding reaction (Huang *et al*., 2004).

Interestingly, according to Arrach and co-workers it was noted that *stm3071* promoter was found to be preferentially activated when *Salmonella* was present within solid tumours. The hypoxic environment of the tumour aligns with the concept that cobalamin is only synthesised under anaerobic conditions. Cobalt is required for this process, thus following on from there, genes involved in cobalt acquisition should be switched on in the same environment (Bobik *et al*., 1997; Jeter, 1990; Jeter *et al*., 1984). Thus, it is possible that low oxygen or an anaerobic environment might be a crucial determinant of the allosteric conformation of STM3071.

Thus, in the next chapter conditions under which promoter of *stm3071* is induced, were examined and discussed.

5 Chapter 5 – Characterisation of *stm3071*

4.4 Introduction

In chapter 4 it was discussed that in order to be able to investigate biological function of the STM3071, precise conditions that induce its expression need to be established.

5.1.1 What induces *stm3071* expression

Interestingly, an intergenic region located upstream from *stm3071* was identified as hypoxia-induced (Arrach *et al.*, 2008). Thus, the main focus of this chapter is verification of conditions that upregulate $P_{stm3071}$.

Salmonella are facultative anaerobic bacteria that naturally accumulate in a wide variety of solid tumours as opposed to normal tissue (Arrach *et al*., 2010; Wall *et al*., 2010; Zhao *et al*., 2007). Necrotic regions of the tumour are hypoxic and relatively acidic compared to normal tissue (Raghunand *et al*., 2003). Avirulent mutant strains, as mentioned earlier were described to prefer tumours over normal tissue in ratios as high as 9,000:1 (Arrach *et al*., 2008) and appear to contribute to tumour reduction in animal models (Zhao *et al*., 2006).

Work carried by Arrach and co-workers (2008) shows the promoter of *stm3071* is preferentially activated within solid tumours. In the study, high-throughput methods were used to screen *Salmonella* promoters selectively expressed in solid tumours. Promoter activity was measured from bacteria grown in spleen tissue and tumours. Out of a range of promoters that were activated in the tumour tissue, 5 were selected and amongst those $P_{stm3071}$ was identified. Those 5 promoters were then analysed *in vivo* in a group of tumour-bearing mice. Results show that although $P_{stm3071}$ produced relatively low signal in tumour, it was undetectable in normal tissue (Arrach *et al.*, 2008). Accordingly, with full understanding of the function and role of STM3071 it can be investigated further whether attenuated *Salmonella* strains with tumour targeting abilities could serve as a novel and selective way of introducing anti-tumour therapeutics under control of selectively induced promoter.

5.1.2 What is the function of *stm3071?*

The availability of complete bacterial genomic sequences has provided the world of molecular biology with enormous insight of genome organisation. Identification of ORFs, intergenic regions and the predictions of gene expression regulation, although still poorly understood, can only be possible through access to entire genome sequence. Applications of mutational analysis have become a major tool of identification of encoding genes and their function. Over the past decade, lambda-red mediated recombination also known as recombineering has found many applications and served as a rapid and relatively simple way of making gene disruption (Mosberg *et al*., 2010).

As discussed in previous chapters it remains unclear whether *stm3071* is a part of putative operon potentially encoding genes involved in cobalt transport. It also remains unclear whether it acts upon those genes as a transcriptional regulator which might also be involved in metal sensing. It is also not known whether STM3071 exists on its own and/or if it interacts with other protein that facilitates DNA-binding. It also remains unanswered under what conditions it is expressed. According to data collated by Arrach and co-workers, (2008), it might be that $P_{stm3071}$ is selectively activated under low oxygen conditions. It is also possible and considering its metal-binding site predictions it might be that its induction is metal-dependent.

In order to address some of the above questions single-gene knock-out strain of *Δstm3071* and $P_{stm3071::lacZ}$ transcriptional fusion were constructed, and tested against a range of conditions.

5.2 *Δstm3071* construction

Δstm3071::cat was constructed previously, directly in SL1344 strain according with published procedure (Datsenko & Wanner, 2000).

To confirm the replacement of *stm3071* with chloramphenicol resistant cassette, PCR was carried out – presented in fig. 5.1 below. Primers used for amplification of this region were designed with approximately 200 bp flanking sequence to allow complete deletion detection. Amplification was carried with *Δstm3071::cat* isolated genomic DNA as a DNA template – to confirm gene replacement; and with SL1344 genomic DNA used as template in control reactions.

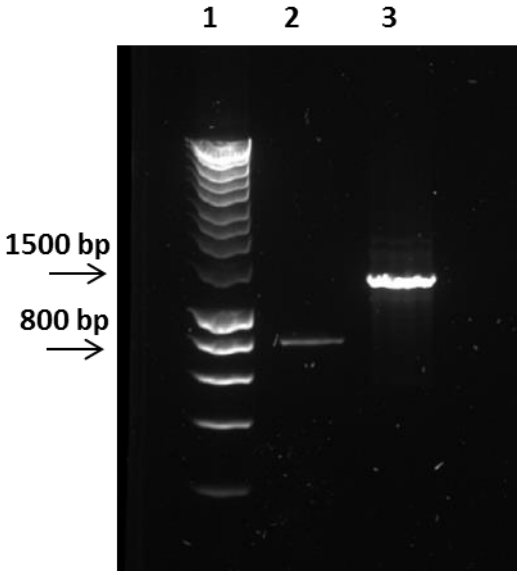


Fig. 5.1 Agarose gel electrophoresis analysis of PCR products – replacement of *stm3071* with *cat* cassette. Lane 1 DNA size marker (Hyperladder 1). Lane 2 PCR product with WT gDNA used as a template – 768 bp; positive control. Lane 3 Confirmation of *cat* cassette replacement – 1500 bp (*cat* cassette size 1100 bp) chloramphenicol resistance gene was inserted in place of *stm3071*. Carried out with 1% agarose gel.

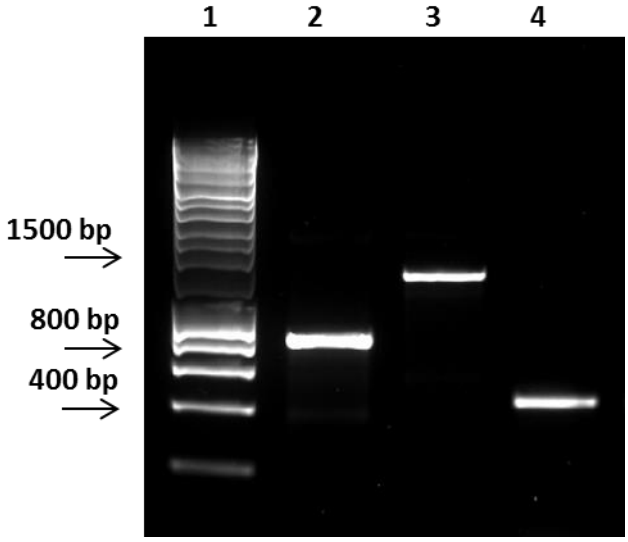


Fig. 5.2 *Δstm3071* construction confirmation. Agarose gel electrophoresis with PCR products amplified with primers designed to amplify flanking region of *stm3071*; Lane 1 Hyperladder I (molecular size marker). Lane 2 g DNA isolated from WT SL1344 was used as a template in PCR. This was to confirm the correct size of the region in the WT – positive control (768 bp). Lane 3 gDNA of *Δstm3071::cat* LB5010a (1500 bp) was used to confirm *stm3071* replacement to cat cassette. Lane 4 *Δstm3071* – complete knock-out confirmation. gDNA of *Δstm3071* LB5010a strain was used as a template in PCR with flanking *stm3071* primers. Corresponding band size (363 bp) confirms complete stm3071 deletion.

To remove cat cassette *Δstm3071::cat* was transformed with pCP20 encoding temperature activated flippase (Datsenko & Wanner, 2000). Elimination of *cat* was confirmed first by growing strain on chloramphenicol and/or ampicillin. PCR carried with selected strain and primers STM3071F and STM3071R and confirmed *stm3071* knock-out from bacterial chromosome (fig. 5.2). Mutation was first generated in LB5010a strain and then transduced with P22 to WT stains SL1344 and LT2 strains.

P22-derived transductants were obtained as described in chapter 2 and screened on green agar plates to obtain lysogen-free colonies and the deletion mutant was transduced back to into a clean wild-type strain. The complete absence of the structural gene was verified by PCR (fig. 5.3) with primers STM3071F and STM3071R listed in table 1, materials and methods (Smith & Levine, 1967).

Following transduction and confirmation fresh overnight liquid culture of *Δstm3071* strain was combined with sterile glycerol to final concentration of 50%. Prepared culture was aliquoted in sterile cryovial tubes and placed at -80°C.

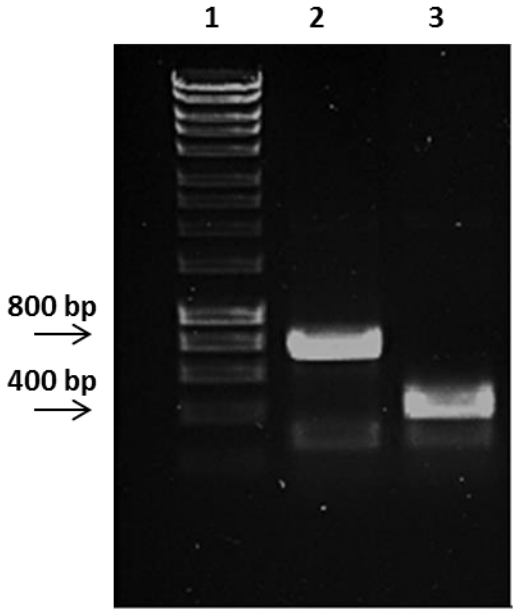


Fig. 5.3 P22 derived transduction confirmation of *Δstm3071* SL1344 strain. Agarose gel electrophoresis with PCR products; Lane 1 Hyperladder I (molecular size marker). Lane 2 gDNA of WT SL1344 used as a template for positive control – *stm3071* in place (768 bp). Lane 3 *Δstm3071* – complete knock-out confirmation (363 bp).

5.3 **Generation of *lacZ* fusions**

Genetic fusions have been widely used in molecular biology to study aspects of gene expression in prokaryotic organisms. Mainly, because they are simple to construct, and the activity of the fused reporter gene can be easily quantified by sensitive biochemical assays. In order to quantify the activity of investigated $P_{stm3071}$ the predicted promoter site was cloned into a promoter-less pRS415 plasmid (Simons *et al.*, 1987).

Arrach and co-workers, (2008) have noted $P_{stm3071}$ to be induced at higher levels in tumour than in spleen. They have also listed a number of other predicte were suggested to be tumour-specific. Amongst those P_{cbiA} was classified.

In 1993, Roth and colleagues described P_{cbiA} region as involved in transcriptional regulation of *cob* operon (B12 biosynthetic genes). Genes encoding biosynthetic pathway of cobalamin are known to respond to intracellular levels of adenosylcobalamin (Escalante-Semerena *et al*, 1990). In *cob* operon *cbiA* encodes for amidase which takes part in a rather late part of the biosynthetic pathway. It was previously described that B12 biosynthesis takes place under anaerobic conditions (Roth *et al*., 1993), thus P_{cbiA} was used as a control in beta-galactosidase analysis which allowed quantifying of promoter activity.

Amongst other promoters that were upregulated under anaerobic conditions, P_{adhE} was described and in 2011 it was defined as tumour selective activated promoter (Chen *et al*., 2011). Amongst several screened proteins, AdhE was found to be up-regulated by over a hundred-fold. P_{adhE} was thus selected as another *lacZ* expression control fusion. However, the aim of this fusion was to test the activity during infection assays in human carcinoma cells to determine tumour specificity and was not used in this study.

