Investigating Bacterial Factors Important for the *Sinorhizobium meliloti*-Legume Symbiosis

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Declaration

I hereby declare that all the work presented in this thesis is my own except where otherwise stated

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Abbreviations

::	Plasmid insertion
Δ	Delta
Φ	Phage
5(6)-FAM, SE	5-(and-6)-carboxyfluorescein, succinimidyl ester
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
Amp	Ampicillin
AMP	Antimicrobial peptide
ATP	Adenosine tri-phosphate
BLAST	Basic Local Alignment Search Tool
BLM	Bleomycin
BODIPY	Dipyrromethene boron difluoride
bp	Base pairs
BSA	Bovine serum albumin
$CaHPO_4$	Calcium phosphate dibasic
cfu	Colony forming units
Cm	Chloramphenicol
CTAB	Hexadecyltrimethylammonium bromide
$CuSO_4.5H_2O$	Copper (II) sulphate pentahydrate
CV	Crystal Violet
DAPI	4',6-Diamidino-2-phenylindol
dH_2O	Distilled water
dNTP	Deoxyribonucleotide triphosphate
DOC	Sodium deoxycholate
DSMO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ENOD	Early nodulin
EPS	Exopolysaccharide
ER	Endoplasmic reticulum
et al.	et allii (and others)

FACS	Fluorescent-activated cell sorting
GC	Gas chromatography
Gm	Gentamycin
H_2O_2	Hydrogen peroxide
H_3BO_3	Boric acid
hALDP	Human adrenoleukodystrophy protein
Hm	Hygromycin
HPLC	High-performance liquid chromatography
IFN	Interferon
Ig	Immunoglobulin
IMP	Increased membrane permeability
IPTG	Isopropylthiogalactopyranoside
K_2HPO_4	Potassium phosphate dibasic
Kan	Kanamycin
kb	Kilobase pairs
LB	Luria-Bertani
LBMC	LB with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$
LPS	Lipopolysaccharide
Mb	Megabase
MES	2-(N-morpholino) ethanesulfonic acid hydrate
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MMS	Methyl methanesulfonate
$MnCl_2.4H_2O$	Manganese (II) chloride tetrahydrate
MS	Mass spectrometry
N	Nitrogen
Na_2HPO_4	Sodium phosphate dibasic
NaCl	Sodium chloride
NaH_2PO_4	Sodium phosphate monobasic
$NaHCO_3$	Sodium bicarbonate
$NaMoO_4.2H_2O$	Sodium molybdate dihydrate
NaOH	Sodium hydroxide

NH_3	Ammonia
OD_{600}	Optical density at 600nm
OD_{605}	Optical density at 605nm
ORF	Open reading frame
OsO_4	Osmium tetroxide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfu	Plaque-forming unit
PHB	Polyhydroxybutyrate
Pi	Phosphate
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
r	Resistant
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
Sm	Streptomycin
Spc	Spectinomycin
TAE	Tris-acetate-EDTA
TB	Trypan blue
Tc	Tetracycline
TCA	Tricarboxylic acid
TE	Tris-EDTA
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
Tn	Transposon
UV	Ultraviolet
v/v	Volume per volume
VLCFA	Very-long-chain-fatty-acid
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

Abstract

In both the legume symbiont *Sinorhizobium meliloti* and the mammalian pathogen *Brucella abortus,* the inner membrane BacA protein is essential for host persistence. In free-living *S. meliloti* and *B. abortus* loss of the BacA protein also results in an increased resistance to the glycopeptide bleomycin and a ~ 50% decrease in the lipopolysaccharide (LPS) very-long-chain-fatty-acid (VLCFA) content. Consequently, it was proposed that BacA may be involved in transport of peptides into the cell and/or that BacA may be involved in the VLCFA modification of the LPS.

During this work it was determined that the increased resistance observed in an *S. meliloti* Δ*bacA* mutant to bleomycin and to the truncated eukaryotic peptide Bac7(1-16), is independent of the VLCFA modification. These data support a model for BacA having multiple non-overlapping functions. Using flow cytometry studies with fluorescently labelled forms of bleomycin and Bac7(1-16) it was found that the BacA protein plays a role in the uptake of bleomycin. However, BacA was shown to be essential for the uptake of Bac7(1-16). Additionally, it was determined that two symbiotically defective *bacA* site directed mutants with known reductions in their VLCFA could still take up Bac7, suggesting that the BacA function that leads to the VLCFA modification could also play a key role in host persistence.

To investigate further the role of BacA in the VLCFA modification and where in the cell envelope the lipid A is modified with the VLCFA, the role of the putative lipid trafficking protein MsbA2 was investigated. Interestingly, it was discovered that *S. meliloti* lacking the MsbA2 protein, is unable to enter host cells and induces a plant defence response more characteristic of a pathogen. To investigate the importance of the VLCFA modification during the symbiosis *S. meliloti* mutants lacking either the AcpXL (VLCFA acyl carrier protein) or LpxXL (VLCFA acyl transferase protein) were characterized in the host. Although not essential for host persistence, loss of each of the proteins did result in distinct defects, suggesting the VLCFA modification is important during the symbiosis. Since there are hundreds of nodule specific cysteine-rich peptides produced by the host plant *Medicago truncatula*, the BacA mediated uptake of one of these peptides

combined with the VLCFA modification may account for the essential role of the BacA protein in the legume symbiosis.

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Chapter 1: Introduction

1.1. Chronic bacterial host interactions

Several bacterial pathogens have evolved a variety of strategies to invade and survive long-term within eukaryotic cells, often resulting in chronic infections within their hosts. Chronic bacterial infections pose serious threats to human health, yet compared to acute bacterial infections, their molecular basis is poorly understood (Monack *et al.*, 2004; Rhen *et al.*, 2003).

Two intracellular pathogens which can cause chronic infections and result in much mortality and morbidity world wide are *Salmonella enterica* serovar Typhi and *Mycobacterium tuberculosis*. *S.* Typhi is a human specific pathogen and currently causes some 21.5 million cases of typhoid fever each year (http://www. cdc.gov/) and around 6% of these patients become chronically infected (Parry *et al.*, 2002). These chronic carriers will continue to shed infective *S.* Typhi (Parry *et al.*, 2002) and are also at an increased risk of developing hepatobiliary cancer (Welton *et al.*, 1979). *M. tuberculosis*, which causes tuberculosis, a chronic disease of humans, results in more than 2 million deaths annually (Domenech *et al.*, 2008). Additionally it is estimated latent infection with *M. tuberculosis* affects more than one-third of the total human population, providing an enormous reservoir for future infections (Young *et al.*, 2008). Today, a growing problem is the emergence of strains of *M. tuberculosis* resistant to drugs, with approximately 400,000 cases of multidrug-resistant tuberculosis occurring each year (Young *et al.*, 2008). Furthermore, there is now the emergence of what is essentially an untreatable form of tuberculosis, known as extensively drug-resistant tuberculosis (Young *et al.*, 2008).

1.2. The alpha-proteobacteria

The alpha-proteobacteria are a large and diverse of group of gram-negative bacteria, many of which establish long term chronic infections within eukaryotic cells (Kersters *et al.*, 2003). The bacteria within this group display a wide range of lifestyles (Batut *et al.*, 2004) and include *Rickettsia* and *Wolbachia* which are obligate intracellular pathogens of mammals and arthropods (Cowan, 2000; Werren *et al.*, 2008). Also included within this group are *Bartonella* and *Brucella* which are both facultative intracellular pathogens of mammals (Dehio, 2005; Moreno &

Moriyón, 2001). However, this group also includes the soil bacteria Rhizobia which actually form a beneficial association with their host, whereby they engage in symbioses with the roots of leguminous host plants (Gibson *et al.*, 2008).

1.3. *Sinorhizobium meliloti*

Sinorhizobium meliloti is one of the best known Rhizobia as a consequence of being studied by a large number of research groups worldwide. *S. meliloti* can either be found free-living in the rhizosphere or in a symbiotic relationship with leguminous plants such as *Medicago sativia* (alfalfa) and *Medicago truncatula*, where the bacteria are found in specialized structures on the root known as nodules (Niner & Hirsch, 1998). Together, *S. meliloti* and the model plant host *M. truncatula* are one of the most important symbiosis model systems (Becker *et al.*, 2008). Various research groups have helped to develop a set of powerful genetic tools for *S. meliloti* (Glazebrook & Walker, 1991) and the *S. meliloti* genome was published in 2001 (Galibert *et al.*, 2001). The *S. meliloti* genome contains over 6000 protein coding genes, distributed into 3 replicons, consisting of a circular chromosome (3.65 Mb) and two megaplasmids, pSymA (1.36 Mb) and pSymB (1.68 Mb).

1.4. The *S. meliloti*-legume symbiosis

The symbiosis between leguminous plants and *S. meliloti* to form a nitrogen fixing nodule is a complex and unique interaction and begins with a specific molecular signal exchange between the legume and free-living *S. meliloti.*

1.4.1. Nod factors

Flavonoid compounds (2-phenyl-1, 4-benzopyrone derivatives) produced by leguminous plants are the first signal exchanged (Perret *et al.*, 2000) and are released when the soil is limited for nitrogen. Flavonoids are recognised by bacterial NodD proteins, which are members of the LysR family of transcriptional regulators (Schell, 1993). The N-terminus of NodD binds to the promoter regions of several of the bacterial nodulation related (*nod*) genes, thereby inducing their expression (Long,

1996; Perret *et al.*, 2000). Nod factors, which are essential signalling molecules, are synthesized by the products of some of these *nod* genes (Oldroyd & Downie, 2004) . Nod factors consist of a backbone of β-1,4-linked N-acetyl-D-glucosamine residues, with a long acyl chain attached to the terminal glucosamine (Perret *et al.*, 2000) (Fig. 1-1). The size and saturation state of this lipid chain varies in a species-specific manner (Gibson *et al.*, 2008) and nod factors can be further modified with a variety of chemical substituents, including acetyl, arabinosyl, fucosyl and sulfuryl additions (Gibson *et al.*, 2008).

Nod factors induce multiple plant responses that are essential for bacterial invasion (Oldroyd & Downie, 2004). One of the first plant responses to occur is a rapid calcium influx in root hairs which is followed by oscillations in the cytosolic calcium concentration (calcium spiking) (Oldroyd & Downie, 2004). This is followed by root hair curling, whereby the bacteria which have attached to the root hair become trapped within tightly curled root hairs (Esseling *et al.*, 2003; Gage, 2004) (Fig. 1-2). At the same time the Nod factors stimulate the initiation of cell division in regions of the root inner cortex. These cells form the nodule primordium and give rise to the cells which will receive the invading bacteria (Foucher & Kondorosi, 2000) (Fig. 1-2).

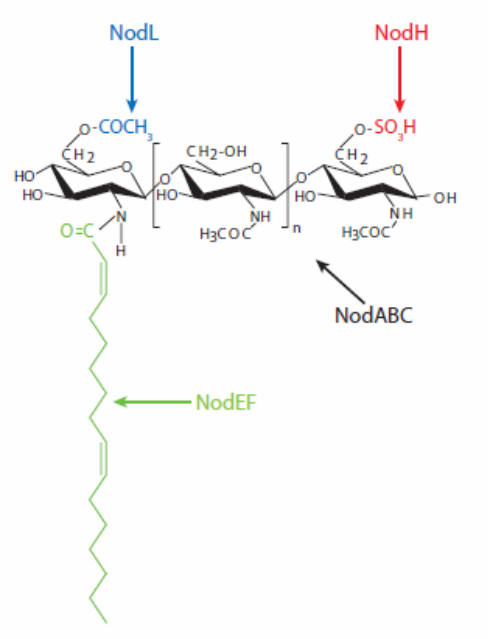

Figure 1-1.The Nod factor produced by *S. meliloti*. Also shown are the Nod proteins involved in Nod factor biosynthesis. (Gibson *et al.*, 2008).

1.4.2. Infection thread development

The bacteria trapped within the curled root hair tip induce the formation of a host derived inwardly growing tubular compartment known as an infection thread (Brewin, 2004; Gage, 2004) (Fig. 1-2). *S. meliloti* produces the exopolysaccharides succinoglycan (also known as exopolysaccharide I) and galactoglucan (exopolysaccharide II), which facilitate infection thread formation (Glazebrook & Walker, 1989; Pellock *et al.*, 2000). The bacteria enter and divide within the infection thread, which traverses the entire root hair and outer cortical cells to reach the inner cortex (Fig. 1-2). The path followed by the infection thread within the cortex is predetermined by cytoplasmic bridges or pre-infection threads (Timmers *et al.*, 1999; van Brussel *et al.*, 1992). The infection thread branches as it grows through the root and enters the nodule primodium, which increases the number of sites from which the bacteria can exit the threads (Gage, 2004) (Fig. 1-2). As an infection thread ceases growth, unwalled droplets of infection thread matrix material (infection droplets) containing the bacteria are formed (Brewin, 2004). These infection droplets extrude from the infection threads and are engulfed by the plant host plasma membrane, forming symbiosomes by an endocytosis like process (Brewin, 2004) (Fig.1-3). After endocytosis the bacteria divide together with the symbiosome membrane, before differentiating into non-dividing nitrogen fixing bacteroids (Robertson & Lyttleton, 1984) (Fig. 1-3).