In chapter 3, it was identified that predicted $P_{stm3071}$ site might involve two consensus sequences. The version of $P_{stm3071}$ used in Arrach study involved a 333 bp upstream region from s*tm3071* location whilst the version predicted by this study analysis involved 404 bp upstream region. Thus, in order to avoid confusion Arrach version was named P_{Arrach} and it refers to shorter version of *stm3071* predicted promoter region.

5.2.1 Molecular cloning of promoters into pRS415 promoter-less vector

Genomic DNA of SL1344 *S.* Typhimurium strain was isolated (fig. 5.4 A) and confirmed by gel electrophoresis. Promoter regions of *stm3071*, *stm3071* (Arrach *et al*., 2008), and *cbiA* were amplified from SL1455 gDNA using primers: Pstm3071F & Pstm3071R, P_Arrach_stm3071F & P_Arrach_stm3071R and PcbiAF & PcbiAR, respectively (primers sequences are listed in table 1, chapter 2). Primers for amplification of promoter regions were designed with EcoRI and BamHI restriction sites overhangs.

Subsequently PCR was conducted for each set of primers in 5 separate reactions. Annealing was carried at 50°C and extension step for 10 min. In order to confirm amplification reactions were tested for presence of a band of correct size using gel electrophoresis. Reactions carried with same set of primers were combined and purified using PCR purification reagents accordingly as described in chapter 2. Promoter-less vector pRS415 and purified PCR products were digested with EcoRI and BamHI restriction enzymes (fig. 5.4 B & C).

Following restriction digest ligation reactions were set up accordingly as described in chapter 2. Colonies that grew overnight in medium with ampicillin were selected, and plasmid DNA was extracted. Plasmid DNA of clones that grew on ampicillin was used as template in PCR reactions (fig 5.5 A, B, and C). Selected successful fusions were then verified by restriction enzyme digest (fig. 5.6).

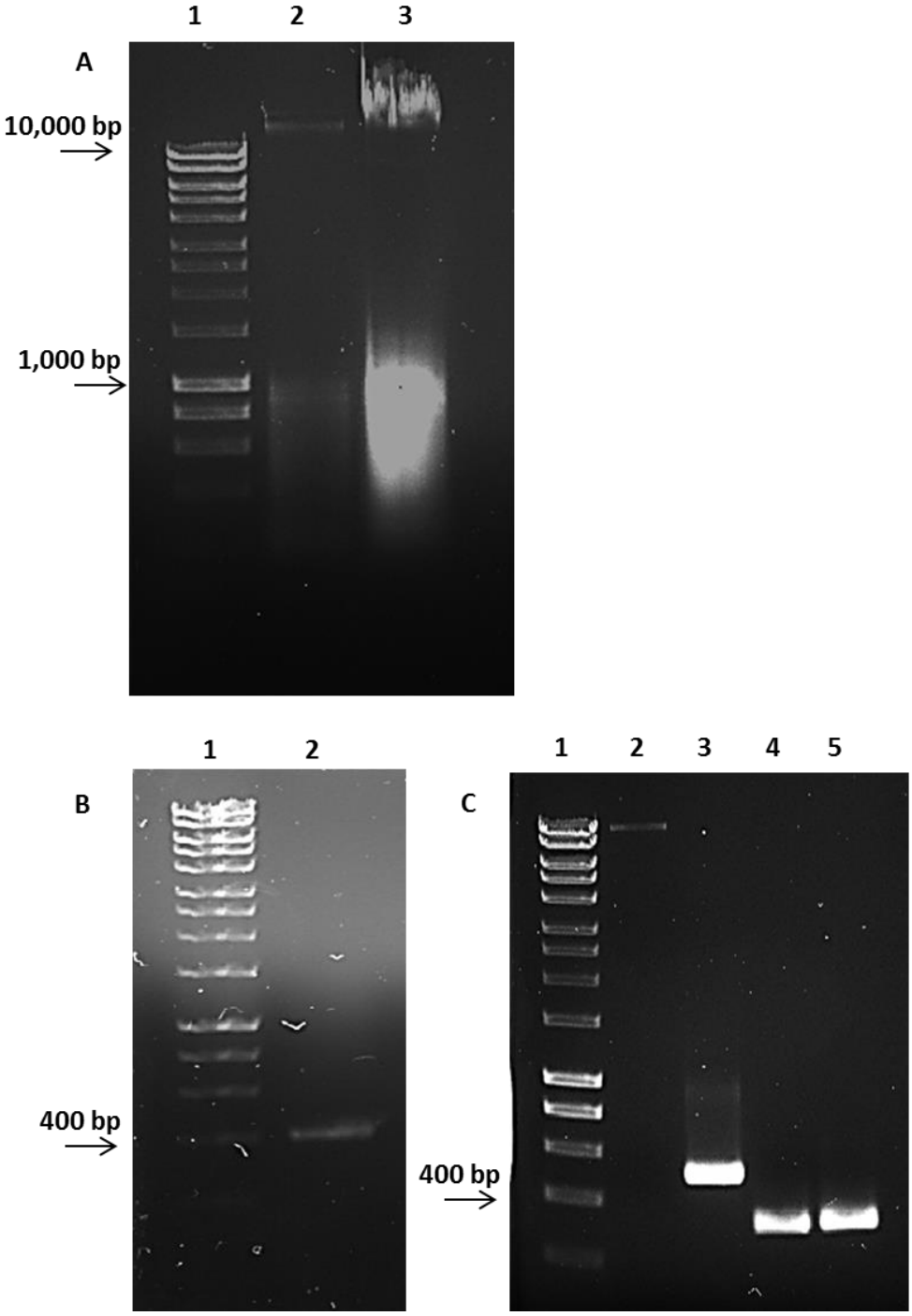


Fig. 5.4. Molecular cloning (A) gDNA of SL144. Line 1 Hyperladder I (DNA size marker). Lane 2 Diluted 1:10 gDNA used as a template in PCR reactions. Line 3 gDNA. **(B)** Lane 1 Hyperladder I (DNA size marker). Lane 2 amplified $P_{stm3071}$ region (404 bp) and digested with EcoRI and BamHI. **(C)** Lane 1 Hyperladder I (DNA size marker). Lane 2 pRS415 digested with EcoRI and BamHI restriction enzymes. Lane 3 amplified P_{adhE} region, not used in this study. Lane 4 amplified P_{cbiA} region (325 bp) and digested with EcoRI and BamHI. Lane 5 amplified P_{Arrach} region (333 bp) and digested with EcoRI and BamHI. Gel electrophoresis performed with 1% agarose gel.

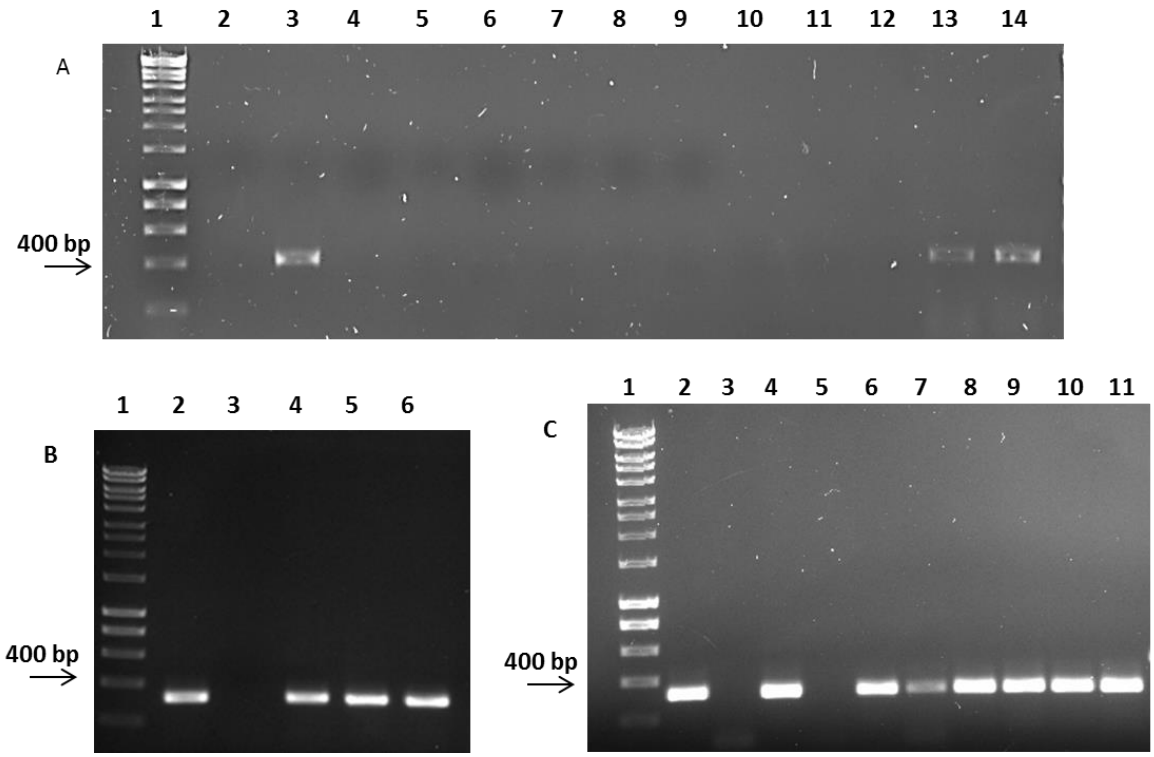


Fig. 5.5. Verification of selected clones - *lacZ* promoter fusions confirmation carried by PCR. Lanes 1 in A, B and C indicate Hyperladder I (DNA size marker). (A) $P_{stm3071}$ clones (404 bp) – cloning successful in lanes 3. 13, and 14. (B) P_{cbiA} clones (325 bp) – successful in lanes 2, 4, 5, and 6. (C) P_{Arrach} clones (333 bp) – successful in lanes 2, 4, 6, 7 ,8 ,9; P_{cbiA} clones (325 bp) – successful cloning in lanes 10 and 11. Gel electrophoresis performed with 1% agarose gel.

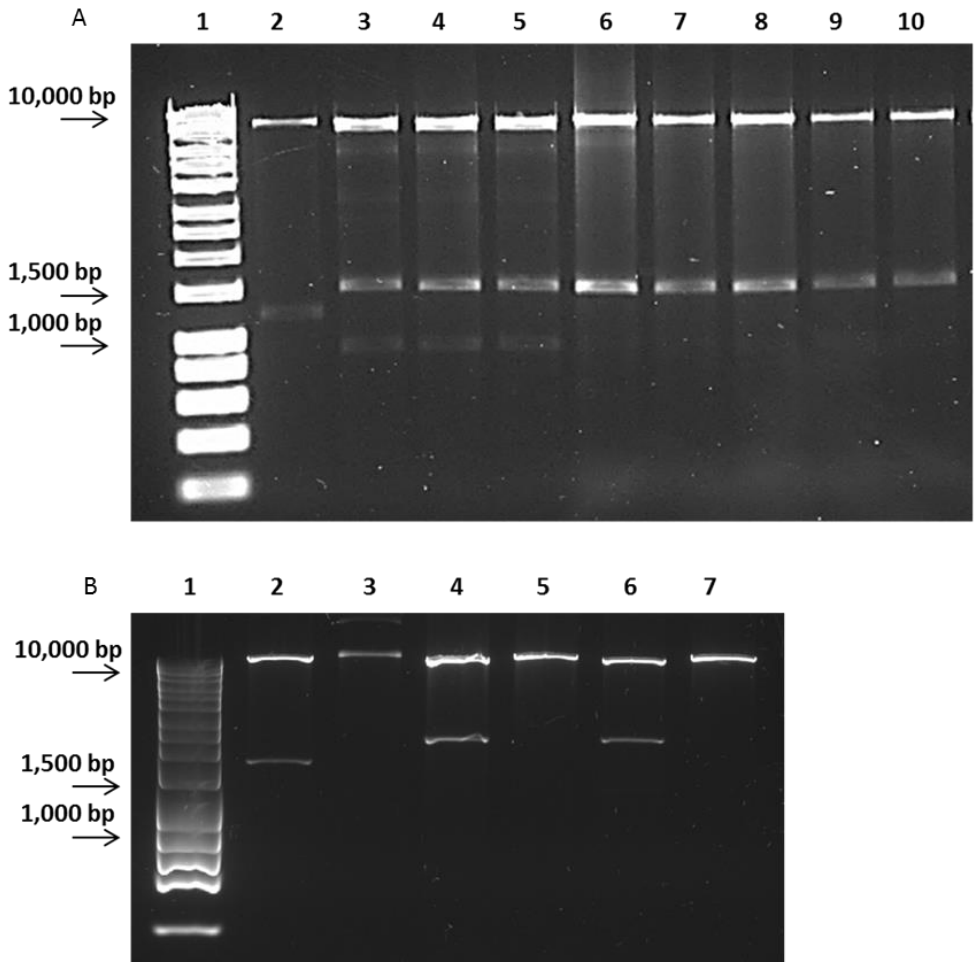
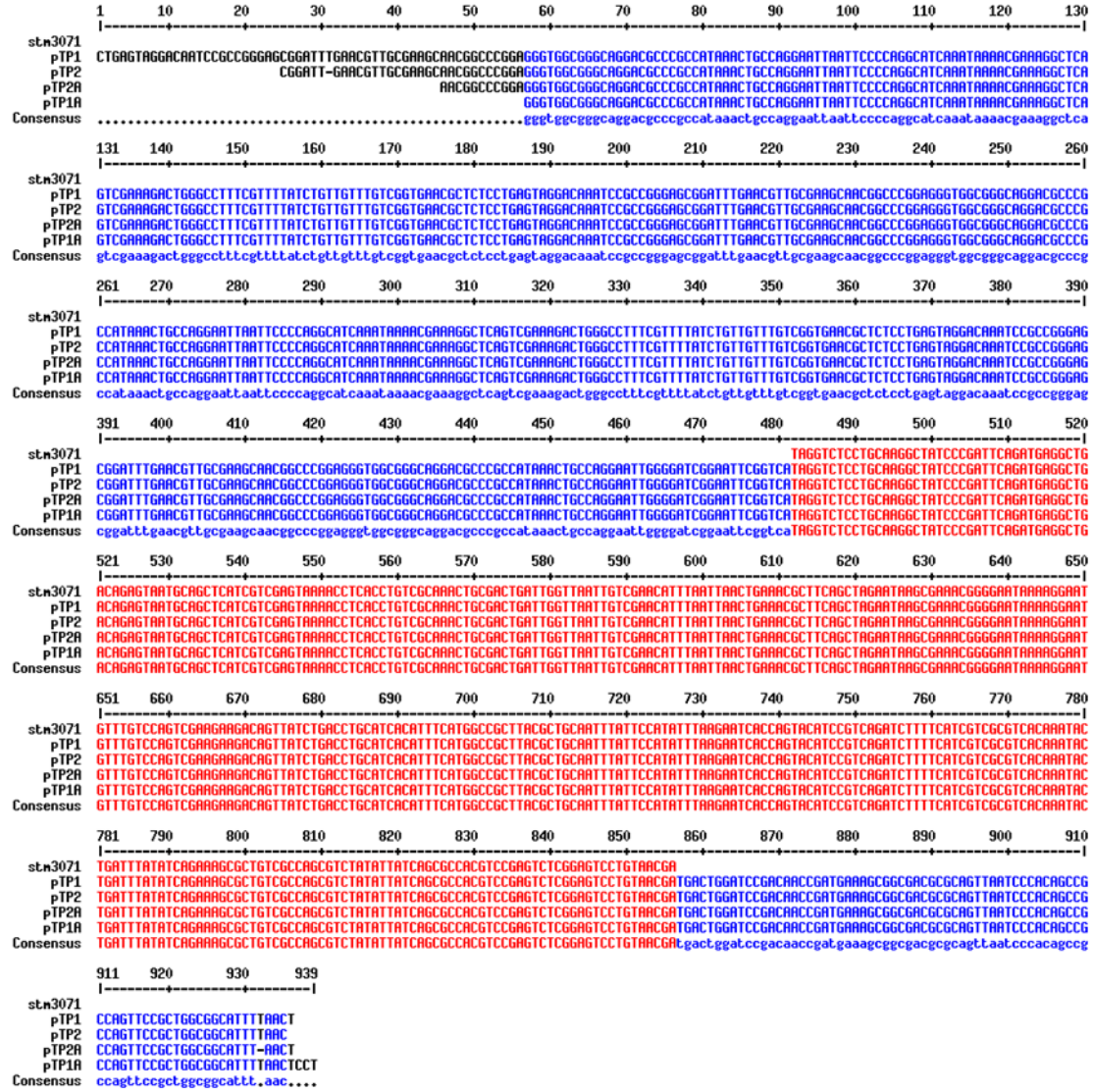


Fig. 5.6 Verification of selected clones - *lacZ* promoter fusions restriction enzyme digests confirmation. Lanes 1 in A, B and C indicate Hyperladder I (DNA size marker). (A) All plasmids were digested with EcoRI and EcoRV. Lane 2 pRS415 (9.5 + 1.3 kb). Lines 3-6 $P_{Arrach::lacZ}$ (pArrach_*stm3071*) (9.5+1.6 kb). Lines 7-9 $P_{cbiA::lacZ}$ (9.5+1.6 kb). (B) Lane 2 pRS415 digested with EcoRI and EcoRV (9.5 + 1.3 kb). Lane 3 Single digested pRS415 with BglII (10.8 kb). Line 4 and 5 EcoRI and EcoRV $P_{stm3071::lacZ}$ (pTP) (9.5+1.6 kb). Lane 5 and 7 BglII single digest, specific restriction site for pTP (1.5 kb). Gel electrophoresis performed with 1% agarose gel.


stm3071
pTP1
pTP2
pTP2A
pTP1A
Consensus

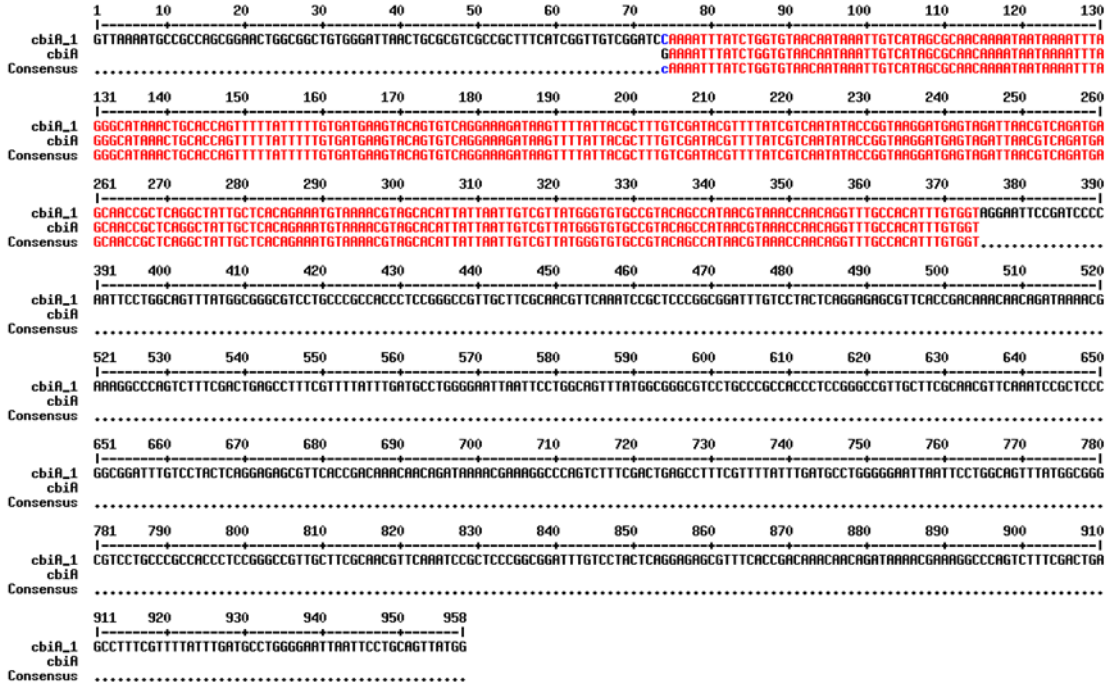


Fig 5.7 Alignment of selected constructs against to the original query sequence from *S.* Typhimurium. Analysis was conducted using Multalin software (http://multalin.toulouse.inra.fr/multalin/) (Corpet, 1988). All selected and sequenced constructs resembled 100% identity with queried regions.

Constructed p*stm3071::lacZ* (pTP), pArrach(*stm3071::lacZ*) (pArrach) and p*cbiA::lacZ* were sequenced to confirm identity of cloned regions. One of each selected constructs were selected, plasmid DNA was extracted and stored at -20°C until further analysis.

5.4 Phenotypic characterisation of *Δstm3071*

In chapter 3 it was hypothesised that *stm3071* is a part of predicted operon that potentially is involved in cobalt transport. It was also proposed that based on sequence identities it may function as a transcriptional regulator. It remains uncertain whether it plays role in the expression regulation of proximal genes or whether it is responsible for regulation of different distal cluster. It is also unclear whether its expression is metal dependent. Sequence similarities and recent research could also suggest that as proposed in chapters 3 and 4, STM3071 might not directly be involved in DNA-binding but it is possible that it coordinates with other protein, similarly to other regulators found in bacteria (Abellón-Ruiz *et al*., 2014). However, as discussed in chapter 3 in order to investigate the function of STM3071, it needs to be understood under what conditions *stm3071* is upregulated.

It was mentioned earlier in this chapter, that Arrach *et al*, (2008) noted $P_{stm3071}$ as preferentially induced in solid tumours. This, however, needs confirmation as to whether promoter was hypoxia-induced or whether that was dependent on other factors only associated with tumour tissue. Hypoxia, is defined as a reduction or complete lack of oxygen in tissues or cells. This happens in the event of oxygen supply reduction or sudden increase of oxygen consumption, which is what is observed in highly proliferating cells such as in solid cancers (Swinson & O'Byrne, 2006). During hypoxia, hypoxia inducible factor alpha (HIF-α) is expressed and it is responsible for regulation of the expression of genes that are involved in vascularisation and higher oxygen demand (Semenza, 2000). Cobalt was described to induce hypoxia in cells by inducing HIF-α (Wu & Yotnda, 2011). This might suggest that hypoxia can be induced by cobalt, and then it is possible that hypoxia-induced genes might be also co-regulated by cobalt. This also aligns with the predicted function of *stm3071* as involved in regulation of the expression of genes potentially involved in cobalt

import. Taking it from there, I might be possible that both hypoxia (anoxic environment) and cobalt are involved in *stm3071* upregulation.