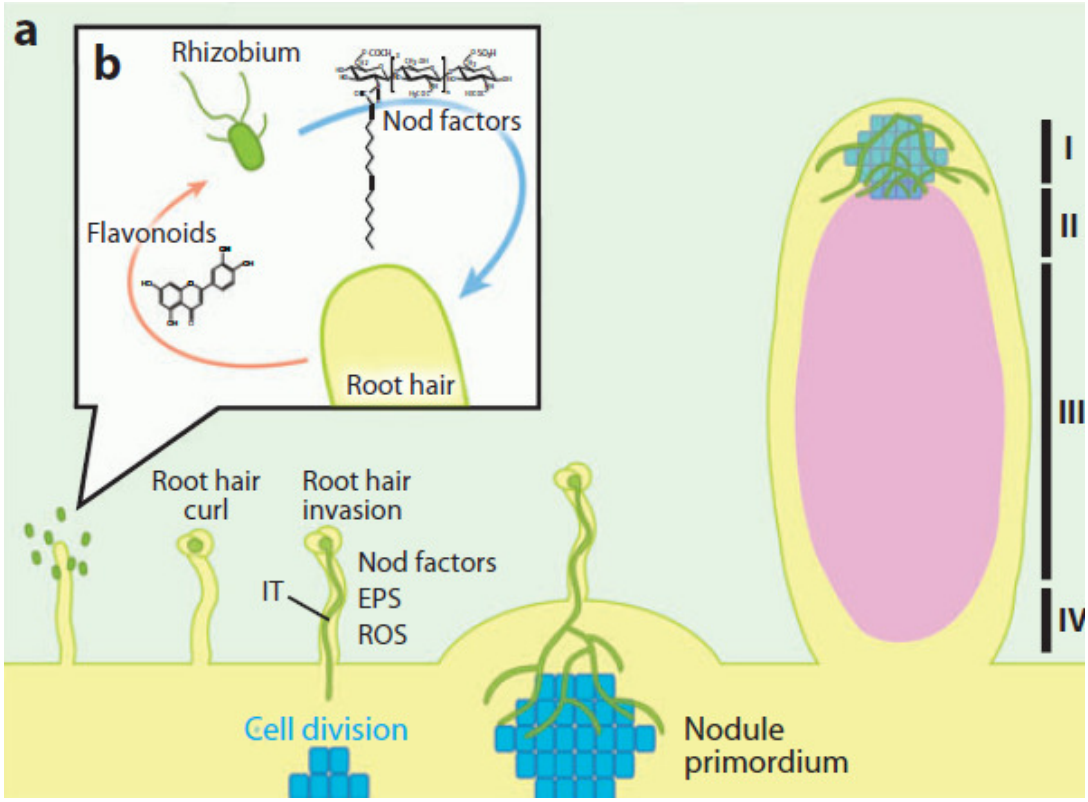

Figure 1-2. Schematic model of nodule development in the *S. meliloti* legume symbiosis. (a-b) The host legume releases flavonoids which trigger bacterial Nod factor production. Nod factor induces various responses in the host legume such as root hair curling which is followed by root hair invasion. In addition to Nod factors and bacterial exopolysaccharide (EPS) root hair invasion requires reactive oxygen species (ROS), which are required for optimal progression of infection threads though the root hair. The Nod factors also induce cell division in the root cortex (shown in blue), which leads to the formation of a nodule meristem. Indeterminate nodules form on alfalfa which originate from the root inner cortex and have a persistent meristem (Zone I). Each nodule also contains an invasion zone (Zone II) and a nitrogen fixing zone (Zone III). In older nodules there is a senescent zone (Zone IV), where both the plant and bacterial cells degenerate (Gibson *et al.*, 2008).

1.4.3. Nitrogen fixation

Expression of the bacterial nitrogenase synthesis (*nif*) and microoxic respiration (*fix*) genes are required for successful nitrogen fixation (Gong *et al.*, 2006). Nitrogenase is the two component enzyme complex responsible for nitrogen fixation and its structure is highly conserved throughout nitrogen fixing bacteria (Dixon & Kahn, 2004). The expression of plant proteins is also necessary to support nitrogen

fixation. One such protein is leghaemoglobin, an oxygen binding protein. Leghaemoglobin is required to maintain a microaerobic environment within the nodule, since the nitrogenase enzyme is irreversibly inactivated by oxygen, yet the bacteria require oxygen for use in respiration (Appleby, 1984). Indeed, it is leghaemoglobin that imparts the pink/red colour displayed by healthy nitrogen fixing nodules. Nitrogenase produced by the bacteroids converts nitrogen (N_2) into ammonia (NH_3). The formula for nitrogen fixation is as follows:

$$N_2 + 8H^+ + 8e- + 16\ ATP \quad \rightarrow \quad 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

In this process, two moles of NH_3 are produced from one mole of N_2 at the expense of 16 molecules of ATP and a supply of electrons and protons (hydrogen ions) (O'Brian, 1996). The ammonia produced by the nitrogenase enzyme is then secreted by the bacteroid, at which point it is thought to be taken up by the plant through NH_3 channels that have been detected in the plant membrane (Day *et al.*, 2001; Prell & Poole, 2006).

Throughout the symbiosis a constant carbon supply is required to provide metabolites and energy for bacteroid differentiation and nitrogen fixation (Day *et al.*, 2001; Prell & Poole, 2006). Polyhydroxybutyrate (PHB) granules produced by *S. meliloti* are degraded during bacteroid differentiation and appear to be preferentially used as a carbon source (Willis & Walker, 1998). The plant host also provides carbon to the bacteroids in the form of dicarboxylic acids, such as malate, though the bacterial dicarboxylate transport (DCT) system (Poole & Allaway, 2000; Ronson *et al.*, 1981), which can then be used for respiration by the bacteria in the tricarboxylic acid (TCA) cycle (Poole & Allaway, 2000). Despite the fact the *S. meliloti*-legume symbiosis is beneficial to both partners, it can be regarded as a chronic infection as the bacteroids persist within the membrane-bound acidic compartments in the plant cells for extensive periods of time (Campbell *et al.*, 2002).

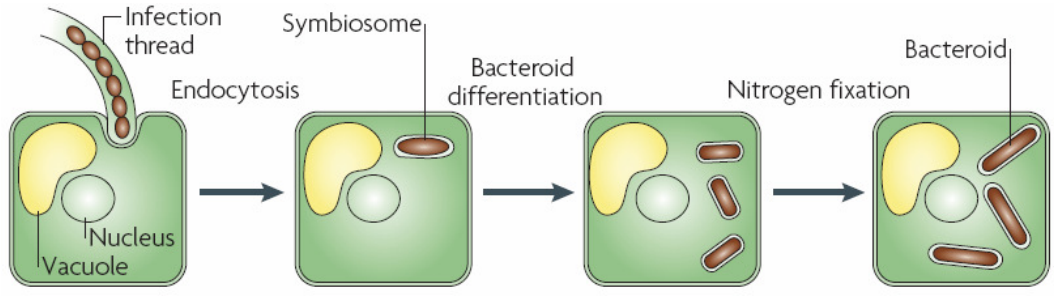

Figure 1-3. Endocytosis of *S. meliloti* into the plant cell and bacteroid differentiation. The bacterium is taken up into the host cell by an endocytosis like process and is engulfed by the plant host plasma membrane, forming a symbiosome. The bacterium will initially divide synchronously with the symbiosome membrane, after which it will differentiate into a nitrogen fixing bacteroid (Jones *et al.*, 2007).

1.5. *S. meliloti*-legume symbiosis as a model system for chronic infection

Although *S. meliloti* establishes an agriculturally important and beneficial symbiosis with leguminous plants (Niner & Hirsch, 1998), the bacteria are closely related to the *Brucella* species, which are chronic mammalian pathogens. This was initially revealed by RNA homology, with *S. meliloti* and *Brucella* species found to differ less than 7% in their 16S RNA sequences (Ugalde, 1999). This close relatedness has more recently been confirmed by genome sequencing of *S. meliloti* and several of the *Brucella* species (DelVecchio *et al.*, 2002; Galibert *et al.*, 2001; Halling *et al.*, 2005; Paulsen *et al.*, 2002). Despite the two very different outcomes of infection, experimental evidence has been accumulating over recent years suggesting parallels exist in the way *S. meliloti* and *B. abortus* survive within their respective hosts (Ferguson *et al.*, 2004; LeVier *et al.*, 2000; Roop *et al.*, 2002).

1.6. The Brucellae

1.6.1. *Brucella* species and brucellosis

Brucellosis is the most common zoonotic infection worldwide (Pappas *et al.*, 2006b) and has a significant impact on both animal and human health. Infections in animals result in abortion and infertility, while human infection will often result in a chronic,

debilitating disease known as undulant fever (Acha & Szyfres, 1980). There are seven species of *Brucella,* based on host specificity; *B. melitensis* (goats), *B. abortus* (cattle), *B. suis* (swine), *B. canis* (dogs), *B. ovis* (sheep), *B. neotomae* (desert mice) and *B. pinnipediae* and *B. cetaceae* (marine mammals) (Pappas *et al.*, 2005). To date, the genomes of *B. melitensis* (DelVecchio *et al.*, 2002), *B. abortus* (Halling *et al.*, 2005), and *B. suis* (Paulsen *et al.*, 2002) have been sequenced and published. Among the four *Brucella* species known to cause disease in humans (*B. melitensis*, *B. abortus*, *B. suis* and *B. canis*), it is *B. melitensis* which is the most prevalent world wide (Dizbay *et al.*, 2007). Additionally this species is also considered to be the most virulent (Eschenbrenner *et al.*, 2002). Humans acquire brucellosis through direct contact with infected animals or animal products, the most common means of transmission is consumption of unpasteurized dairy products such as milk and soft cheese (Nicoletti, 1989; Pappas *et al.*, 2006a). In areas of the world where brucellosis has not been eradicated but successful eradication programs are in effect, human brucellosis has become primarily an occupational hazard for animal handlers, slaughterhouse workers and veterinarians. Contrastingly in regions where brucellosis is endemic in goats, sheep and cattle, human brucellosis remains a serious public health problem. The most common symptom of brucellosis is one of a flu-like illness with fever, malaise, anorexia and muscle weakness (Young, 1989). Additionally, serious complications such as endocarditis (Hadjinikolaou *et al.*, 2001) and neurological disorders may also occur (Shakir *et al.*, 1987).

1.6.2. Treatment and prevention of brucellosis

Treatment of human brucellosis is difficult due to the intracellular nature of the infection, and prolonged antibiotic therapy is necessary (Young, 1989). The development of a brucellosis vaccine suitable for humans would be an ideal solution to the problems of inadequate veterinary control in many countries and the problems associated with antibiotic treatment. Unfortunately, the live attenuated vaccines that have been essential components of the successful eradication programs in farm animals, made from *B. abortus* strain 19 and *B. melitensis* Rev1 strain, are virulent for humans (Spink *et al.*, 1962). Additionally, the virulence of the newly instituted bovine vaccine strain *B. abortus* RB51 is presently unknown (Kahler, 1998).

Consequently there is no safe effective vaccine available for use in humans, and control of human disease therefore relies on preventing exposure.

1.6.3. The Brucellae as bio-warfare/terrorism agents

There are also several features of the brucellae that make them a threat as potential bio-warfare/terrorism agents (Valderas & Roop, 2006) Firstly, they are highly infectious by the aerosol route, with an infectious dose estimated to be at approximately 10 to 100 organisms (Franz *et al.*, 2001). Secondly, since brucellosis results in low mortality, it can be very effectively used as an incapacitating agent and could soon overwhelm hospitals and other medical care facilities if used as an agent (Valderas & Roop, 2006). Thirdly, as discussed previously successful treatment of human brucellosis requires prolonged antibiotic therapy (Young, 2000), and relapse rates after apparently successful treatment are reported to be as great as 30% (Franco *et al.*, 2007). Finally, as previously stated there is no safe and effective vaccine available for use in humans. Due to the risk of *Brucella* species being used as agents of bio-warefare/terrorism, *B. melitensis*, *B. suis* and *B. abortus* are included on the class B list of select agents as defined by the Centers for Disease Control and Prevention (Valderas & Roop, 2006).

1.6.4. Survival of *Brucella* species within the host

Brucella infections in both their natural animal hosts and in humans are characterized by their chronic nature (Baldwin & Roop, 1999; Enright, 1990) with many natural hosts remaining infected for life (Enright, 1990). The brucellae primarily reside within macrophages in their animal and human hosts (Kohler *et al.*, 2003; Roop *et al.*, 2004). It is the capacity for the brucellae to survive within these host phagocytes that is responsible for their chronic nature (Baldwin & Roop, 1999). Upon ingestion by host macrophages the brucellae reside in an acidified compartment that fuses with components of the early endosomal pathway (Celli *et al.*, 2003; Porte *et al.*, 2003), at which point the bacteria are also exposed to the oxidative burst (Jiang & Baldwin, 1993; Phillips & Roop, 2001). Although opsonisation of the bacteria with specific IgG or activation with IFN-γ has been shown to enhance the bactericidal action of

cultured macrophages (Eze *et al.*, 2000; Jiang & Baldwin, 1993), virulent strains of *Brucella* can still resist killing by these cells and in time display net intracellular replication. The progression of *Brucella*-containing vacuoles down the endosomal-lysosomal pathway is limited (Celli *et al.*, 2003) and virulent *Brucella* strains are trafficked to intracellular compartments that are favourable for intracellular survival and replication. The composition of these intracellular compartments, known as replicative phagosomes is enriched in membrane components originating from the endoplasmic reticulum (ER) of the host macrophages. The expression of the *Brucella* type IV secretion system appears to be essential for maintaining this continual interaction between the *Brucella* containing vacuole and the ER (Celli *et al.*, 2003). Although the replicative phagosome is a favourable environment for intracellular survival, *Brucella* still need to make major physiological adaptations to withstand the environmental stresses during long term residence in the host macrophage (Valderas & Roop, 2006).