The anaerobicity aspect as mentioned previously in this thesis, is associated with *S.* Typhimurium ability to synthesise vitamin B12 (Jeter *et al.*, 1984). Thus, if cobalt is mainly required for B12 biosynthesis then homeostasis regulation of this toxic metal is likely to take place under anaerobic conditions. Vitamin B12 paradox however, is based on the fact that cobalamin is synthesised in the absence of oxygen, and bacteria require cobalamin for 1,2- propanediol utilisation. This can only occur when the final electron acceptor is provided, and usually oxygen plays that role, hence the paradox (Price-Carter *et al*., 2001). On the other hand, as described by Winter *et al.*, (2010) *Salmonella* during invasion in the intestinal epithelium in order to outcompete the resto of the gut microbiota induces inflammation. During the inflammation, thiosulfur compounds are converted into tetrathionate, which can be used as a final electron acceptor (Winter *et al.*, 2010). Thus, it suggests that bacterium itself evolved with the ability to modulate the host environment and therefore to out-compete other pathogens, other symbiotic bacteria and to establish infection.

5.4.1 Exposure to cobalt

In order to address whether the mutation affects the ability to grow in the presence of cobalt and to determine the concentration that limits growth of *S.* Typhimurium, growth experiments were conducted in the presence of increasing concentrations of cobalt. Experiments were carried both under aerobic and anaerobic conditions and in minimal media. Minimal media limited the access of potential metals chelators that are naturally existing in rich media. Minimal media were prepared in acid washed glassware to reduce the possibility of other metals contamination. Exposure to cobalt in minimal media shows the same trend for both SL1344 and *Δstm3071.* Mutated strain did not grow in any different manner than WT. In experiments carried in M9 media (fig. 5.8), anaerobic growth was less supported in comparison to anaerobic growth in NCE glucose media (fig. 5.9). Interestingly under anaerobic conditions both strains exhibit greater tolerance to cobalt. However, this was more observable in M9 media. Viable count of cultures exposed to cobalt was performed for experiment carried in M9 media in order to compare observations from OD measurements.

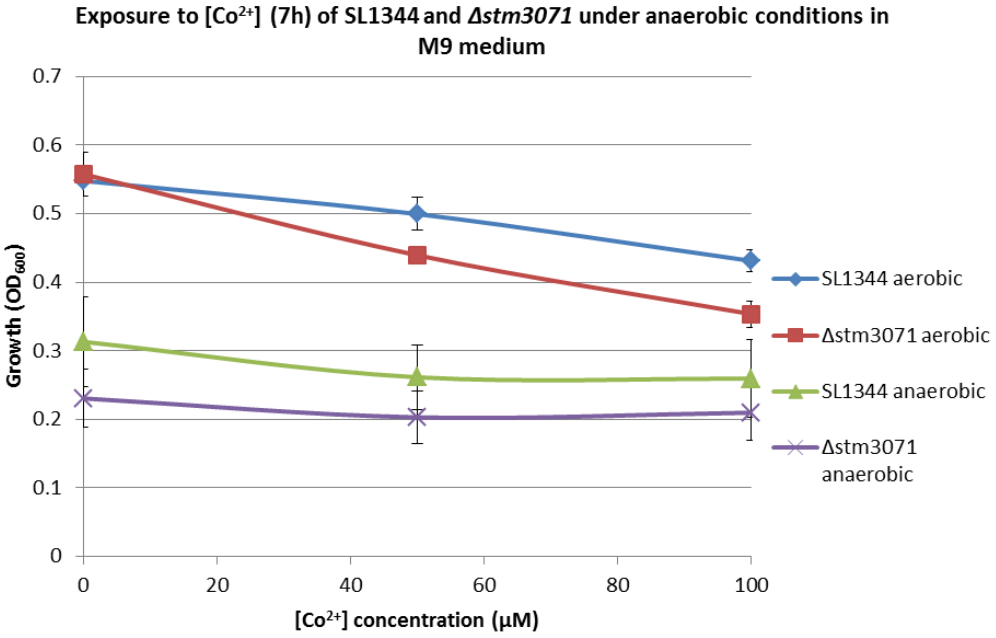


Fig 5.8 Loss of *stm3071* does not influence tolerance to cobalt (M9 medium). Overnight cultures of SL1344 and *Δstm3071* grown in M9 with glucose medium were diluted 1:50 into fresh M9 media with increasing concentrations of cobalt. Aerobic cultures were incubated at 37°C, 200 rpm for 4-5 hours, until OD_{600} reached ~0.4-0.6. Anaerobic cultures, also incubated at 37°C, 80 rpm were measured after 5-6 h until OD_{600} reached ~0.2-0.4. For anaerobic experiments, media were pre-equilibrated overnight in anaerobic cabinet. Data points represent the mean of 3 independent experiments performed in triplicate. Error bars represent standard error of the mean.

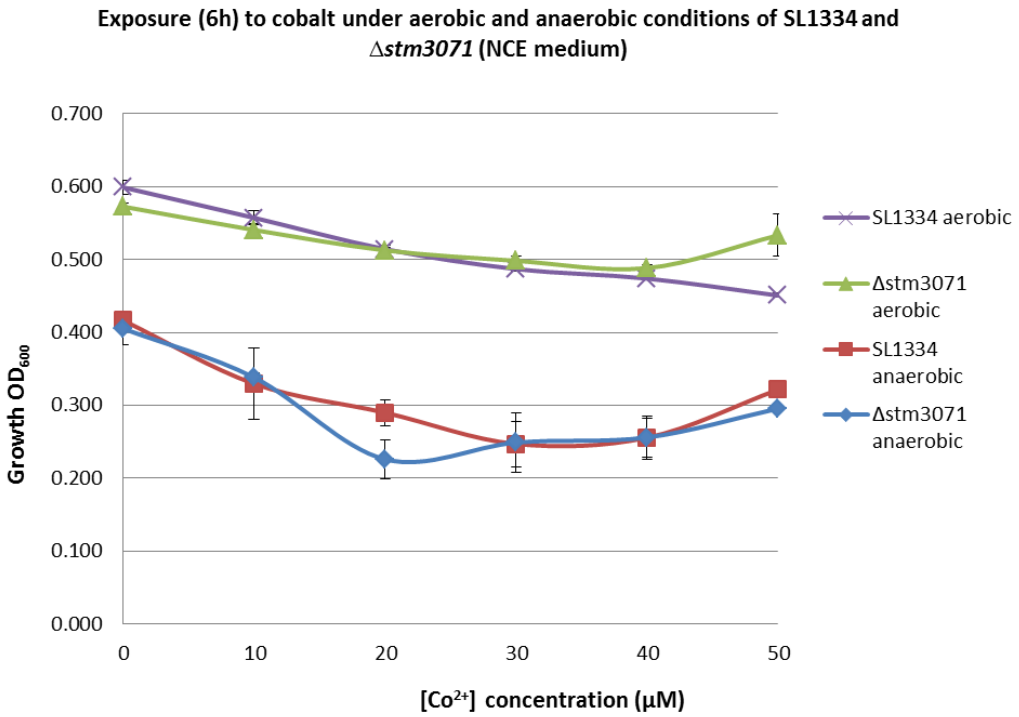


Fig 5.9. Loss of *stm3071* does not influence tolerance to cobalt (NCE medium). Overnight cultures of SL1344 and *Δstm3071* grown in NCE with glucose medium were diluted 1 in 50 into fresh NCE + glucose media with increasing concentrations of cobalt. Aerobic cultures were incubated at 37°C, 200 rpm for 4-5 hours, until OD_{600} reached ~0.4-0.6. Anaerobic cultures, also incubated at 37°C, 80 rpm were measured after 5-6 h until OD_{600} reached 0.4-0.6. For anaerobic experiment, media were pre-equilibrated overnight in an anaerobic cabinet. Data points represent the mean of 3 independent experiments performed in triplicate. Error bars represent standard error of the mean.

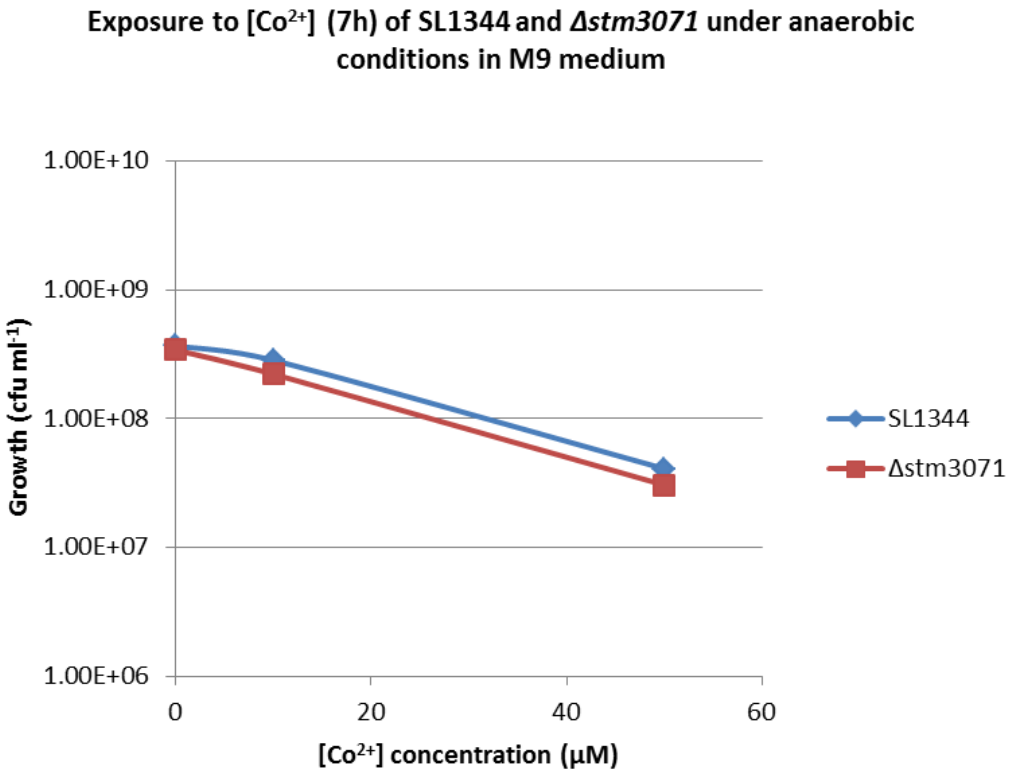


Fig 5.10 Loss of *stm3071* does not influence tolerance to cobalt (M9 media, viable count). Overnight cultures of SL1344 and *Δstm3071* grown in M9 medium were diluted 1:50 into fresh M9 media with increasing concentrations of cobalt. Media were pre-equilibrated overnight in an anaerobic cabinet. Cultures were incubated for 7h, at 37°C, 80 rpm. Following exposure, cell count was determined using viable count as described in chapter 2. Data points represent the mean of 3 independent experiments performed in triplicate. Error bars represent standard error of the mean.

S. Typhimurium SL1344 and mutant strain *Δstm3071* were exposed to titrated cobalt concentrations under aerobic and anaerobic conditions. Loss of *stm3071* does not influence the ability to tolerate higher concentrations of cobalt. It was noted, that under anaerobic conditions toxic effect of cobalt was subtler. There was an observed fold-change reduction of growth at concentration of 50 µM [Co^{2+}], the same for both strains (fig 5.10).

5.4.2 Anaerobic growth with 1,2 propanediol

Scavenging cobalt is required for biosynthesis of vitamin B12 (Jeter *et al*., 1984). Cobalamin is required for 1,2-propanediol utilisation (Adkins *et al*., 2006). During inflammation, *S.* Typhimurium modulates lumen environment and is able to accommodate tetrathionate as a final electron acceptor in the process of propanediol utilisation which at the same time give it an advantage over the rest of the microbiota present in the gut (Winter *et al*., 2010).

Knowing that 1.2-pronanediol can only be utilised when cobalamin is provided; and taking onto account that a final electron acceptor must be present in order for propanediol to be metabolised, *S.* Typimurium SL1344 and *Δstm3071* *a*erobic growth was monitored in the presence or absence of cobalamin with 1,2-propanediol as the only carbon source. This was carried out in NCE medium supplemented with 50 mM concentration 1,2-propanediol. Overnight cultures, grown in NCE glucose medium were prepared anaerobically to allow cobalamin synthesis.

This was to test whether *Δstm3071* ability to produce cobalamin was affected. Growth in medium supplemented with cyanocobalamin (150 nM CNB12) and propanediol was used as a positive control. Positive controls showed no difference between WT or *Δstm3071* strain. There was also no observable difference in growth between two strains when CNB12 was not added. However, more growth was observed when medium was supplemented with CNB12 (fig. 5.11).

The same experiment was also carried out under anaerobic conditions, however no growth was observed as the alternative electron acceptor was not provided (fig 5.12).

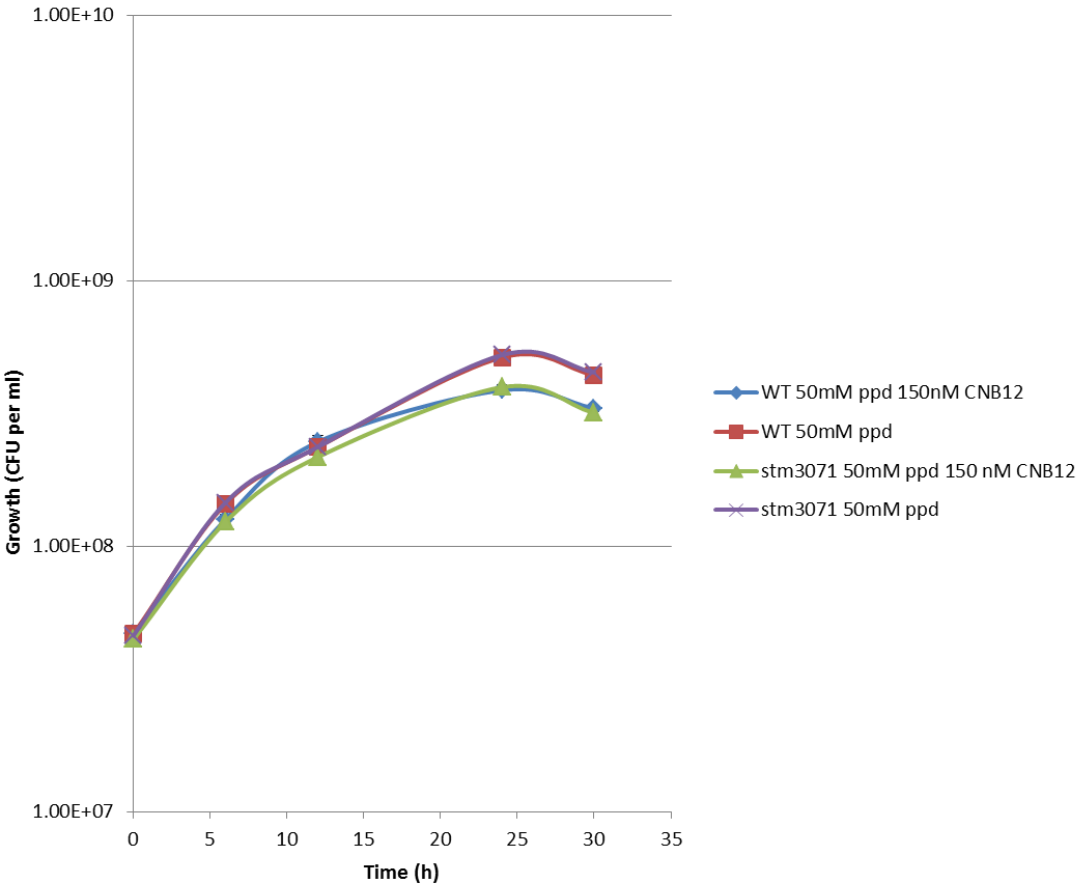


Fig. 5.11 Growth of SL1344 vs *Δstm3071* with 1,2-propanediol with or without CN-B12 under aerobic conditions. Loss of *stm3071* does not affect the ability to produce cobalamin. Overnight cultures, grown in NCE glucose medium were prepared anaerobically to allow cobalamin synthesis. Cultures were then incubated aerobically (final electron acceptor was not added) for 30h, at 37°C, 200 rpm. 1,2- propanediol was added as carbon source. Data points represent the mean of 3 independent experiments performed in triplicate. Error bars represent standard error of the mean.

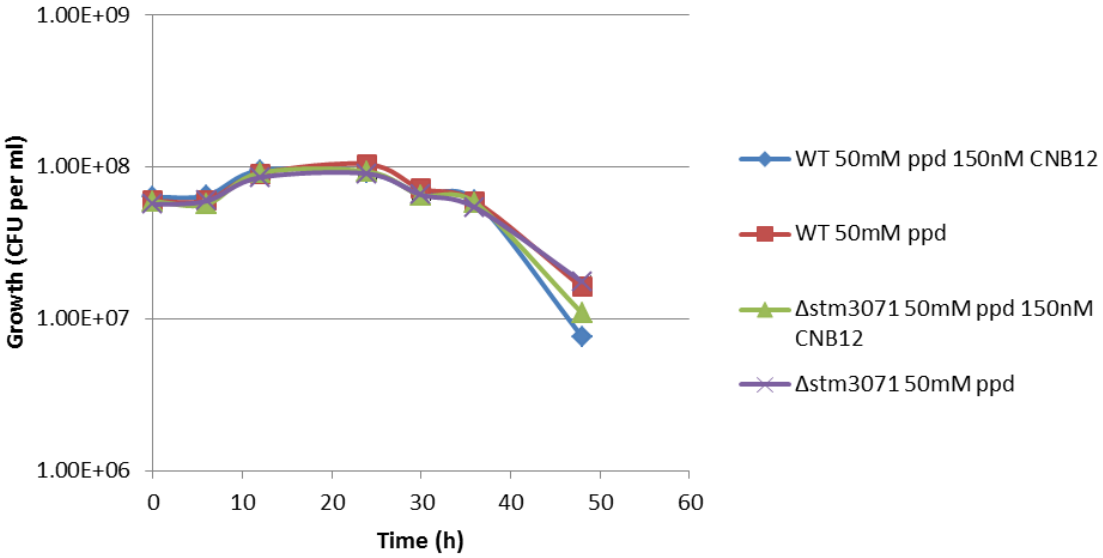


Fig. 5.12 Growth of SL1344 vs *Δstm3071* with 1,2-propanediol (ppd) with or without cyanobobalamin (CN-B12) under anaerobic conditions. Anaerobic growth with 1,2-propanediol as a carbon source is not supported when the electron acceptor is not provided. Overnight cultures, grown in NCE glycerol medium were prepared in aerobic conditions. Growth medium was pre-equilibrated to anaerobic conditions overnight. Cultures were inoculated with overnight culture 1:50 and incubated at 37°C, 80 rpm anaerobically. 1,2-propanediol was added as carbon source. Data points represent the mean of 3 independent experiments performed in triplicate. Error bars represent standard error of the mean.

It was observed (fig. 5.12) that in the anaerobic environment 1,2-propanediol cannot be utilised without the presence of an alternative electron acceptor, even when CNB12 was already provided. Thus, sodium tetrathionate was added to a final concentration of 40 mM to test whether loss of *stm3071* affected *S.* Typhimurium ability to utilise propanediol. Exceptionally precise preparation of the media for this experiment was required. Media were pre-equilibrated under anaerobic conditions. Overnight cultures were set up in NCE glycerol to avoid any carryover growth from aerobic to anaerobic conditions (glycerol is not metabolised anaerobically). Each experiment was carried out with positive controls (NCE glucose) to test medium in case no growth with 1,2-propanediol occurred (fig. 5.13).

Obtained results suggest that the mutant strain was unable to survive under anaerobic conditions using 1,2-propanediol. However, it was not clear if that was due to inability to produce cobalamin.

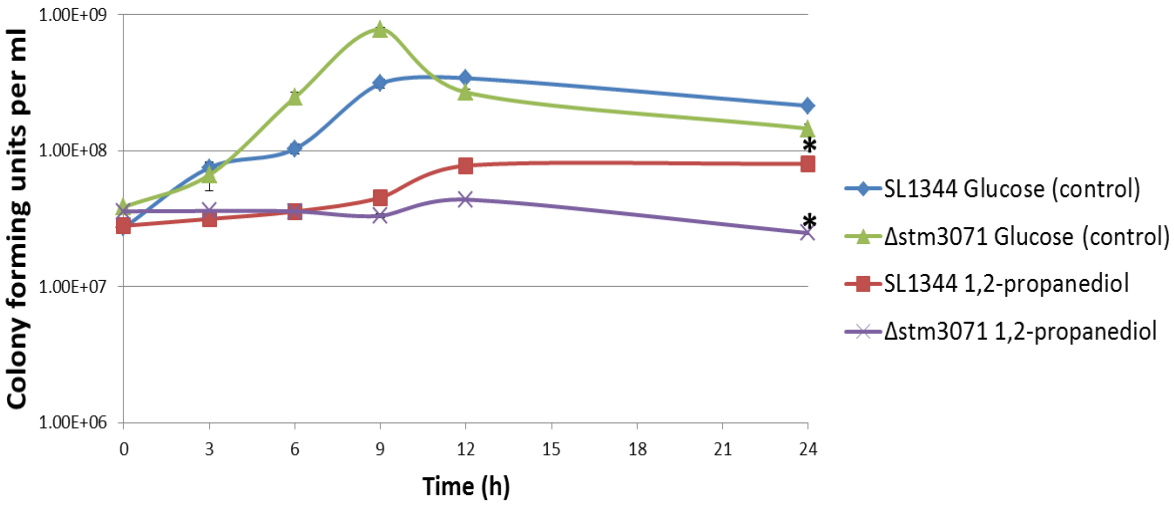


Fig 5.13 Growth curve of SL1344 & *Δstm3071* under anaerobic conditions on 1,2-propanedion as a source of carbon. Loss of *stm3071* affects growth on 1,2-propanediol under anaerobic conditions. Overnight cultures, grown in NCE glycerol medium were prepared in aerobic conditions. Growth medium was pre-equilibrated to anaerobic conditions overnight. Sodium tetrathionate was provided as an alternative final electron acceptor with medium supplemented with 1,2-propanediol. Strains cultured anaerobically with 50mM 1,2-propanediol or 11mM glucose in NCE medium. Error bars represent standard error of the mean. Paired t-test was conducted to compare 1,2-propanediol utilisation of SL1344 and *Δstm3071*, with $p \leq 0.05$ showing statistically significant difference in growth.

5.4.3 B12 assay

To test the ability to produce cobalamin, a B12 assay was carried out. MacConkey base agar plates were prepared as described in chapter 2. Plates contained 1,2-propanediol and cobalt for test controls. For negative controls, media were prepared without cobalt. Medium was inoculated 1:50 from the aerobic overnight culture and incubated anaerobically overnight. Then, bacteria were cultured on MacConkey base agar plated and incubated at 37°C for 24h. If growth occurred and 1,2-propanediol was used as a carbon source pH indicator turned colonies pink.

Number of positive and negative controls was designed to confirm the reliability of this assay. Personal communication with John Roth enabled to address number of solutions in terms of growth with propanediol. Additionally, Roth lab kindly provided reference strains previously described and characterised as cobalamin-defective. WT strains were used in this assay as negative controls - TR10000 (*S*. Typhimurium LT2, Roth lab) and SL1344. TT17104 and was used as positive control – *pocR* defective strain, cannot grow on propanediol and should not be able to synthesise B12. TT10327 also used as positive control – this strain cannot synthesise B12.