1.7. Host invasion parallels between *S. meliloti* and *Brucella* species

Both *S. meliloti* and *Brucella* species persist in eukaryotic cells within a host derived membrane bound acidic compartment (LeVier *et al.*, 2000; Mellor, 1989; Niner & Hirsch, 1998). As previously stated, experimental evidence has been accumulating over recent years suggesting parallels exist in the way *S. meliloti* and *B. abortus* survive within their respective hosts (Ferguson *et al.*, 2004; LeVier *et al.*, 2000; Roop *et al.*, 2002). For example the production of periplasmic cyclic β-glucans is required for *S. meliloti* to adhere to root hairs and *B. abortus* species to survive within their intracellular compartments in the host macrophage (Arellano-Reynoso *et al.*, 2005; Dickstein *et al.*, 1988). There is also conservation of regulatory genes between *Brucella* species and *S. meliloti* which are important for either invasion or persistence within their respective hosts. For example, the *B. abortus bvrS/bvrR* sensor kinase/response regulator system is necessary for the intracellular survival of the bacteria (Sola-Landa *et al.*, 1998). Although it has not been possible to make null mutants in the *S. meliloti* homologues of these genes, demonstrating they are essential (Cheng & Walker, 1998; Keating, 2007), an *S. meliloti* mutant that

produces a constitutively active ExoS sensor kinase is compromised in its ability to infect root hairs (Cheng & Walker, 1998; Yao *et al.*, 2004; Zhang & Cheng, 2006). One major parallel in the lifestyle of *S. meliloti* and *B. abortus* bacteria is the requirement for the bacterial encoded BacA protein, which is critical for both bacteria to chronically infect their respective hosts.

1.8. The BacA protein

1.8.1. The BacA protein is essential for chronic infection of both *S. meliloti* and *B. abortus*

The *S. meliloti bacA* mutant Rm8368 was initially identified by a Tn*phoA* mutagenesis screen, as a mutant defective in establishing a symbiosis with alfalfa plants (Long *et al.*, 1988). The *S. meliloti bacA* Rm8368 mutant could infect and invade the root hairs and developing nodule though the infection thread and be successfully endocytosed into the host cell. However, unlike the Rm8002 parent which successfully differentiated into a nitrogen fixing bacteroid (Fig. 1-4A), the *bacA* mutant was unable to successfully differentiate and was found to undergo senescence (Fig. 1-4B) (Glazebrook *et al.*, 1993). Thus, the mutated locus strain was named *bacA* (bacteroid development factor A).

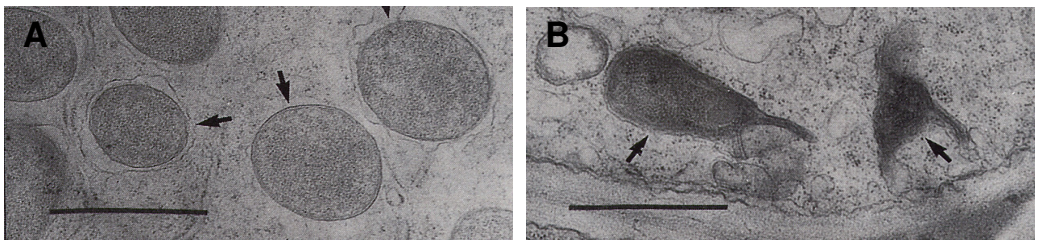

Figure 1-4. Transmission electron micrographs of nodule sections induced by the *S. meliloti* parent strain and the *bacA* mutant. (A) In nodules induced by the *S. meliloti* Rm8002 parent strain, healthy regular shaped bacteroids are visible, as highlighted by the arrows. (B) Contrastingly, in nodules induced by the *S. meliloti bacA* mutant Rm8386 the bacteroids highlighted by the arrows appear very intensely stained and irregularly shaped indicating they are senescent (Glazebrook *et al.*, 1993). Bars, 1 µm.

Subsequently, an isogenic *B. abortus bacA* mutant was constructed (LeVier *et al.*, 2000). The mutant, which was designated KL7, initially colonized the livers and spleens of experimentally infected BALB/c mice up to 2 weeks post infection (LeVier *et al.*, 2000). However, after this time the KL7 mutant was rapidly cleared from mice, while the *B. abortus* parent strain could successfully maintain chronic infection in these organs (LeVier *et al.*, 2000). Additionally the *B. abortus* KL7 mutant appeared much less resistant to cultured murine macrophages, relative to the parental strain (LeVier *et al.*, 2000). Thus, these studies demonstrated that although both the *S. meliloti* and *B. abortus bacA* mutants can establish an intracellular infection within their hosts, neither can maintain a long term residence in their intracellular niche. Interestingly a recent study found that disruption of the *Rv1819* gene in the *M. tuberculosis,* which encodes a BacA related protein (see section 1.8.2) resulted in the bacteria being unable to maintain chronic infection in mice (Domenech *et al.*, 2008).

1.8.2. *bacA* encodes an inner membrane protein

The *S. meliloti bacA* gene encodes a 420 amino acid inner membrane protein, which is predicted to have seven membrane spanning domains (Glazebrook *et al.*, 1993) (Fig. 1-5) and shows 82% similarity and 67% identity to the *B. abortus* BacA protein (LeVier *et al.*, 2000). The BacA homolog in *E. coli* SbmA is 79% similar and 64% identical to *S. meliloti* BacA (Ichige & Walker, 1997). It was suggested some years ago by Southern blot analysis, that the genomes of several bacteria encode proteins that appear to be related to the *S. meliloti bacA* gene (Glazebrook *et al.*, 1993). In subsequent years, BLAST searches of sequence databases have revealed there is a second class of proteins related to BacA/SbmA, referred to as BacA related proteins (LeVier & Walker, 2001). The BacA related proteins are more diverged from *S. meliloti* BacA (38-59% similarity) but do show large blocks of similar residues. These proteins are also around 200 amino acids longer than BacA/SbmA, with highly conserved motifs common to bacterial ATP binding cassette (ABC transport) proteins (Fath & Kolter, 1993). The BacA/SbmA gene products themselves are classified as one of the subunits of a putative transport protein belonging to the ABC superfamily (Mattiuzzo *et al.*, 2007). ABC-transport systems are widely distributed

in all living organisms and mediate the uptake or export of a wide variety of substances across cell membranes (Biemans-Oldehinkel *et al.*, 2006; Davidson & Chen, 2004; Locher, 2004).

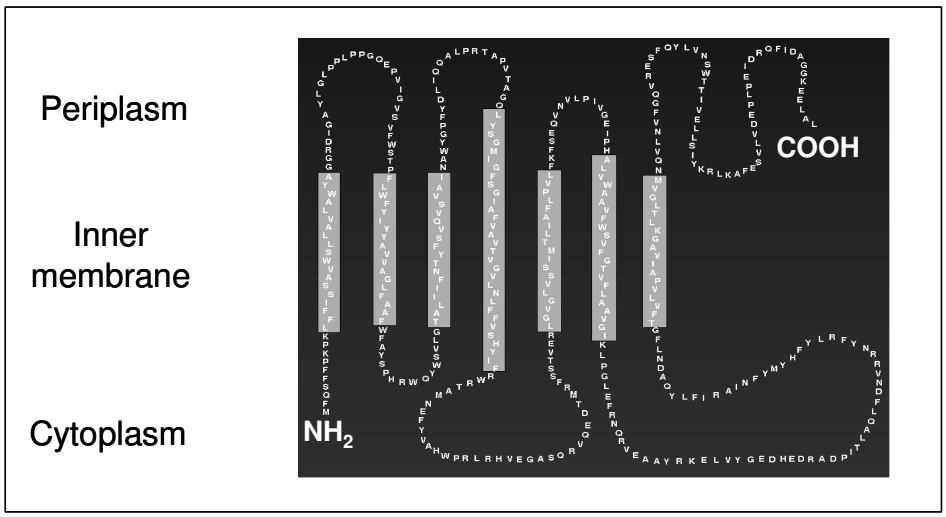

Figure 1-5. The proposed topology of the *S. meliloti* BacA inner membrane protein (Glazebrook *et al.*, 1993).

1.8.3. BacA is proposed to be involved in peptide uptake

E. coli sbmA was first identified as a gene that mediated sensitivity of bacteria to the microcins B17 and J25 and the glycopeptide antibiotic bleomycin (Lavina *et al.*, 1986; Salomon & Farias, 1995; Yorgey *et al.*, 1994). These observations led to the proposal that the SbmA protein could be a peptide transporter. It was subsequently found that the *S. meliloti* and *B. abortus bacA* mutants displayed an increased resistance to the glycopeptide bleomycin (Ferguson *et al.*, 2002; Ichige & Walker, 1997; LeVier *et al.*, 2000). Additionally, the finding that *E. coli sbmA* deficient mutants could be complemented by the *S. meliloti bacA* gene (refer to section 3.1 for more details) suggested a functional similarity between the two proteins (Ichige & Walker, 1997). Hence, it was proposed that like SbmA, BacA could be involved in the transport of peptides, such as bleomycin, into the cell. Interestingly, a more recent study found that the *E. coli* SbmA protein is involved in the uptake of Bac7, which is a proline-rich antimicrobial peptide (AMP) of mammalian origin (Mattiuzzo *et al.*, 2007). Two gene families (glycine rich proteins and cysteine rich peptides), which encode secreted AMPs, have been identified to have nodule-specific

expression in *M. truncatula,* a legume host of *S. meliloti* (Alunni *et al.*, 2007; Kevei *et al.*, 2002; Mergaert *et al.*, 2003; Mergaert *et al.*, 2006). It has been suggested previously that these peptides could play a role in initiating bacteroid development (Mergaert *et al.*, 2003; Mergaert *et al.*, 2006). Hence, it may be possible that BacA could be involved in the uptake of a host derived peptide essential for bacteroid differentiation (Fig. 1-6).

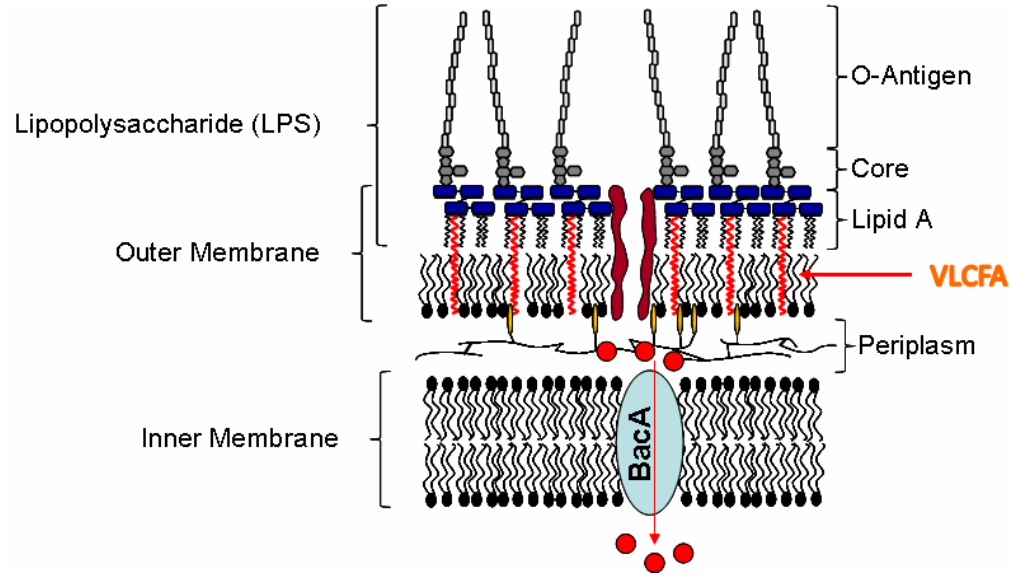

Figure 1-6. Proposed model for BacA function. Once peptides have reached the periplasmic space they could be directly transported across the cytoplasmic membrane by the BacA protein. A schematic of the *S. meliloti* cell envelope is shown.

1.8.4. BacA also affects the lipid A VLCFA modification

Free-living *S. meliloti bacA* mutants were also shown to display an increased sensitivity to detergents and cell envelope-disrupting agents (Ferguson *et al.*, 2002). This finding supports a model whereby the function of BacA could affect the integrity of the bacterial cell envelope. Consistent with this model, the BacA protein was found to be distantly related to a family of peroxisomal-membrane proteins, including the human adrenoleukodystrophy protein (hALDP) (Ferguson *et al.*, 2004). Several members of this family are proposed to be involved in the transport of either very-long-chain fatty acids (VLCFAs) or long chain fatty acids out of the cytoplasm into the peroxisome, where they can then be degraded (Braiterman *et al.*, 1999;

Footitt *et al.*, 2002; Verleur *et al.*, 1997; Zolman *et al.*, 2001). One member of this family, hALDP is affected in patients with X-linked adrenoleukodystrophy. This neurological disorder is caused by a mutation in the *ABCD1* gene (which encodes hALDP), which results in defects in peroxisomal β-oxidation (Braiterman *et al.*, 1999) and the accumulation of VLCFAs in all tissues of the body (Valianpour *et al.*, 2003). Interestingly, it was also determined that four site directed mutations of the BacA protein that prevent *S. meliloti* from forming a successful symbiosis with alfalfa are also conserved amino acids in hALDP (Ferguson *et al.*, 2004). This finding combined with the free-living phenotypes of the *S. meliloti* and *B. abortus bacA* mutants (Ferguson *et al.*, 2002; Roop *et al.*, 2002) led to the discovery that the BacA protein affects the VLCFA lipid A content in both *S. meliloti* and *B. abortus* (Ferguson *et al*., 2004).

The lipid A is a component of the lipopolysaccharide (LPS), which forms the outermost leaflet of the outer membrane in gram negative bacteria (Fig. 1-6). The LPS is composed of the O-chain polysaccharide, the core oligosaccharide and the lipid A which is the moiety that anchors the molecule into the outer leaflet of the outer membrane (Fig. 1-6). The lipid A of the LPS of both *S. meliloti* and *B. abortus* parent strains in the free living state is modified with a VLCFA of either 27-OHC28:0, 27-O (βOmeC4:O) C28:0 or 29-OHC30:0 (Bhat *et al.*, 1991; Ferguson *et al.*, 2004) (Fig. 1-7). Contrastingly, the *S. meliloti* and *B. abortus bacA* mutants produce a mixture of lipid A molecules with and without the VLCFA modification (Ferguson *et al.*, 2004). Therefore, in the absence of the BacA protein *S. meliloti* and *B. abortus* can only transfer the VLCFA onto a portion of the lipid A molecules. This led to the proposal of a model whereby the BacA protein transports VLCFAs out of the cytoplasm into the periplasm, where they then can be used to modify the lipid A in the outer membrane (Ferguson *et al.*, 2004) (Fig.1-8).

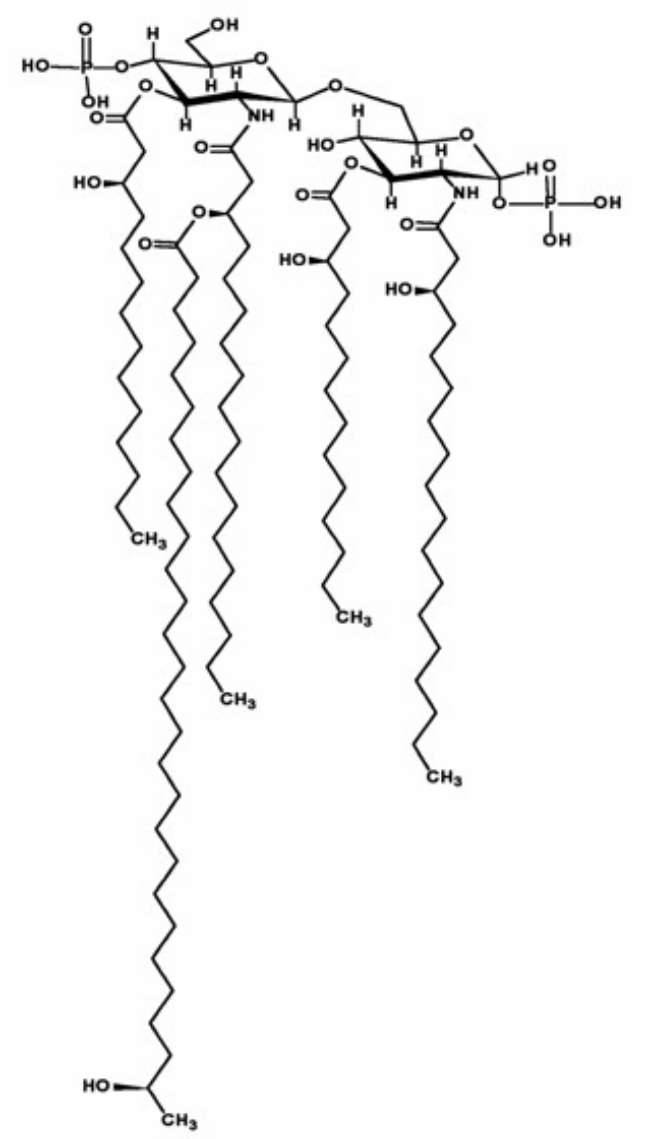

Figure 1-7. Major lipid A species in free-living *S. meliloti.* The lipid A molecule shown is modified by 27-OHC28:0, which can be replaced by 27-O (βOMeC4:0) C28:0 (forming another major species) or 29-OHC30:0 (forming a minor species) (Ferguson *et al.*, 2004).

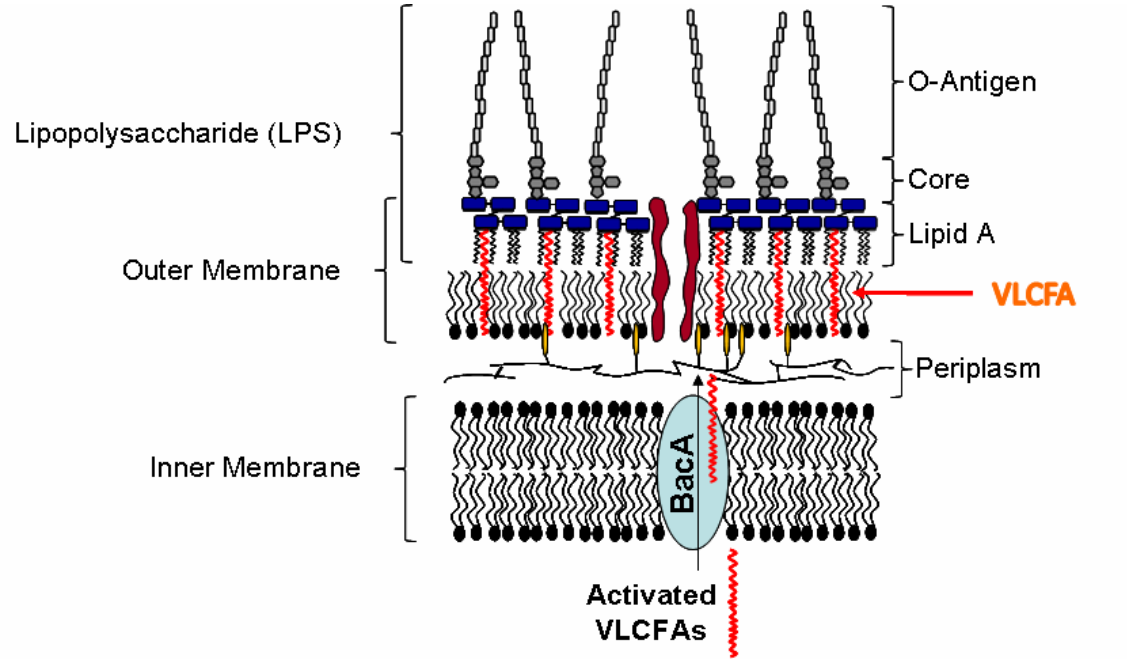

Figure 1-8. Second proposed model for BacA function. The BacA protein could transport VLCFAs out of the cytoplasm into the periplasm, where they then can be used to modify the lipid A in the outer membrane.

1.9. Biosynthesis of VLCFA lipid A modifications

To determine if the reduction in the VLCFA modification was responsible for the host persistence defect in the *S. meliloti bacA* mutant and to gain a better understanding of the role of VLCFA modifications in the symbiosis, the biosynthesis of the VLCFA-modified LPS was investigated. Mutants were constructed with disruptions in the *acpXL* and *lpxXL* genes (Ferguson *et al.*, 2005) which encode proteins directly involved in the biosynthesis of VLCFA-modified LPS. The VLCFAs are synthesized while attached to a cytoplasmic C28-acyl carrier protein (AcpXL) (Brozek *et al.*, 1996) and transferred onto a 3-deoxy-D-manno-octulosonic acid $(Kdo)_2$-lipid IV_A by an inner membrane-associated C28-acyl transferase (LpxXL) protein (Basu *et al.*, 2002). Although the LPS of the *acpXL* and *lpxXL* free-

living mutants completely lacks the VLCFA modification in complex media, it was determined the mutants were able to form a chronic infection with alfalfa (Ferguson *et al.*, 2005). This suggested that unlike the BacA protein, these proteins were not essential for the alfalfa symbiosis.

1.10. The *S. meliloti acpXL* and *lpxXL* mutants show defects in the alfalfa symbiosis

Although the AcpXL and LpxXL proteins were not essential for the alfalfa symbiosis (Ferguson *et al.*, 2005), *S. meliloti* lacking these proteins were found to be substantially less competitive in alfalfa co-inoculation experiments with the parent strain. Additionally, the *S. meliloti acpXL* mutant showed a delay in nodulation, relative to the parent strain (Ferguson *et al.*, 2005; Sharypova *et al.*, 2003). Work performed on the related bacterium *Rhizobium leguminosarum,* found that cells lacking the AcpXL protein were delayed in the onset of nitrogen fixation, relative to the parent strain (Vedam *et al.*, 2004). Additionally, transmission electron microscopy (TEM) of *R. leguminosarum acpXL* mutant infected pea nodules showed the bacteroids had major defects, compared to the parent strain, whereby the bacteroids were oddly shaped and multiple bacteroids could be found within a single symbiosome (Vedam *et al.*, 2004). Therefore, despite not being essential for the symbiosis, these data would suggest the VLCFA modification could still play a key role. Furthermore VLCFAs are produced by a number of other bacterial species, which also form persistent bacterial-host interactions. Hence, it has previously been hypothesized that VLCFAs may play an important role in host persistence (Bhat *et al.*, 1991; Vedam *et al.*, 2003).

1.11. Could additional host induced VLCFA modifications be occurring in *S. meliloti*?

However, there still remains the unresolved question as to why the *bacA* mutant, which only has a partial reduction in the VLCFA lipid A content, is defective in the symbiosis, whereas the *acpXL* and *lpxXL* mutants which completely lack the modification are able to persist within the plant. One possibility is that *S. meliloti*

LPS is further modified with VLCFAs during the legume symbiosis and that the BacA protein, not AcpXL and LpxXL, could be essential for these host-induced LPS changes (Ferguson *et al.*, 2005). Evidence for this stems from work performed in *R. leguminosarum*, where it was found that despite lacking the VLCFA modification in the free-living state, the lipid A of the *acpXL* mutant was partially modified with a VLCFA when extracted from the pea plant (Vedam *et al.*, 2006). Furthermore, experimental evidence has also shown that the LPS hydrophobity increases in *S. meliloti* during the alfalfa symbiosis, consistent with a increase in VLCFAs (Ferguson *et al.*, 2005). Additionally, the genome of *S. meliloti* encodes multiple acyl carrier and transferase genes whose products could potentially be involved in host induced LPS changes (Geiger & Lopez-Lara, 2002). Intriguingly the *S. meliloti smb20651* gene encodes a potential acyl carrier protein which is located in an operon with putative long-chain fatty acid CoA ligase (Geiger & Lopez-Lara, 2002) (for more details refer to section 6.2.1.5). Thus, Smb20651 could be a potential candidate for host induced lipid A changes in *S. meliloti*. In addition to the BacA protein playing some role in host induced lipid A changes, it is possible that one or more of these additional proteins could partially compensate for the loss of AcpXL and LpxXL *in planta*.

1.12. LPS transport

However, if the proposed model that BacA plays some role in the transport of activated VLCFAs out of the cytoplasm onto the lipid A in the outer membrane (Ferguson *et al.*, 2004) is correct, then the LPS would need to be transported across the inner membrane before the lipid A could be modified with a VLCFA. However, the process by which LPS and other lipid containing macromolecules are transported from their site of synthesis on the inner face of the inner membrane to the outer membrane is still a poorly understood process (Doerrler, 2006; Ruiz *et al.*, 2006).

1.12.1. The MsbA protein is involved in LPS transport in *E. coli*

In *E. coli*, the transport of newly synthesized rough LPS (containing lipid A and the core oligosaccharide) from the inner to the outer membrane is dependent upon the

inner membrane ABC transporter MsbA protein by a proposed flip-flop mechanism (Doerrler *et al.*, 2001; Zhou *et al.*, 1998) (Fig.1-9). The *msbA* gene was first identified in *E. coli* as a multicopy suppressor of a mutation in the *htrB* (*lpxXL)* gene, which encodes an enzyme involved in a late step of the biosynthesis of the lipid A (Clementz *et al.*, 1996; Karow & Georgopoulos, 1993). Although this protein is essential for *E. coli* growth (Doerrler *et al.*, 2001), a temperature-sensitive *msbA* mutant has been shown to accumulate LPS in the inner membrane at a non-permissive temperature (Doerrler *et al.*, 2001; Doerrler *et al.*, 2004). The *E. coli* MsbA protein is also proposed to be involved in the transport of phospholipids across the inner membrane (Doerrler *et al.*, 2001). Additionally in the bacterium *Neisseria meningitidis* deletion of the sole *msbA* gene prevents LPS transport, although in this case phospholipid transport was not found to be affected. Therefore, there is still some debate regarding the role of MsbA proteins in phospholipid transport (Tefsen *et al.*, 2005). A protein implicated in the LPS targeting to the outer membrane is the IMP (increased membrane permeability) protein, a β-barrel outer membrane protein, the IMP protein has been shown to be essential in *E. coli* and its depletion results in abnormalities in outer membrane assembly (Braun & Silhavy, 2002). Additionally, it has been proposed that two *E. coli* essential genes *lptA* and *lptB* participate in LPS biogenesis. These two genes have been proposed to play a role in transport of LPS from the outer face of the inner membrane to the outer membrane (Sperandeo *et al.*, 2007).

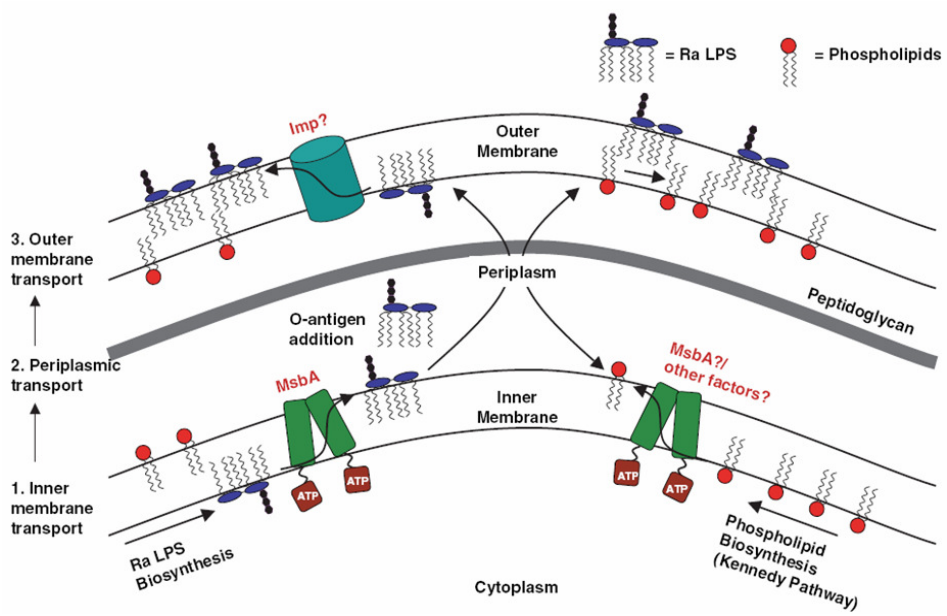

Figure 1-9. Schematic showing steps of lipid movement through the envelope of *E. coli*. Phospholipids and rough LPS (Ra LPS) are synthesized in the cytoplasm and at the inner leaflet of the inner membrane. These molecules are then transported across the inner membrane. The MsbA protein plays a major role in this process. LPS is transported across the inner membrane by an ATP dependent transport mechanism involving MsbA. The O-antigen is added to Ra LPS on the periplasmic face of the inner membrane. The transport mechanism across the periplasm is still unclear. Once LPS reaches the outer membrane it is thought to be transported to the extracellular side of the outer membrane by an ATP independent transport system, presumably involving the IMP protein (Doerrler, 2006).