Results show (fig. 5.14) that the loss of stm3071 does not affect the ability to produce B12. WT controls were also able to produce B12. Positive controls TT17104 & TT10327 were unable to produce vitamin B12 – as expected. TT17104 strain is *pocR* defective mutant of LT2 hence it was not expected to utilise 1,2-propanediol. TT10327 has a defective *cbiD* hence it cannot make B12 and so it is unable to utilise 1,2-propanediol (Bobik *et al*., 1992).

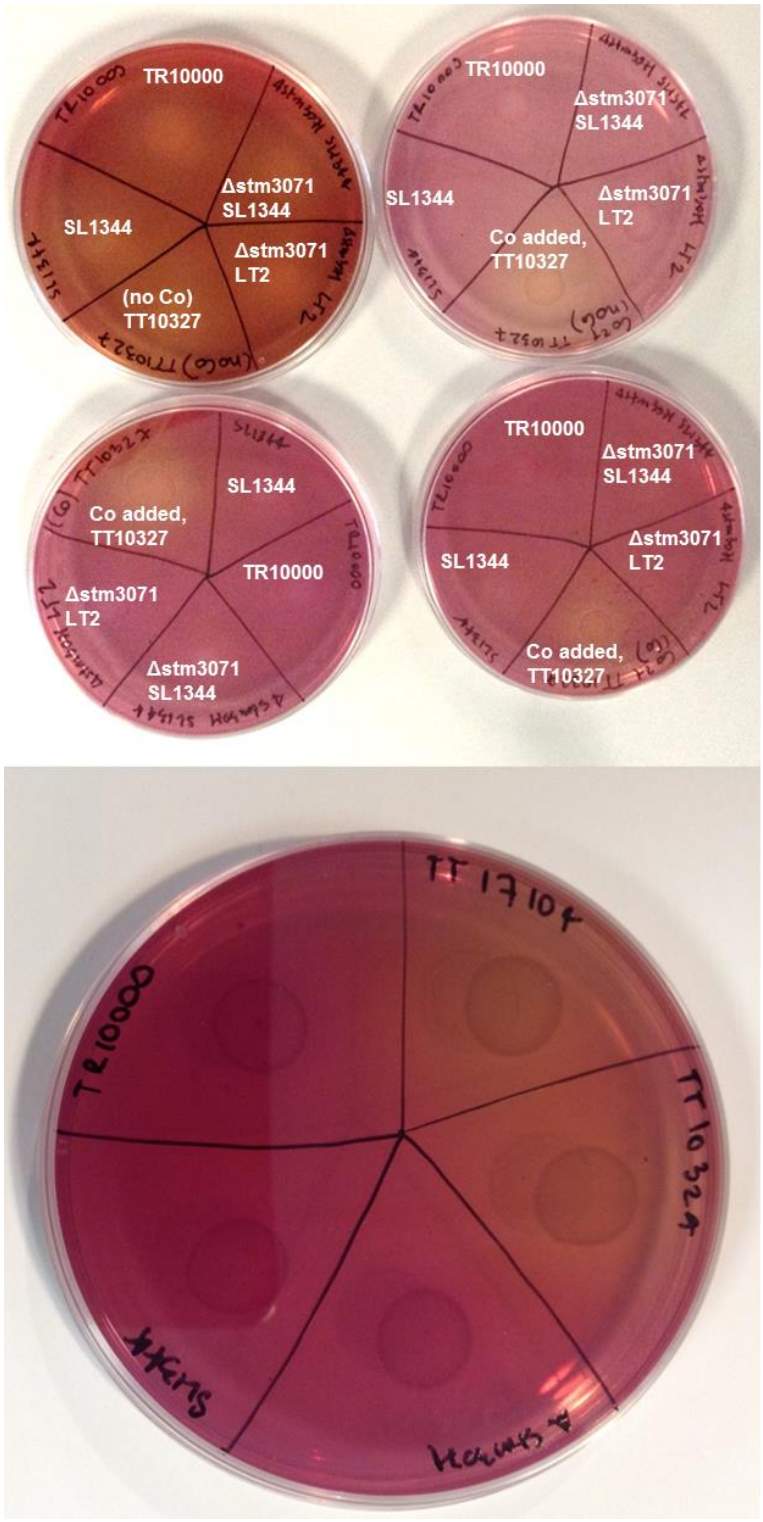


Fig 5.14 Loss of *stm3071* does not affect the ability to synthesise vitamin B12. B12 assay carried out with bacterial cells cultured overnight anaerobically. Cells were then plated out and incubated with plates containing pH indicator and 1,2-propanediol as a carbon source. Pink indicates the ability to produce cobalamin and hence use propanediol. Bacteria that were unable to produce B12 indicate in yellow. Growth did not occur only with defined mutant strains that is deficient in B12 synthesis; *Δstm3071* was able to produce B12.

5.4.4 β-galactosidase activity of $P_{stm3071}$ in response to cobalt

Earlier in this chapter it was described that concentration of 50 µM of cobalt has a certain level of inhibitory effect on *S.* Typhimurium SL1344 and *Δstm3071*. There was no observed difference in cobalt tolerance between the two strains.

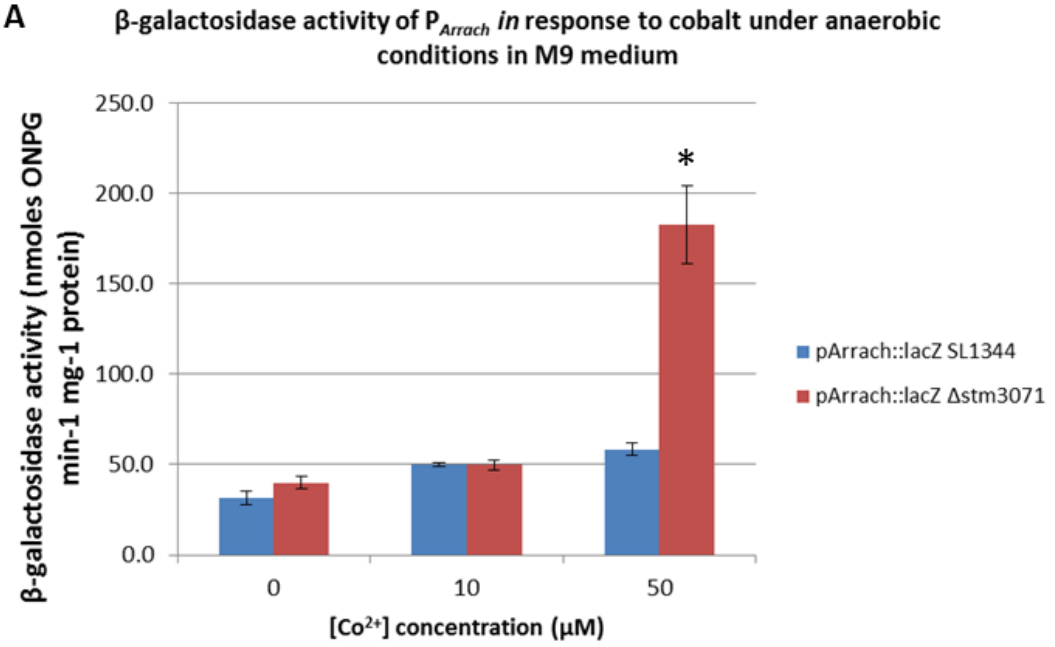
Constructs containing promoter region of $P_{stm3071}$, Arrach *et al.*, (2008) version of $P_{stm3071}$ and P_{cbiA} were electroporated into LB5010a. LB5010a is a restriction minus, modification plus *S.* Typhimurium strain that allows more efficient manipulations. Selected ampicillin resistant transformants were cultured overnight at 37°C. Plasmid DNA was isolated, confirmed by restriction digest and electroporated into *S.* Typhiurium SL1344 and *Δstm3071*.

This was to test conditions in which $P_{stm3071}$, is expressed. Additionally, as it is predicted that *stm3071* might function as a transcriptional regulator of downstream genes, construct was introduced into *Δstm3071* strain as well as SL1344.

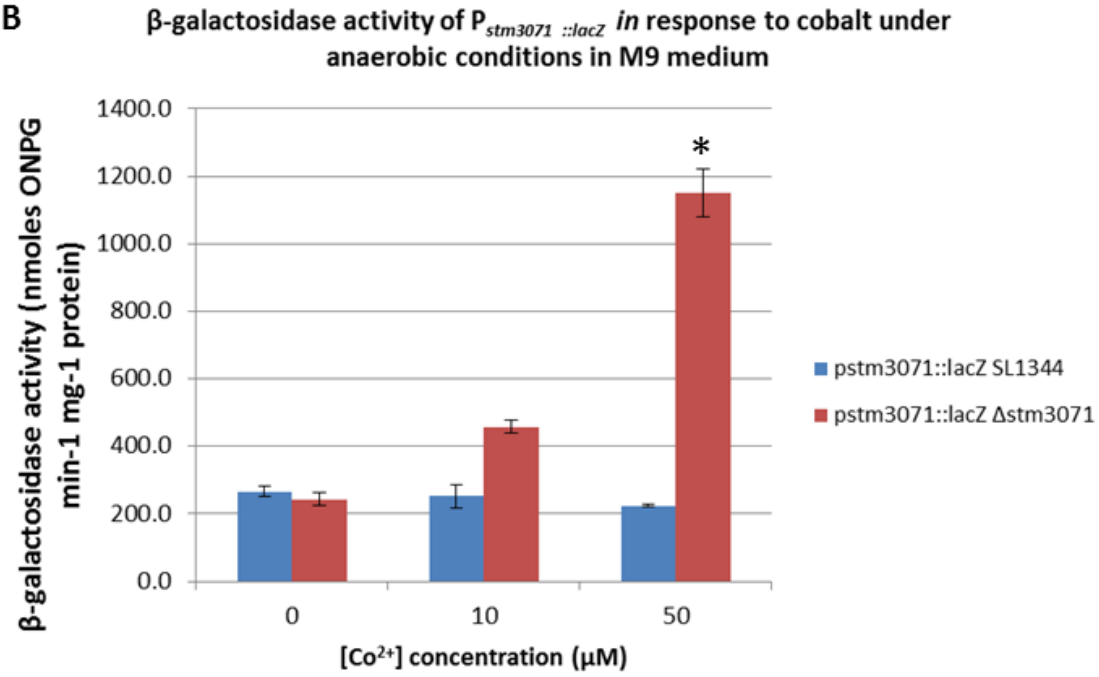
β-galactosidase assays were carried out with 0, 10 and 50 µM [Co^{2+}] under anaerobic conditions in M9 medium. Constructed p*cbiA::lacZ* control was used as control - predicted the region as slightly upregulated in anaerobic environment.

Elevated beta-galactosidase activity of p*stm3071::lacZ* (pTP), pArrach (*stm3071::lacZ*) (pArrach) was observed in in response to cobalt but only in the mutant strain. *pcbiA::lacZ* appears marginally switched on and not cobalt dependent.