1.12.2. The *S. meliloti* genome encodes multiple MsbA like proteins

Interestingly, the *S. meliloti* Rm1021 genome (Galibert *et al.*, 2001) encodes multiple proteins which share between 26-34% identity (47-58% similarity) and 23-32% identity (44-54% similarity) over their entire length with the *E. coli* and *N. meningitidis* MsbA proteins respectively (Beck *et al.*, 2008). It has previously been shown that the *S. meliloti* MsbA like protein, ExsA affects the molecular weight distribution of the exopolysaccharide (EPS) succinoglycan and so has been proposed to be a transporter of succinoglycan across the inner membrane (Becker *et al.*, 1995). Although the *S. meliloti exsA* mutant can form a symbiosis with legumes, in *B. abortus* loss of the *exsA* gene resulted in decreased survival of the bacteria in BALB/c mice relative to the parent strain (Rosinha *et al.*, 2002) demonstrating that

the ExsA protein is critical for full bacterial virulence. Additionally, the *exsA* mutant strain induced improved protective immunity in *B. abortus* infected mice compared to the protective immunity induced by the current commercially available S19 vaccine strain (Rosinha *et al.*, 2002). Furthermore, the *S. meliloti* and *B. abortus* MsbA like potein, NdvA (known as Cgt in *B. abortus*) is involved in the transport of cyclic β−(1,2) glucan and is essential for the host interaction (Dickstein *et al.*, 1988; Roset *et al.*, 2004; Stanfield *et al.*, 1988). Together these findings raise the possibility that other *S. meliloti* MsbA-like proteins could also be playing a role in the transport of polysaccharide or lipid-containing polysaccharide such as LPS and these processes could play an important role in the host interaction. Additionally by investigating lipid trafficking in *S. meliloti* and by characterizing the role of the *S. meliloti* MsbA like proteins more could potentially be learnt regarding the possible role of the BacA protein in the VLCFA modification of the lipid A and where in the cell envelope this process may occur.

1.13. Loss of the BacA protein in *S. meliloti* may result in other lipid A independent changes

In addition to the proposed roles for the BacA protein in peptide uptake and VLCFA transport (Figs 1-6 & 1-8), there also remains the possibility that loss of BacA could result in other alterations to the cell. For example the free-living *S. meliloti bacA* mutant has an increased sensitivity to the hydrophobic dye Crystal Violet (Ferguson *et al.*, 2002). To date this phenotype has not been shown to be due to the VLCFA alteration and could be consistent with other alterations occurring in the *S. meliloti* cell envelope. It has been shown that the BacA homologue SbmA in *Salmonella enterica* serovar Typhimurium is encoded in an operon with *yaiW,* which is thought to encode a lipoprotein (K.Tan and G.P. Ferguson, unpublished data). Although *S*. Typhimurium SbmA is not essential for chronic persistence, YaiW is essential (S. Eriksson and G.P. Ferguson, unpublished data). While BacA is not part of an operon (refer to section 7.1 for more details) and no YaiW homologue has been found in *S. meliloti*, there are multiple lipoproteins encoded in the genome of *S. meliloti* (Galibert *et al.*, 2001). This raises the possibility that in addition to affecting the VLCFA modification, BacA could also effect the lipid modification of another cell

envelope component. Interestingly it has also been shown in *E. coli* that a functional SbmA protein is required for full efficiency of the tetracycline exporter TetA (de Cristobal *et al.*, 2008). This finding too is consistent with the idea that BacA function may similarly affect the activity of one or more membrane proteins.

1.14. Aims of PhD project

The overall aim of this PhD was to use the *S. meliloti* legume symbiosis as a model system to learn more about bacterial factors that are important for chronic infection.

The specific aims of this project were:

1. To investigate if the *S. meliloti* BacA protein is involved in peptide uptake and determine if this may be linked to the essential role of BacA in chronic infection

2. To investigate the role and the biosynthesis of LPS in free-living and symbiotic *S. meliloti* to determine the importance of the VLCFA modification in chronic infection

3. To determine if there are any other alterations occurring in *S. meliloti* upon loss of the BacA protein, which could also be linked to the host persistence defect

Chapter 2: Materials and Methods

2.1. Bacterial strains and plasmids

All *S. meliloti* and *E. coli* bacterial strains used in this work are described in tables 2-1 and 2-2, respectively. Plasmids used in this work are described in table 2-3.

2.2. Growth conditions and media

Luria-Bertani (LB) (Sambrook *et al.*, 1989) broth was prepared by dissolving 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl, in a final volume of 1 litre of distilled water. The pH of the solution was then adjusted to 7.5, followed by autoclaving. Muller Hinton broth (Mueller & Hinton, 1941) containing 2.0 g of meat infusion, 17.5 g of casein hydrolystate and 1.5 g of starch per litre was purchased from Merck and prepared according to the manufacturer's instructions. To prepare LB and Muller Hinton agar, 15 g of Bacto agar was added per litre of broth, prior to autoclaving. For experiments, unless otherwise stated *S. meliloti* strains were grown from -80°C frozen stocks and incubated at 30°C with aeration at 200 rpm, in LB broth supplemented with 2.5mM $CaCl_2$ and 2.5mM $MgSO_4$ (LBMC) for 48 hours. *E. coli* strains were grown from - 80ºC frozen stocks and incubated in either LB or Mueller Hinton broth at 37°C with aeration at 200 rpm for 16 hours. When required antibiotics were used at the defined concentrations (table 2-4), unless stated otherwise. Prior to use, antibiotics were filter sterilized and stored at -20ºC. Both *S. meliloti* and *E. coli* strains were stored long-term at -80ºC with 10% v/v DMSO.

Table 2-1. *S. meliloti* strains used in this study

***S. meliloti* strains**	**Relevant characteristics**	**Source and reference**
Rm1021	Sm^r derivative of SU47	(Meade *et al.*, 1982)
Rm8002	Wild type; Rm1021 (SU47 Sm^r) *pho*	(Long *et al.*, 1988)
Rm8654	Rm8002 *bacA654*::Spc^r	(Ichige & Walker, 1997)
SmGF1	Rm1021, *bacA654*::Spc^r	(Ferguson *et al.*, 2002)
SmGF4	Rm1021, *acpXL*::pK18mobGII Nm^r	(Ferguson *et al.*, 2005)
SmGF5	Rm1021, *bacA654*::Spc^r, *acpXL*::pK18mobGII Nm^r	(Ferguson *et al.*, 2005)
SmGF6	Rm1021, *lpxXL*::pHJ104 Nm^r Hm^r	(Ferguson *et al.*, 2005)
SmGF7	Rm1021, *bacA654*::Spc^r, *lpxXL*:: pHJ104 Nm^r Hm^r	(Ferguson *et al.*, 2005)
SmGF8	Rm1021, *acpXL*::pK18mobGII, *lpxXL*:: pHJ104 Nm^r Hm^r	(Ferguson *et al.*, 2005)
SmGW1	Rm1021, *recA*::Tn*5*::*233* Gm^r Spc^r	G. Walker
SmVM1	Rm1021, *bacA654*::Spc^r, *recA*::Tn*5*::*233* Gm^r Spc^r	This study
ALR30B1	Rm1021, *smb20651*::Spc^r	(Ramos-Vega *et al.*, 2009)
SmVM2	Rm1021, *smb20651*::Spc^r, *acpXL*::pK18mobGII Nm^r	This study
SmgshA	Rm1021, *gshA*::pJHgshA Nm^r	(Harrison *et al.*, 2005)
SmgshB	Rm1021, *gshB*::pCMgshB Tc^r	(Harrison *et al.*, 2005)
SmgshB2	Rm1021, *smb21586*::pK19mob2ΩHMB Nm^r	A. Becker
SmVM3	Rm1021, *bacA654*::Spc^r, *gshA*::pJHgshA Nm^r	This Study
SmVM4	Rm1021, *gshB*::pCMgshB Tc^r, *bacA654*::Spc	This study
SmVM5*	Rm1021, *gshA*::pK19mob2ΩHMB Nm^r	This Study
SmVM7	Rm1021, *gshB*::pCMgshB Tc^r *smb21586*::pK19mob2ΩHMB Nm^r	This study
SmVM8*	Rm1021, *smb21275*::pK19mob2ΩHMB Nm^r	This Study
SmVM9*	Rm1021, *smb21273*::pK19mob2ΩHMB Nm^r	This Study
SmVM10	Rm1021, *Smc04266*:: pHJ104 Nm^r Hm^r	This study
SmVM11	Rm1021, *Smc04266*:: pHJ104 Nm^r Hm^r, *bacA654*::Spc	This study
SmSB1	Rm1021, *msbA2*::pHJ104 Nm^r Hm^r	(Beck *et al.*, 2008)
SmSB1	Rm1021, *msbA1*::pHJ104 Nm^r Hm^r	S. Beck
SmVM12	Rm1021, *msbA2*::pHJ104 Nm^r Hm^r, *bacA654*::Spc	This study
SmVM13	Rm1021, *msbA1*::pHJ104 Nm^r Hm^r, *bacA654*::Spc	This study

^{r} denotes antibiotic resistance

* Insertional mutation not yet confirmed by PCR

Table 2-2. *E. coli* strains used in this study

E. coli strains	Relevant characteristics	Source or reference
DH5α	*supE44*Δ*lac*U169 (Φ80*lacZ*ΔM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Bethesda Research Laboratory
MT616	MM294A *recA*56 (pRK600) Cm^r	(Finan *et al.*, 1986)
MM294A	*pro*-82 *thi*-1 *endA*1 *hsdR*17 *supE*44	(Finan *et al.*, 1986)
One Shot® TOP10 competent cells	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Sm^r) *end*A1 *nup*G	Invitrogen
NEB 5-alpha competent cells	*huA2* Δ(*argF-lacZ)U169 phoA glnV44* Φ*80*Δ (*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
S17-1	*thi pro hsdR*$^+$ *recA*/RP4-2 Tc::Mu, Km::Tn*7* (RP4 to 2 =RP4ΔTn*1*)	(Simon *et al.*, 1983)
MJF274	F2$^-$ Δ*kdpABC5 thi rha lacI lacZ trkD1*	(Elmore *et al.*, 1990)
MJF335	MJF276 *gshA*::Tn*10* Kan^r	(Miller *et al.*, 1997)
RYC1000	MC4100 Δ*rbs-7 recA56 gyrA*	(Genilloud *et al.*, 1984)
RYC1001	RYC1000 *sbmA* (spontaneous)	F. Moreno

^{r} denotes antibiotic resistance

Table 2-3. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pRK404	Broad host range control plasmid Tc^r	(Ditta *et al.*, 1985)
pJG51A	pRK404 carrying the Sm*bacA* gene	(Glazebrook *et al.*, 1993)
pAI351	pRK404 carrying the Ec*SbmA* gene under control of the *S. meliloti bacA* promoter	(Ichige & Walker, 1997)
K8G	pRK404 with *bacA* K8G	(LeVier & Walker, 2001)
W57G	pRK404 with *bacA* W57G	(LeVier & Walker, 2001)
S83G	pRK404 with *bacA* S83G	(LeVier & Walker, 2001)
W87G	pRK404 with *bacA* W87G	(LeVier & Walker, 2001)
Y120G	pRK404 with *bacA* Y120G	(LeVier & Walker, 2001)
N159G	pRK404 with *bacA* N159G	(LeVier & Walker, 2001)
H165G	pRK404 with *bacA* H165G	(LeVier & Walker, 2001)
W182G	pRK404 with *bacA* W182G	(LeVier & Walker, 2001)
Q193G	pRK404 with *bacA* Q193G	(LeVier & Walker, 2001)
R194G	pRK404 with *bacA* R194G	(LeVier & Walker, 2001)
D198G	pRK404 with *bacA* D198G	(LeVier & Walker, 2001)
F223G	pRK404 with *bacA* F223G	(LeVier & Walker, 2001)
S231G	pRK404 with *bacA* S231G	(LeVier & Walker, 2001)
T259G	pRK404 with *bacA* T259G	(LeVier & Walker, 2001)
R284G	pRK404 with *bacA* R284G	(LeVier & Walker, 2001)
Q332G	pRK404 with *bacA* Q332G	(LeVier & Walker, 2001)
K350G	pRK404 with *bacA* K350G	(LeVier & Walker, 2001)
F363G	pRK404 with *bacA* F363G	(LeVier & Walker, 2001)
pMM100	Derivative of pACYC184 that expresses the LacI repressor Tc^r	(Lavina *et al.*, 1986)
pWSK29	pSC101/*bla*/- , low copy number vector Amp^r	(Wang & Kushner, 1991)
pWSK29-MtbacA	pWSK29, carrying the *M. tuberculosis bacA* gene under the control of the *lac* promoter	(Domenech *et al.*, 2008)
pJN105	*ara*C-P_{BAD} casette cloned in pBBR1MCS5, Gm^r	(Newman & Fuqua, 1999)
p*msbA*2G97A	pJN105 carrying the entire *msbA2* gene containing a G97A mutation and 90 bps upstream Gm^r	(Beck *et al.*, 2008)
pRK600	pRK2013 *npt* ::Tn*9* Cm^r	(Finan *et al.*, 1986)
pRF771	pTE3 with an alternative polylinker Tc^r	(Wells & Long, 2002)
pJG176	pRF771, P*trp*::*msbA2*	(Griffitts *et al.*, 2008)
pJH104	*S. meliloti* suicide plasmid Nm^r Hm^r	(Davies & Walker, 2007)
pCR 2.1-TOPO	TA PCR cloning vector containing *lacZ*, Amp^r kan^r/Nm^r	Invitrogen
pTOPO-*smc04266*in	pCR 2.1-TOPO carrying a 267 bp *smc04266* internal fragment	This study
pJH104-*smc04266*in	pJH104 carrying a 267 bp *smc04266* internal fragment	This study
pBBR1MCS-5	Derivate of pBBR1-MCS Gm^r	(Kovach *et al.*, 1994)
pgshAc	pBBR1MCS-5 with a 1,500 bp PCR fragment of *gshA*	(Harrison *et al.*, 2005)
pK19mob2ΩHMB -*smb21275*in	pK19mob2ΩHMB carrying 309bp *smb21275* internal fragment Nm^r	A. Becker

pK19mob2ΩHMB -*smb21273*in	pK19mob2ΩHMB carrying 334 bp *smb21273* internal fragment Nm^r	A. Becker
pK19mob2ΩHMB -*gshA*in	pK19mob2ΩHMB carrying 330 bp *gshA* internal fragment Nm^r	A. Becker

[r] denotes antibiotic resistance

Table 2-4. Antibiotics used in this study.

Antibiotic	Final concentration (µg.ml^{-1})		Solvent
	E. coli	*S. meliloti*	
Ampicillin	50	-	Water
Chloramphenicol	12.5	20	100% Ethanol
Gentamycin[a,b]	5	50	Water
Hygromycin	-	100 (in LB) 150 (in LBMC)	Water
Kanamycin	50	-	Water
Neomycin[c]	-	200	Water
Spectinomycin[a]	20	100	Water
Streptomycin	100	500	Water
Tetracycline	10	10	50% Ethanol

[a] When used in combination for *S. meliloti*, gentamycin was used at a final concentration of 20 µg.ml^{-1} and spectinomycin was used at a final concentration of 50 µg.ml^{-1}

[b] When used for the *S. meliloti gshA* & pgshAc and *S. meliloti gshA* & pBBR1MCS-5 mutants a concentration of 10 µg.ml^{-1} was used.

[c] When used for the *S. meliloti gshA* & pgshAc and *S. meliloti gshA* & pBBR1MCS-5 mutants a concentration of 100 µg.ml^{-1} was used.

2.3. Molecular techniques

2.3.1. Plasmid Purification

Early stationary phase *E. coli* cultures (OD_{600} ~2.5) containing the desired plasmid were harvested by centrifugation at 2264 x g for 10 minutes. The plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen) and a microcentrifuge according to the manufacturer's instructions, with the exception that in the final step the DNA was eluted into 30 µl of molecular grade water (Sigma). The plasmid DNA was then stored at -20ºC.

2.3.2. Agarose gel electrophoresis

Purified DNA was analysed on 1.0% (w/v) agarose (Seakem LE agarose, Cambrex) gels in 1 x TAE buffer (50 x TAE buffer: 242 g Tris, 100 ml of 0.5 M Na_2 EDTA [pH 8.0] and 57.1 ml glacial acetic acid, adjusted to 1 litre with distilled water). Five parts DNA were typically added to one part 6 x loading buffer (30% v/v glycerol, 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol), prior to loading the gel. A 1 kb or 100 bp DNA ladder (both, New England Biolabs) were used according to the manufacturers instructions to size the DNA products. Each gel was run at 80V, until adequate migration had occurred, as determined by the position of the leading dye front. The gel was then stained with ethidium bromide (0.5 $\mu g.ml^{-1}$) for 30 minutes, the DNA fragments were visualized using a UV light source and then photographed. When required DNA was extracted from gels and purified using the QIAquick Gel Extraction Kit (Qiagen) and a microcentrifuge according to the manufacturer's instructions.

2.3.3. Restriction digests

Restriction digests were performed to digest the pHJ104 vector prior to ligation with the *smc04266* internal gene fragment and additionally to check the pCR-2.1 TOPO and pJH104 vectors for presence of the *smc04266* insert. The *XhoI* and *AvrII* restriction enzymes were used (New England Biolabs). Reactions were prepared according to the manufacturer's instructions using 1 µl of each restriction enzyme, 2 µl of 10 x NEBuffer 2, 100 $\mu g.ml^{-1}$ of BSA, the appropriate amount of DNA and the reaction was made up to a final volume of 20 µl with molecular grade water (Sigma). The reaction was incubated at 37ºC for 2 hours, after which the products were run on an agarose gel and if necessary the products were immediately gel purified to eliminate the enzymes.

2.3.4. Polymerase chain reaction (PCR)

Primers used in this study are detailed in section 2.3.6. Primers were either designed by hand or by using MacVector software, version 9.5 and were purchased from MWG-Biotech AG. To amplify DNA, Taq polymerase (New England Biolabs) was used according to the manufacture's protocol. A typical PCR reaction was set up as follows:

2.5 µl of forward primer (10 pmol/µl)
2.5 µl of reverse primer (10 pmol/µl)
2 µl of 10 x ThermoPol buffer
1 µl of dNTPs (10 mM each, Roche)
1 µl of template (bacterial colony diluted in 20 µl of molecular water)
1 µl of *Taq* DNA polymerase (5 units)
10 µl of molecular water (Sigma)

The PCR reactions were run on an Eppendorf mastercycler gradient PCR machine and PCR conditions were as follows:

1. 94 ºC for 4 minutes (initial denaturation)
2. 94 ºC for 1 minute (denaturation)
3. 54 ºC for 50 seconds (annealing)
4. 72 ºC for 1 minute (extension)

Steps 2-4, 30 cycles

5. 72 ºC for 10 minutes (final extension)

Samples were then held at 4 ºC, until required.

The samples were then run on an agarose gel to determine that the PCR product was of the correct size. When required DNA was extracted from gels and purified (section 2.3.2)

2.3.5. Transformation of DNA into bacterial cells

Plasmid DNA was transformed into Top 10 (Invitrogen) and NEB-5-alpha cells (New England Biolabs) according to the enclosed protocols. DNA was incubated with the cells on ice for 30 minutes and then heat shocked at 42ºC for 30 seconds and

placed on ice for 5 minutes. The mixture was then incubated with SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) at 37ºC for 1 hour with shaking, before being plated onto LB plates with the appropriate antibiotics and X-gal (40 $\mu g.ml^{-1}$) if necessary.

2.3.6. Construction of the *S. meliloti smc04266* insertional mutant

2.3.6.1 Gene amplification

A 267 bp *smc04266* internal fragment was amplified by PCR (section 2.3.4) using the primers Smc04266-8F-*Avr II* (5'-TAACCTAGGGCAAAGGTAACGAAAAC G-3') and Smc04266-267R-*XhoI* (5'-GGCCTCGAGGGAAGGTGAAGATAGGC TTG-3'). The *Taq* DNA polymerase enzyme (New England biolabs) was used to ensure 3' adenine overhangs for subsequent cloning into the TOPO vector. The PCR product was confirmed as being of the correct size by gel electrophoresis and was gel purified (section 2.3.2).

2.3.6.2. TOPO TA cloning

The purified PCR fragment was ligated into the pCR-2.1 TOPO vector provided with the TOPO TA cloning kit (Invitrogen), according to the manufacturer's instructions. The ligation mixtures were transformed into one shot Top 10 cells, provided with the kit (section 2.3.5) and plated onto LB plates containing kanamycin and X-gal (40 µl of a 40 $mg.ml^{-1}$stock per plate). White colonies were picked and grown in LB and kanamycin and the pCR-2.1 TOPO vector was extracted from the clones using the QIAprep Spin Miniprep Kit (Qiagen). The purified vector was then digested with *AvrII* and *XhoI* (section 2.3.3), to identify positive clones. Subsequently the *smc04266* PCR fragment was released from the pCR-2.1 TOPO vector by digestion and gel purified. Use of the TOPO TA cloning kit ensured that the PCR fragment was completely digested, prior to its ligation into the pJH104 vector.

2.3.6.3. Ligation, screening and conjugation

The pJH104 plasmid was purified from DH5α cells using the QIAprep Spin Miniprep Kit (Qiagen) kit. Then the plasmid was digested using *AvrII* and *XhoI* (section 2.3.3), followed by gel purification. The plasmid was next treated with Antarctic phosphatase (New England Biolabs), according to the manufacturer's instructions, followed by further gel purification. The restricted *smc04266* internal gene fragment and pHJ104 vector were ligated using T4 DNA ligase (Promega), according to the guidelines supplied. A 10 µl reaction was set up containing 100 ng of pHJ104 vector, 17 ng of the *smc04266* internal gene fragment, 1 µl of the 10 x ligase buffer and 1 µl of T4 DNA ligase. The reaction was incubated overnight at 4ºC. In addition controls were set up with the gene fragment alone and with vector minus the gene fragment. The ligation reaction was subsequently transformed (section 2.3.5) into 5-alpha competent *E. coli* (New England Biolabs). Potential positive clones were then purified on LB, plus kanamycin and positive clones were next identified. This was achieved by a PCR screen of the clones and restriction digest of the extracted plasmid. For the PCR screen the Smc04266-8F-*Avr II* (5'-TAACCT AGGGCAAAGGTAACGACA AACG-3') primer was used, specific for the internal gene fragment and the pJH104 gusR (5'-GAGTTTTTTGATTTCA CGGGTT-3') primer was used specific for the pJH104 vector. With this primer pair positive clones where identified by a PCR product of 309 bp. For the restriction digest, the plasmid was extracted from the purified clones and the plasmid was digested with *XhoI* and *AvrII* to check for presence of the insert.

The pJH104 vector containing the *smc04266* insert (pJH104-*smc04266*in) was then transferred into Rm1021 using tri-parental mating (section 2.4). The pJH104 vector is able to replicate in *E. coli* but not in *S. meliloti,* so the *smc04266* genomic DNA is disrupted by homologus recombination upon transfer of pJH104-*smc04266*in into Rm1021. The disrupted *smc04266* gene was confirmed by PCR using the primers Sm04266-297F (5'-ACGGCGACGAGTGGATCG-3'), designed 297 bp upstream of the *smc04266* gene and pJH104 gusR (5'-GAGTTTT TTGATTTCA CGGGTT-3', specific for the vector. Disruption of the gene was confirmed by a PCR product of 640 bp using this primer pair.

2.4. Conjugation of plasmids into *S. meliloti* Rm1021 by triparental mating

The *S. meliloti* Rm1021 recipient strain was grown up for 48 hours to late exponential phase (OD_{600} ~3.0), and the *E. coli* donor and MM294A helper strain (pRK600) were grown for 16 hours to early stationary phase (OD_{600} ~2.5). Prior to setting up the conjugation reaction all strains were washed in LB media 3 times, to remove all antibiotics. A 40 µl aliquot of each culture was then mixed in a 1:1:1 ratio and the mixture (120 µl) was spotted on the centre of an LBMC plate. Additionally, as a control 40 µl of each of the cultures were spotted individually onto an LBMC plate. The plates were incubated for 16 hours at 30 ºC, after which the mating mixture and the 3 negative controls were purified onto LBMC agar with the appropriate antibiotic. Additionally to avoid contamination with the *E. coli* donor strains, a final concentration of 1 $mg.ml^{-1}$ streptomycin was used in the selection agar.

2.5. *S. meliloti* mutant library

Construction of the Rm1021 *smb21275*, *smb21273* and the *gshA* insertional mutants was achieved by use of a *S. meliloti* plasmid integration mutant library, purchased from the University of Bielefeld, Germany (Rüberg and Becker; Capela *et al*., unpublished). The library contains *E. coli* S17-1 clones carrying the mobilizable vector pK19mob2ΩHMB (Luo *et al.*, 2005b), each containing an *S. meliloti* internal gene fragment (200-350 bp in length). The pK19mob2ΩHMB vector can replicate in *E. coli* but is unable to replicate in *S. meliloti* and integrates into the target gene by homologous recombination. To conjugate the plasmids into *S. meliloti* Rm1021, a biparental mating was performed, since *E. coli* S17-1 contains a chromosomally integrated copy of RP4 that supplies the transfer functions (Simon *et al.*, 1983), so a helper strain is not necessary. The biparental matings were performed exactly as described for the triparental mating, with the exception that a 1:1 ratio of the Rm1021 recipient and *E. coli* S17-1 strains were used.

2.6. Preparation of *S. meliloti* phage lystates

This procedure was performed as described previously (Finan *et al.*, 1984), with minor modifications. A late exponential phase culture (OD_{600} ~3.0) of the desired *S. meliloti* strain was diluted 10-fold in 5 ml total of LBMC, incubated at 30°C for 2 hours and then 100 µl of M12 phage (~1 x 10^9 pfu.ml^{-1}) was added to the culture, which was incubated for 16 hours at 30°C. Upon lysis of the *S. meliloti* cells 10% $CHCl_3$ was added, followed by centrifugation at 2264 x g for 10 minutes to remove cellular debris. The prepared lysate was stored at 4°C with 10% $CHCl_3$.