A


β-galactosidase activity of P Arrach in response to cobalt under anaerobic conditions in M9 medium
β-galactosidase activity (nmoles ONPG min-1 mg-1 protein)
250.0
200.0
150.0
100.0
50.0
0.0
*
0
10
50
[Co2+] concentration (µM)
pArrach::lacZ SL1344
pArrach::lacZ Δstm3071

B


β-galactosidase activity of P stm3071 ::lacZ in response to cobalt under anaerobic conditions in M9 medium
β-galactosidase activity (nmoles ONPG min-1 mg-1 protein)
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
*
0
10
50
[Co2+] concentration (µM)
pstm3071::lacZ SL1344
pstm3071::lacZ Δstm3071

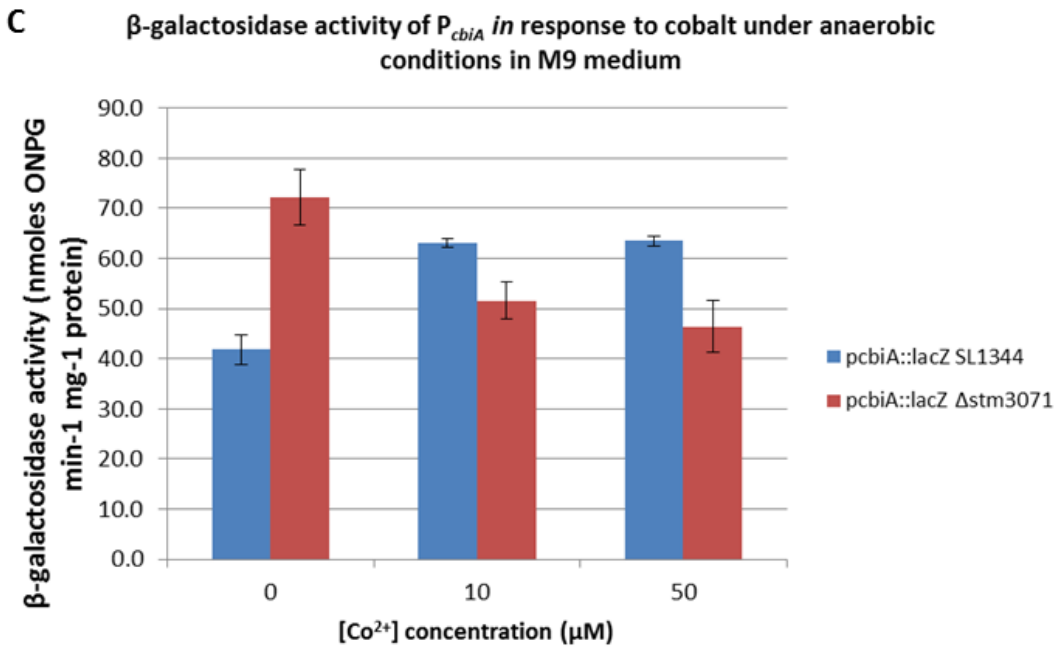


Fig. 5.15 β-galactosidase activity measured in SL1455 and *Δstm3071*. All experiments were carried under the same conditions (anaerobic, 37°C until OD_{600} 0.3 was reached) in triplicate on three different occasions. Error bars represent standard error. Paired t-test was carried out to compare β-galactosidase of p*stm3071::lacZ* SL1344 and p*stm3071::lacZ Δstm3071,* with p≤0.05 showing statistically significant difference in β-galactosidase activity – this indicated with an *.

5.4 **Summary**

In this chapter the work was addressed from two separate angles. Firstly, in order to investigate the importance of *stm3071* a deletion mutant was constructed. Secondly, $P_{stm3071}$ was tested against a range of conditions to determine the conditions that induce the expression of *stm3071*.

Considering the close location to predicted metal transport genes and its predicted function as a transcriptional regulator, a constructed deletion strain was tested in the presence of cobalt. Due to the well described fact that *Salmonella* needs to scavenge cobalt for *de novo* biosynthesis of vitamin B12 (Adkins *et al*., 2006) it was hypothesised that in the absence of an anaerobically-induced transcriptional regulator, anaerobic growth might be affected. It was observed that concentration of 50 µM [Co^{2+}], has an inhibitory effect on bacterial growth. However, under anaerobic conditions bacteria does not seem to react to metal toxicity in as severe way as under aerobic environment. It is possible that since cobalt is required more anaerobically then bacteria has evolved with mechanisms that allow it to withstand higher concentrations of this toxic metal.

Although there was no obvious phonotype observed in terms of cobalt tolerance it was hypothesised that if a hypothetical operon encodes for an importer, the ability to produce vitamin B12 might be abolished. As described earlier, vitamin B12 is necessary for utilisation of 1,2-propanediol. Hence, a mutant strain was tested for the ability to both produce vitamin B12 and 1,2-propanediol.

Although we could observe that the loss of *stm3071* affected the ability to grow in defined medium with 1,2-propanediol under anaerobic conditions. Conversely, in medium supplemented with glucose and cobalt, under anaerobic conditions mutant strain *Δstm3071* was still able to produce vitamin B12. This however might be due to the compensational effect of a different gene, possibly *cob* operon. The observed difference might be due to the fact that in a defined minimal medium it is more likely to see distinctive changes. Expression experiments show that $P_{stm3071}$ promoter was cobalt responsive but only in *Δstm3071.* This was difficult to explain. However, it is possible that there are differences of intracellular metal levels between mutant and the wild type strain. Those differences might be subtle and might not have a direct effect on survival.

6 Chapter 6 – Discussion

6.1 Occupying the niche

Salmonella is a facultative anaerobic and pathogenic organism that in response to the environment, adapted and evolved in an excellent way that give it advantages over the competing microbiota (Raffatellu *et al*., 2009). It thrives in the inflamed gut and utilises substrates that cannot be utilised by other organisms (Bäumler & Sperandio, 2016). It was described earlier that that during the infection reactive oxygen species convert thiosulfate to tetrathionate which in turn serves as an alternative electron acceptor in 1,2-propanediol utilisation (Winter *et al*., 2010).

The ability to utilise 1,2-propanediol takes place in an adenosylocobalamin-dependent manner (Adkins *et al*., 2006). B12 synthesis only occurs under anaerobic environment and it requires cobalt (Jeter *et al*., 1984). Cobalt on the other hand is toxic when present in excess; however it is essential for B12 synthesis. In order to overcome competing microbiota, *Salmonella* occupies niches that allow this smart pathogen to utilise resources that nobody else competes over (Winter *et al*., 2010).

Constructed *Δstm3071* did not behave differently to a wild type strain in a fluctuating metal environment. However, in precisely calibrated minimal media supplemented with 1,2-propanediol as the only carbon source, *Δstm3071* was not able to grow conversely to the wild type strain. It remains unclear why it continued to synthesise cobalamin. The strain should not be able to grow on 1,2-propanediol if vitamin B12 is not synthesised. It is hypothesised however, that the very subtle phenotype difference can only be observed in a defined environment, where other genes that play compensational role are upregulated. It is thus proposed that in order to see more distinctive phenotypical change, *stm2021* and *stm3073*, encoding for ABC-type cobalt transporter should be eliminated and tested for the ability to produce cobalamin and utilise 1,2-propanediol under anaerobic conditions (Fig. 6.2).

5.5 Metal transport

The mechanisms of which bacterial cells are trafficking metals are still not very well understood. Identified *stm3071* was proposed to be responsible for

upregulation of proximal downstream cluster predicted to encode cobalt transport genes. Similar organisation of the identified genes was also noted in other organisms associated with gut colonisation. The sequence similarities of investigated operon suggest that they encode for the ECF cobalt transporter. The abundance of this system might suggest they are likely to be functional since the course of evolution did not eliminate them. Additionally, it was also hypothesised, that STM3071 might act as a transcriptional regulator in other more distant cluster. The question as to whether STM3071 functions as a DNA-binding protein remained unanswered. The conducted EMSA experiment had limitations due to the possibility of improper secondary or tertiary structure of STM3071. It is possible that in order to achieve appropriate conformation that determined functionality it needed overexpression and purification under anoxic conditions. NsrR, a nitric oxide oxygen-sensitive transcriptional repressor from *Bacillus subtilis* was shown to expose greater affinity towards consensus site when overexpressed under anaerobic conditions (Kommineni *et al.*, 2010). It is also possible that the binding site of STM3071 is distant from the operon. It is also possible the protein is only functional in the presence of metal. Thus, metal-binding assay should be conducted to determine whether STM3071 has the affinity towards cobalt or/and possibly nickel (Rodionov *et al.*, 2006). lt also needs clarification whether the system is metal specific. It was previously determined experimentally that the substrate specificity for the S component of the CbiMN ECF system is particularly precise. Metal specificity assay showed strong preference for Co^{2+} over Ni^{2+} (Rodionov *et al.*, 2006). Other similar bacterial systems such as NikMN (Ni^{2+}), BioY (biotin) (Hebbeln *et al.*, 2007), RibU (riboflavin) (Duurkens *et al*., 2007) were described to have substrate specific S component. ECF system, as mentioned in chapter 3, class I of ECF transporters is encoded by operons that have two genes encoding for A unit, T unit and specific S component unit. This suggest strong specificity towards particular substrate (Rodionov *et al*., 2009). Interestingly, amongst class I of ECF components there were not found significant similarities (with maximum 11-21% identity) between transporters from different subfamilies. The only exception that showed similarities were the homologous CbiM and NikM families. Thus CbiM and NikM transporters were subdivided into separate subfamilies of cobalt and nickel transporters (Rodionov *et al*., 2006).

Based on similarities identified in amino acid sequence of STM3071 it was hypothesised that STM3071 possibly has a similar mode of action to CarD/CarG (Garcia-Moreno *et al*., 2010) complex that interacts with another protein, which contains specific AT-gook binding motif. Due to the presence of three conservative His residues and putative zinc-binding site it is possible that STM3071 plays role in DNA-binding and it is involved in transcription regulation of the putative cobalt uptake system. Another possible limitation of the performed EMSA experiment was that regions used as probes could have overwhelmed the protein with their size and as a result, no shift was observed. Overall, for future EMSA experiments, there need to be established: 1) whether metal is required for proper folding of STM3071; 2) the protein should be overexpressed under anaerobic conditions; 3) probes should be redesigned and two predicted consensus sequences should be tested at first instance (Kommineni *et al.*, 2010; Osman *et al*., 2015; Sydor *et al.*, 2013).

5.6 **Metal sequestering**

Cellular proteins assist in metal transport. Those are specific however these proteins tend to select essential divalent cations with a ranked in order of preference defned by Irving-Williams series: Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} (IRVING & WILLIAMS, 1948). Thus, competitive metals must be kept out of binding sites for the weaker ions. It often occurs that in vivo metal preferences do not match the absolute metal preferences. As reported, InrS (nickel-responsive efflux derepressor), CoaR (cobalt-responsive efflux activator), ZiaR and Zur (zinc-responsive efflux derepressor and influx activator, respectively) do not match the Irving rule (Foster et al., 2012; Rutherford, Cavet, & Robinson, 1999; Thelwell, Robinson, & Turner-Cavet, 1998; Tottey et al., 2012). For instance, even though nickel correlated with higher activity, it was zinc that coupled DNA-binding to zinc binding. CoaR for was found to preferentially bind cobalt and activate *coaT* operator-promoter region of cobalt efflux system. There is evidence that CoaR is membrane-associated and cobalt acquisition may involve channelling through cobalamin biosynthetic complex, also membrane-associated. CoaR is likely not to be able to sense cobalt directly but intermediates in the cobalamin biosynthetic pathway. (Rutherford *et al.*, 1999). This suggests that proteins have the specific affinity towards specific metals. STM3071 as a predicted ECF importer possibly requires to metallate cobalt or zinc to allow transcription derepression or repression. Concluding from the beta-

galactosidase experiments, overexpression of $P_{stm3071}$ was cobalt responsive, thus it confirms to be cobalt specific. It requires clarification whether it would also be nickel or possibly zinc responsive. The promoter was cobalt responsive however only in the mutant strain. It is then possible that in the absence of STM3071 the transcription of the cobalt uptake system is constitutively derepressed resulting in continuous transcription of higher more effective influx of cobalt in the mutant strain. This remained unanswered, however conducting metal content assay of cells exposed to cobalt anaerobically could provide with the explanation.