2.7. *S. meliloti* transductions using M12 phage

S. meliloti phage transductions were performed based on a modification of a previously described method (Finan *et al.*, 1984). A late exponential phase culture (OD_{600} ~3.0) of the recipient strain was mixed at a 1:1 ratio with undiluted, $10^{-1}$and 10^{-2} dilutions of the appropriate phage lysate, in a final volume of 200 µl LB. After gentle mixing the samples were incubated at 30°C for 2 hours, followed by incubation at room temperature for 30 minutes. The bacterial cells were then pelleted by centrifugation at 9447 x g for 3 minutes, washed and then re-suspended in the same volume of LB. The cells were then recovered by incubation at 30°C and 200 rpm for 1.5 hours. The transduced cells were plated onto LB agar containing the appropriate antibiotics and incubated at 30ºC for 72 hours or until single colonies appeared. The colonies were purified twice, initially on LB agar plus antibiotics followed by the third purification performed using LBMC agar with antibiotics.

2.8. Preparation of Bac7(1-16) peptides

The N-terminal fragment 1-16 of Bac7 was synthesized by Monica Benincasa, at the University of Trieste, Italy as previously described (Benincasa *et al.*, 2004). A fluorescently-labelled version of Bac7(1-16), $Bac7_{1-16}$-BY, was prepared by the linkage of the thiol reactive dye BODIPY®FL N-(2-aminethyl)maleimide

(Invitrogen) to an additional C-terminal cysteine residue as reported previously (Scocchi *et al.*, 2008). This work was also performed by Monica Benincasa. When the peptides were received they were dissolved in sterile distilled water and stored at -20^{o}C until use.

2.9. Preparation of fluorescently labelled bleomycin A_5 (F-BLM)

2.9.1. Coupling reaction and agarose gel electrophoresis

F-BLM was prepared as described previously (Aouida *et al.*, 2004) with some modifications. 100 µl aliquot of 2.1 mM of the fluorescent molecule 5-(and-6)-carboxyfluorescein, succinimidyl ester [5(6)-FAM, SE] (Molecular Probes, C-1311) prepared in 0.2 M $NaHCO_3$ (pH 9.0) was added to 300 µl of 0.6 mM Bleomycin A_5 (LTK labs USA), prepared in 0.2 M $NaHCO_3$ (pH 8.3). The mixture was incubated for 2 hours at 25°C and the reaction was stopped by the addition of 10 µl of 1.5 M hydroxylamine (pH 8.5). The reaction products were resolved on a 1% (w/v) agarose (Seakem LE agarose, Cambrex) gel (2 hours, 80 V) using 40 mM 2-(N-morpholino) ethanesulfonic acid hydrate (MES) buffer, pH 6.0 (Sigma). F-BLM (the fluorescent band, which migrates towards the cathode) was visualized by UV light (Fig. 2-1). Since the free bleomycin is positively charged and will migrate towards the cathode and completely out of the gel and the free 5(6)-FAM, SE is negatively charged and will migrate towards the anode (Fig. 2-1), the F-BLM would not be expected to be contaminated with any starting products.

2.9.2. Purification of F-BLM from agarose

In each case the F-BLM band was carefully excised from the gel and the agarose removed following a modification of a previously published procedure (Tautz, 1983). The excised agar band was cut into small pieces and frozen at -80°C in glass wool plugged (~5 mm depth) 0.5 ml tubes. The F-BLM was collected in a 1.5 ml tube by centrifugation (9447 x g, 10 minutes at 4ºC) of the pierced 0.5 ml tube. This step was repeated several times until only the agarose was left in the glass wool plug.

The F-BLM was then freeze-dried and re-suspended in sterile water to the appropriate concentration.

2.9.3. Quantification of F-BLM

Electrospray ionisation-mass spectrometry analysis revealed that the F-BLM produced contains a 2:1 ratio of 5(6)-FAM, SE linked per bleomycin A_5 molecule (M. Scocchi and G. P. Ferguson, unpublished data). The F-BLM was quantified by making a series of 5(6)-FAM, SE standards diluted in 0.2 M $NaHCO_3$ (pH 9.0). These standards were read in a fluostar optima plate reader (BMG Labtech) (Excitation 495 nm and Emission 520 nm) and from these readings, 5(6)-FAM, SE standard curves were constructed to calculate an approximate concentration of the F-BLM stocks (Fig. 2-2A &B). Over the course of this work two independent stocks of F-BLM were produced; since the second stock was at a much higher concentration than the first, two calibration curves were produced. The reading for the first F-BLM stock produced was 22500 fluorescence units and so reading from the calibration curve (Fig. 2-2A) and taking into account the 2:1 ratio of 5(6)-FAM, SE, per bleomycin molecule the concentration of F-BLM stock 1 was found to be 4 $\mu g.ml^{-1}$. The reading from the second stock made (diluted 1 in 6) was 18700 fluorescence units and so the concentration of the second stock, as read from the calibration curve (Fig. 2-2A) was found to be 17.4 $\mu g.ml^{-1}$. After quantification F-BLM was aliquoted and stored at −20°C, until required. The preparation and purification of F-BLM was performed in the dark and F-BLM was kept on ice wherever possible.

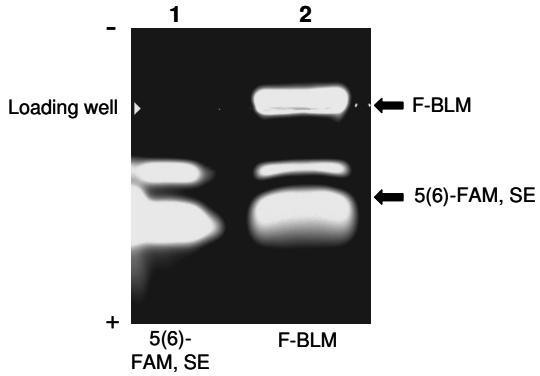

Figure 2-1. Resolution of F-BLM by agarose gel electrophoresis. After the conjugation reaction was stopped the product was loaded into a 1% agarose gel in 40 mM MES buffer and run for 2 hours at 80 V. The bands were then detected by UV light. The white arrow head shows the position of the loading well and the filled arrows show the position of the products resolved. Since the uncoupled bleomycin is positively charged it migrates towards cathode and completely out of the gel.

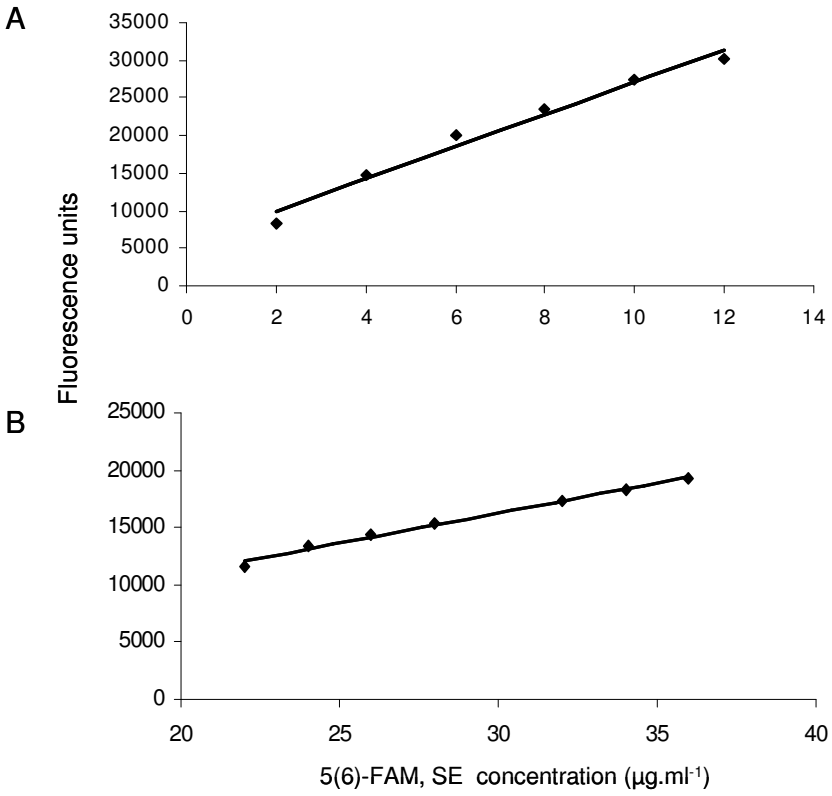

Figure 2-2. Calibration curves used to quantify the F-BLM stocks. Standards of 5(6)-FAM, SE were prepared and measured in a BMG LABTECH Fluostar optima 96 well plate reader (Excitation 495 nm and Emission 520 nm). These samples were either measured neat (A) or diluted 1/6 (B). The fluorescence readings obtained were then used to produce a calibration curve.

2.10. Stress assays

2.10.1. Filter disc assays

The required *S. meliloti* strains were grown in LBMC to late exponential phase (OD_{600} ~3.0) for 48 hours and the *E. coli* strains were grown to early stationary phase (OD_{600} ~2.5) for 16 hours. All strains were then washed 3 times in LB and re-suspended to an OD_{600} of 0.2. 100 µl of culture was added to 3ml of LB soft agar (0.8 g of agar per 100 ml LB broth), cooled to ~ 45ºC and poured onto LB plates (25 ml). Where stated in the text the bottom layer of LB was supplemented with 2.5mM $CaCl_2$ and 2.5mM $MgSO_4$. After 30 minutes, a sterile paper disc (6-mm diameter; Becton Dickinson) was applied to the centre of each plate and 5 µl of the agent to be tested was applied. The plates were then incubated at 30°C for 72 hours for *S. meliloti* and 37°C for 16 hours for *E. coli*, and the diameter of growth inhibition was recorded. The growth inhibition zone from at least three plates for each strain and each set of conditions was measured. The results were then averaged, and the error bars shown represent the standard deviation from the mean for one experiment.

2.10.2. Gradient assays

For the sodium deoxycholate (DOC) gradient assay *S. meliloti* cells were grown and prepared exactly as described for the filter disc assays. Large square plastic plates were used (22.5 cm in length) and 200 ml of LB media was used for preparation of each layer. The bottom layer, which was poured on a slant, contained no DOC. To create gradients of DOC, the top layer of agar contained 24 mM DOC. Where appropriate the LB was supplemented with 2.5mM $CaCl_2$ and 2.5mM $MgSO_4$, in this case to avoid precipitation, LBMC agar used for the top layer was cooled to 45ºC prior to DOC addition. The cultures to be tested were streaked (10 µl-aliquots) evenly across the plates. The plates were then incubated at 30°C for 72 hours, and the length of the growth inhibition zone was then recorded. The growth inhibition zone from at least three plates for each strain and each set of conditions was

measured. The results were then averaged, and the error bars shown represent the standard deviation from the mean for one experiment.

2.10.3. Liquid viability assays

The liquid viability assays, unless stated otherwise, were performed with late exponential phase *S. meliloti* cultures (OD_{600} ~3.0) and early stationary phase OD_{600} ~2.5) *E. coli* cells. The cells were washed 3 times in LB and diluted to an OD_{600} of 0.1 in LB medium. After addition of the defined agent at the appropriate concentration, the *S. meliloti* cultures were incubated at 30°C and the *E. coli* cultures at 37°C. At defined times, samples were removed, serially diluted in LB medium, then 10 µl aliquots plated in triplicate on LB agar plates or LBMC agar plates where stated in the text. Colony forming units (cfus) were calculated after 16 hours at 37°C for *E. coli* and 72 hours at 30°C for *S. meliloti*. The mean cfu ml^{-1} in the three 10 µl aliquots was plotted and the error bars represent the standard deviation from the mean for one experiment.

2.11. Bac7(1-16), $Bac7_{1-16}$-BY and F-BLM sensitivity assays

The required *S. meliloti* strains were grown to late exponential phase (OD_{600}~3.0) for 48 hours and the *E. coli* strains were grown to early stationary phase (OD_{600} ~2.5) for 16 hours. The strains were then sub–cultured so they would be in mid-exponential phase (OD_{600} ~0.9) prior to the assay. The defined mid-exponential phase strains were harvested, washed 3 times in LB and diluted to an OD_{600} of 0.05 in fresh LB medium. After the addition of either the Bac7(1-16), $Bac7_{1-16}$-BY or F-BLM to the defined concentrations, the *S. meliloti* cultures were incubated at 30°C and *E. coli* cultures were incubated at 37°C. At defined times, samples were removed, serially diluted in LB medium, then 10 µl aliquots plated in triplicate on LB agar plates. Colony forming units (cfus) were calculated after 72 hours at 30°C for *S. meliloti* and after 16 hours at 37ºC for *E. coli*. The mean cfu ml^{-1} in the three 10 µl aliquots was plotted and the error bars represent the standard deviation from the mean.

The sensitivity of the *E. coli* RYC1001 strain carrying the pWSK29-MtbacA vector (expressing *M. tuberculosis* BacA) and the appropriate control strains to Bac7 (1-16) was assessed as follows: Stationary phase cells were diluted to an OD_{600} of 0.1 in Mueller Hinton broth with the appropriate antibiotics and the cells were then grown at 37ºC to an OD_{600} of 0.3, at which point IPTG (0.4 mM) was added to induce transcription of the M. *tuberculosis* BacA protein, which is under control of the *lac* promoter. Cells were induced for 2 hours, and were then re-suspended to an OD_{600} of 0.035 (with 0.4 mM IPTG and the appropriate antibiotics) and the Bac7 (1-16) peptide was added at the defined concentration and the cultures were incubated at 37°C. At defined times, samples were removed, serially diluted in Mueller Hinton medium, then 10 µl aliquots plated in triplicate on Mueller Hinton agar plates. Colony forming units (cfus) were calculated after 16 hours at 37°C.