5.7 **Operon prediction**

As summarised in chapter 3, it is likely that *stm3071/3072/3073/3074/3075* represent a putative operon. Genes arranged in a tandem, delimited by promoter-operator site located upstream from the beginning, are classified as promoters (Chen *et al.*, 2004). Co-existance and similar organisation within other organisms suggests strong evidence in the operon prediction (Rogozin *et al*., 2004). Transcription orientation, genes encoding proteins of related function, conservancy amongst other *Salmonella* strains gives the additional indication that analysed tandem of genes is likely to be an operon. High conservancy amongst other species associated with the gut suggest that this type of putative importer of cobalt is specific for intracellular survival. It was also found to be flanked with conserved genes at most instances amongst other species, such as *Citrobacter*. Similar clusters can be found mostly in *Salmonella* strains, but also in other pathogens, such as *Shigella* or *Klebsiella*, which may serve as a direct suggestion that they are required for full virulence and intracellular survival. From the phylogenetic tree browser analysis it can be concluded that the conserved COG1661 (*stm3071*) was acquired by horizontal gene transfer. Predicted proteins share high conservancy especially analogues of *stm3074* and *stm3075* suggesting how metal transport is essential for organisms associated with the gut. It is possible that predicted operon exists as a copy for *cbiMNQO*. However, it is also likely that those are functional under the completely different conditions. Additionally to the evidence provided in chapter 3, RNAseq data was obtained from (Srikumar *et al*., 2015). This RNAseq data shows that conditions that switch the expression of both queries are similar.

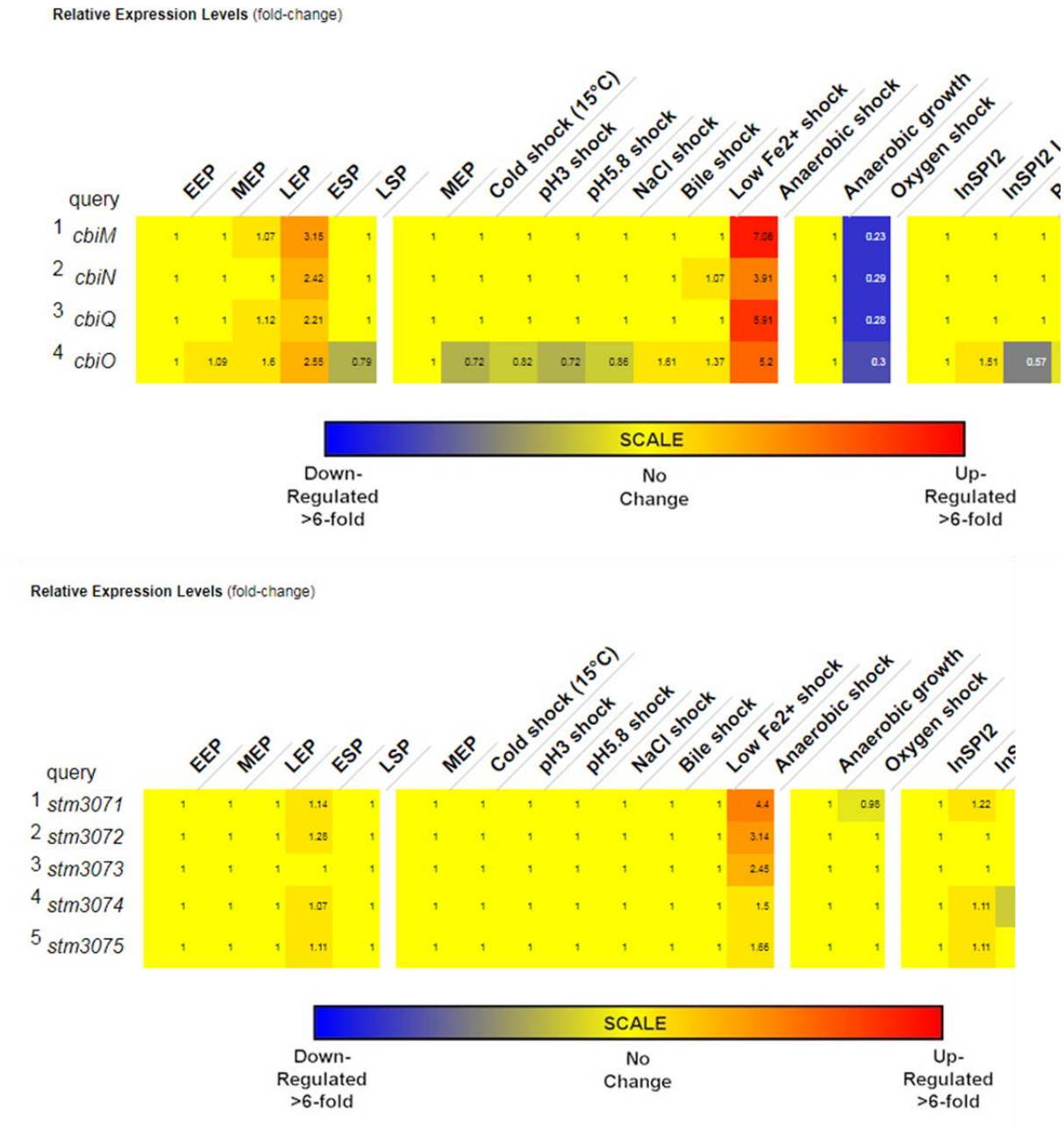


Fig. 6.1 Visual representaito of the RNAseq data showing relative expression levels of *stm3071/3072/3073/374/3075* and *cbiMNQO*. Both operons are expressed under the anaerobic shock conditions intracellularly (Srikumar *et al.*, 2015).

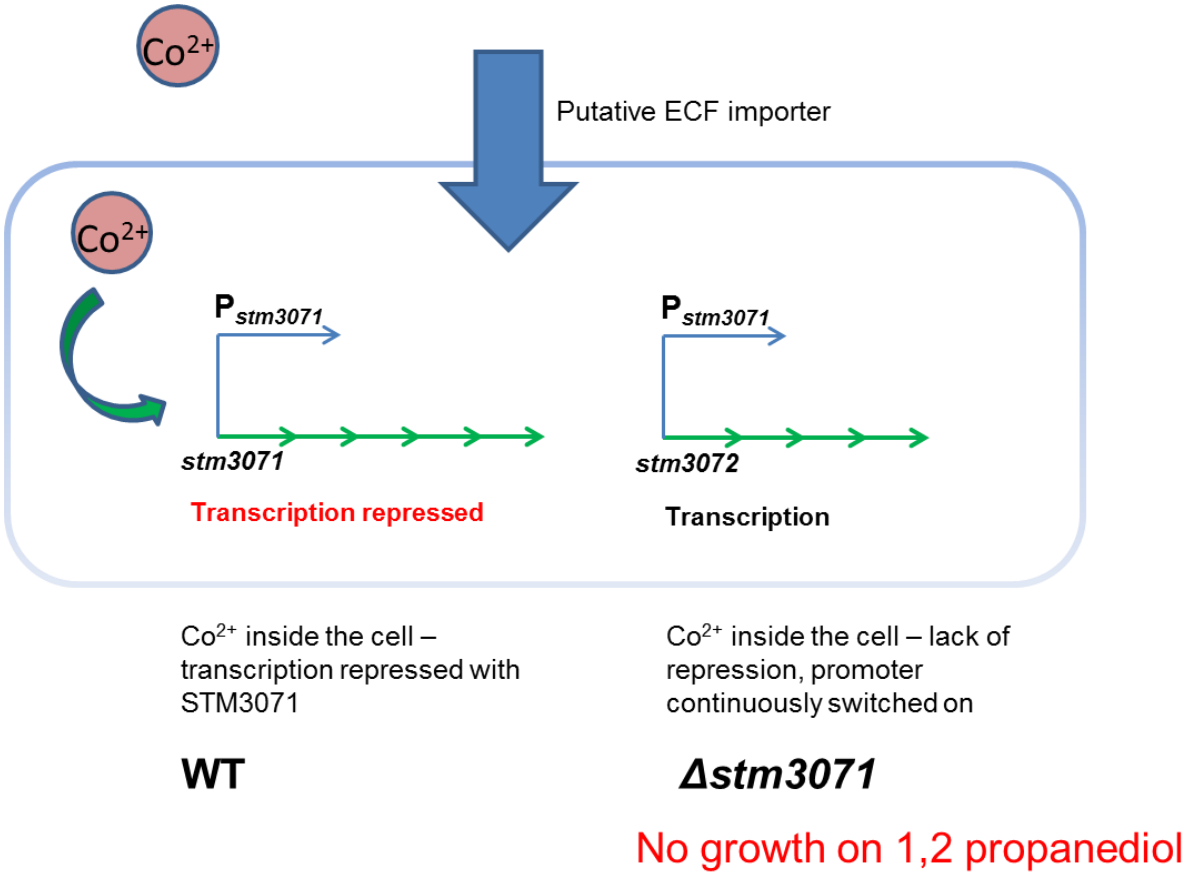


Fig. 6.2 Schematic representation of current understanding and hypothesis of the role of STM3071 in *S.* Typhimurium. Currently based on the findings of this work it can be understood, that under conditions when cobalt is needed, STM3071 derepresses the transcription, ECF importer is expressed and cobalt is transported inside the cell. However, when Co^{2+} is present inside the cell, STM3071 represses the transcription. In the event where *Δstm3071* is not present, it is possible that $P_{stm3071}$ is expressed constitutively and additionally responds to cobalt elevated levels, resulting in higher expression levels. It was found that *Δstm3071* does not utilise 1,2-propanediol under anaerobic conditions. B12 assay however showed that the mutant strain was able to produce B12. Thus, it can be predicted that in a limited setting the phenotype might be subtle, as the complementary operon might not be expressed under defined conditions.

5.8 **Antitumor aspect**

In work presented by Arrach *et al*, (2008), promoter region of *stm3071* was noted to be preferentially activated within solid tumours. *Salmonella* as a facultative anaerobic bacterium has a natural ability to accumulate in niches of solid tumors (Arrach *et al*., 2010; Wall *et al*., 2010; Zhao *et al*., 2007). Necrotic regions of tumours are hypoxic. Hypoxia, which means low oxygen or anaerobicity which is similar to conditions required for activation of *cob* and *pdu* operons. Tumour environment was found to act upon $P_{stm3071}$ expression. In this study $P_{stm3071::lacZ}$ fusion was constructed to determine what conditions induce its expression.

Constructed expression fusion of $P_{stm3071}$ promoter was shown to be cobalt responsive; however it was only observed when the expression was induced in *Δstm3071.* It remains unanswered as to whether this activity is cobalt specific. It is possible that STM3071 regulates genes involved in cobalt transport, and this possible accumulation of the metal inside the cell resulted with elevated expression levels.

Accordingly, with full understanding of the function and role of STM3071 it can be investigated further whether attenuated *Salmonella* strains with tumour targeting abilities could serve as a novel and selective way of introducing anti-tumour therapeutics under control of selectively induced promoter. Therefore, attenuated *Salmonella* strains with tumour-targeting abilities could serve as a novel delivery system of anticancer therapeutics under control of promoters selectively induced in tumours (Arrach *et al.*, 2009). This strategy can provide a therapeutic tool of reduced highly toxic for normal tissue side effects and become selective approach for metastatic cancer.

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