2.12. $Bac7_{1-16}$-BY and F-BLM uptake assays

2.12.1. Peptide treatment

Prior to performing each assay, fresh 50 mM sodium phosphate buffer was made, since this buffer was shown to deteriorate with time. Initially, a 0.1 M sodium phosphate buffer stock was made as follows: A 1 M stock of Na_2HPO_4 and a 1 M stock of NaH_2PO_4 were made, after which 57.7 ml of 1 M Na_2HPO_4 and 42.3 ml of NaH_2PO_4 were added into final volume of 1 litre of distilled water. This was then diluted 1 in 2 to make a 50 mM stock, the pH of the buffer was then checked to ensure it was 7.0 and the buffer was filter sterilised.

For the assay mid-exponential phase cultures (OD_{600} ~0.9) of the defined strains were harvested, washed 3 times and re-suspended to an OD_{600} of 0.05 in fresh filtered LB medium. After the addition of the defined fluorescently labelled peptide at the appropriate concentration, the *S. meliloti* cultures were incubated at 30°C and *E. coli* at 37°C, both in a water bath. At the defined times the cells were washed twice in LB filtered medium to remove extracellular labelled peptide and then re-suspended in filtered 50 mM sodium phosphate buffer pH 7.0. To account for extracellular binding of the labelled peptide to the cells, the cultures were treated

with and without 1 $mg.ml^{-1}$ of the extracellular quencher of fluorescence Trypan blue (Sigma, 4 $mg.ml^{-1}$ filter sterilized stock) for 10 minutes at room temperature, prior to flow cytometry analysis.

2.12.2. Flow cytometry

A Beckon Dickinson (BD) LSR II flow cytometer equipped with a 488 nm laser was used to measure the fluorescence parameter of single cells after treatment with the F-BLM and $Bac7_{1-16}$-BY labelled peptides. Both 5(6)-FAM, SE and BODIPY® FL maleimide (505/513) fluorescence were measured using a 530/30 nm band pass filter (FL1). To assess 5(6)-FAM, SE fluorescence for the F-BLM uptake experiments the following parameters were used: forward scatter set to 894 V (linear scale), side scatter set to 341 V (linear scale) and FL1 set to 500 V (logarithmic scale) with the FSC threshold set to 5,000. To assess BODIPY® FL maleimide fluorescence for the $Bac7_{1-16}$-BY uptake experiments the following parameters were used: forward scatter set to 500 V (logarithmic scale), side scatter set to 250 V (logarithmic scale) and FL1 set to 450 V (logarithmic scale) with the FSC and SSC thresholds set to 900 and 200, respectively. In both cases 10,000 events were collected for each analysis. Data were acquired and analyzed using the BD FACSDiva and FlowJo (Tree Star Inc.) software respectively.

2.13. *S. meliloti* bleomycin treatment to assess DNA damage

The required strains were grown to late exponential phase (OD_{600} ~3.0) for 48 hours. The strains were then sub–cultured (5 ml volume) the day prior to the bleomycin treatment so they would be in mid-exponential phase of growth when required (OD_{600} ~0.9). The mid-exponential phase cultures were harvested, washed 3 times with LB and the cell pellet was re-suspended in a final volume of 2 ml of LB (OD_{600} ~2.3). For each strain to be assessed the 2 ml culture was then split into two tubes (1 ml per tube) and to one tube bleomycin A_5 (20 $\mu g.ml^{-1}$) was added and the second tube served as an untreated control. All tubes were incubated at 30ºC for two hours with shaking. After incubation the cells were then washed once in LB and recovered

by centrifugation at 2264 x g (10 minutes). The cell pellets were then frozen at -80ºC until the genomic extraction was performed.

2.13.1. Genomic DNA Extraction

This method was a modification of a previously published protocol (Wilson, 1987). Prior to performing the assay, the Hexadecyltrimethylammonium bromide (CTAB) /NaCl solution was made as follows: 4.1 g of NaCl was dissolved in 80 ml of distilled water, the solution was heated to 65ºC and 10 g of CTAB was slowly added with stirring. When the CTAB had dissolved, the final volume was adjusted to 100 ml.

To perform the DNA extraction the cell pellets were removed from -80ºC and thawed on ice, then each cell pellet was re-suspended in 567 µl of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5) by pipetting, then 30 µl of 10% (w/v) SDS and 3 µl of proteinase K (20 $mg.ml^{-1}$) were added. The samples were then mixed and incubated for 1 hour at 37ºC. 100 µl of 5 M NaCl was added and mixed well, followed by 80 µl of the prepared CTAB/NaCl solution and the samples were then incubated for 10 min at 65ºC. Next an equal volume of chloroform was added, mixed well and the sample centrifuged at 9447 x g for 5 mins. After centrifugation, the top layer was carefully moved into to a fresh tube. An equal volume of 1:1 phenol:chloroform was added, mixed well and centrifuged as before for 5 mins. The top layer was then moved into a clean tube and the genomic DNA was then precipitated with a 0.6 volume of isopropanol (mixed by inversion). The sample was then centrifuged at 9447 x g for 5 mins to pellet the DNA. After carefully removing the isopropanol the DNA pellet was washed once by centrifugation (9447 x g, 2 minutes) with 70% ethanol. The DNA pellets were then air dried and re-suspended in 25 µl of molecular water (Sigma) and the DNA quantified by UV spectrometry using a Beckman DU 650 spectrophotometer.

2.13.2. 4',6-diamidino-2-phenylindole (DAPI) staining of *S. meliloti* genomic DNA

Cells were treated with and without bleomycin A_5 exactly as for the genomic DNA preparations (section 2.13) except that following the final centrifugation step, the cell pellet was re-suspended in 100 µl of 1% (v/v) toluene, vortexed and stored at 4ºC. The DAPI assay was performed following the modification of a previously published protocol (Johnson, 1994). Cells were diluted to an OD_{600} of 0.08 in dilution buffer (10 mM NaCl, 6.6 mM Na_2SO_4, 5 mM HEPES pH 7.0) and 100 µl of each sample was placed in a 96 well plate and DAPI was added at a final concentration of 0.1µg ml^{-1} and the samples were incubated overnight at 4ºC. Following incubation the fluorescence intensity of each sample was measured using a Fluostar optima plate reader (BMG Labtech) with excitation and emission at 350 and 450 nm respectively.

2.14. Transmission electron (TEM) microscopy of bleomycin treated cells

Cells were treated with and without bleomycin A_5 exactly as for the genomic DNA preparations (section 2.13), except after the final centrifugation step, the bacterial pellet was re-suspended in 1 ml of fixative (2.5% w/v gluteraldehyde in 0.1 M cacodylate buffer pH 7.4) for 1 hour. The cells were washed twice in 0.1 M cacodylate buffer pH 7.4, re-suspended in 500 µl of this buffer and stored at 4ºC. The fixed cells were then given to Alastair McKinnon (Electron Microscopy Facility, University of Aberdeen) who processed the samples as follows: The samples were pelleted by centrifugation at 9447 x g for 3 min, then 2% (w/v) low gelling point agar (dissolved at ~ 70ºC) was cooled and added to the cell pellet at 35ºC. The agar cell suspension pellet was left to fix overnight at 4ºC then trimmed to EM block size (~ 2x2mm x 1mm thick) before loading onto a Leica EMTP tissue processor. In the tissue processor the pellets received a secondary fix in 1% OsO_4, dehydration in graded concentrations of ethanol to 100%, followed by immersion in increasing concentrations of acetone/spurr mixture before completing the infiltration with pure spurr resin. The pellets were next embedded in fresh spurr in 8mm polyethylene truncated pyramid capsules (TAAB Laboratory Equipment) and then polymerised in

a vacuum-embedding oven at 60ºC for 24 hours. Semi-thin (0.5 µm) light microscopy survey sections were cut using a Reichert Ultracut microtome and stained with 1% (w/v) Toluidine blue (hotplate at ~ 80ºC). Areas were selected for ultra-thin sectioning (silver/gold-80nm) using a Diatome diamond knife on a Leica UC6 ultramicrotome. The sections were collected onto 200 mesh fine bar copper grids and stained to provide electron contrast using uranyl acetate and lead citrate. The ultrathin sections were examined with a Philips CM10 TEM and imaged with a Gatan Bioscan CCD camera.

2.15. Lipolysaccharide (LPS) preparations

2.15.1 Extraction of LPS by SDS lysis

Isolation of *S. meliloti* LPS was performed using late exponential phase cultures (OD_{600} ~ 3.0). 1 ml of the bacterial culture was pelleted by centrifugation at 9447 x g for 3 minutes and the pellet re-suspended in 30 µl of lysis buffer (1M Tris-HCL pH 6.8, 2% w/v SDS, 4% v/v β-mercaptoethanol, 10 % v/v glycerol and 0.005% w/v bromophenol blue). The sample was then boiled at 100°C for 10 minutes, then cooled to room temperature at which point 10 µl of proteinase K (2.5 $mg.ml^{-1}$ stock in lysis buffer) was added and the samples were incubated at 60°C for 1 hour. Two volumes of sample buffer (120mM Tris-HCL pH 6.8, 3% w/v SDS, 9% v/v β-mercaptoethanol, 30% v/v glycerol and 0.03% w/v bromophenol blue) were then added, at which point the sample was either loaded into the SDS-PAGE gel immediately or stored at -80°C, until use.

2.15.2. Analysis of LPS by SDS-PAGE

SDS-PAGE gel analysis was performed using the Bio-rad mini-protean II electrophoresis cell system according to the manufacturer's instructions. The gel buffer contained 3M Tris and 0.3% (w/v) SDS, pH adjusted to 8.45. The gel solutions (to make two gels) were made up as follows:

Separating/resolving gel	Concentrating gel
(15.5 % acrylamide)	(2.3 % acrylamide)
2.3 ml H_2O	4.2 ml H_2O
3.3 ml gel buffer	1.5 ml gel buffer
1 ml glycerol	500 µl acrylamide/bisacrylamide
3.3 ml acrylamide/bisacrylamide	30 µl ammonium persulphate (10% w/v)
40 µl ammonium persulphate (10% w/v)	7.3 µl TEMED
4 µl TEMED	

The Acrylamide/Bisacrylamide solution contained 93 g of an acrylamide solution and 6 g of bisacrylamide solution per 200 ml of distilled water.

The running buffers were prepared as follows:
Anode buffer: 0.2 M Tris-HCl, pH 8.9
Cathode Buffer: 0.1 M Tris, 0.1 M Tricine and 0.1% w/v SDS
Gels were run at approximately 100 V until adequate migration had occurred.

2.15.3. Fixing and staining of LPS SDS-PAGE gels

For sodium-m-periodate staining, the gel was fixed overnight in fixative solution (40% v/v ethanol and 5% v/v glacial acetic acid). For alcian blue staining, the gel was fixed overnight in alcian blue (0.05% w/v alcian blue in fixative solution). After fixing, for sodium-m-periodate staining, the gel was incubated in 50 ml of oxidiser (0.7% w/v sodium-meta-periodate in fixative solution) for 5 mins. For both staining procedures the gels were washed three times for 15 mins in distilled water. The gels were then incubated in silver stain (2 ml 25% ammonium hydroxide solution, 128 µl 10 N NaOH and 140 ml of distilled water; 1 g silver nitrate semi-dissolved in 5 ml

dH_2O added dropwise for 5 mins). This was followed by three, 10 minute washes with distilled water. The gels were then incubated with development solution (0.02% w/v formaldehyde and 50 $\mu g.ml^{-1}$citric acid) until the bands appeared (approximately 2-10 mins). Once the bands were clear the reaction was stopped with 0.5 % acetic acid for 1 min.

2.16. Preparation of *S. meliloti* cells for two-dimensional gel analysis

The *S. meliloti* cells were prepared for two-dimensional (2D) gel analysis using an empirical protocol from the University of Aberdeen proteomics facility. A 5 ml late exponential phase culture (OD_{600} ~3.0) was washed 3 times with and finally re-suspended in 1 ml of PBS. The OD_{605} of the cells (diluted 1/50 in PBS) was measured and the 1 ml culture (in PBS) was pelleted by centrifugation at 9447 x g for 3 minutes and re-suspended in a volume of lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.3% dithiothreitol, 1% v/v pH 4-7 IPG buffer [GE healthcare] and 0.002% w/v bromophenol blue), that was equal (in ml) to the OD_{605} reading i.e 0.270 ml would be used for an OD_{600} reading of 0.270. The cell pellet re-suspended in lysis buffer was left on ice for 30 minutes and then pelleted by centrifugation at 9447 x g for 5 minutes and the supernatant was collected and stored at -20°C until use by the proteomics facility.

2.17. *S. meliloti*-alfalfa interaction experiments

2.17.1 Germination of seedlings

Alfalfa (*Medicago sativa* cv. Iroquois) seedlings were surface sterilized for 15 minutes in 50% v/v bleach (Unilever UK), followed by several washes with sterile water, until the smell of bleach was no longer detectable. The seedlings (approximately 20 per plate) were then placed onto the top half of a 1% Bacto agar plate with 1 ml of sterile water. The plates were then wrapped with parafilm and covered in foil and the seedlings left to germinate upright in the dark at 22°C for 72 hours.

2.17.2. Inoculation of alfalfa plants

The three day old seedlings had their seed coats carefully removed with sterile forceps and were then placed onto a petri dish containing 40 ml of Jensen's agar (section 2.17.4). Prior to inoculation each culture was grown to late exponential phase (OD_{600} ~3.0) in LBMC, washed 3 times and re-suspended to an OD_{600} of 0.05 in sterile water. The seedlings were inoculated with 1 ml of the appropriate culture or 500 µl of each culture for competition assays. Notches had previously been melted into the edge of the Petri dish and lid, through which the plant would emerge. The plates were wrapped in parafilm, making sure the notch was not covered and the plants were then incubated at 22°C on a 16 hour day cycle. Plant growth and nodule characteristics were determined after a four week period and in addition when required nodules were harvested from the growing plants. Nitrogen fixation and successful symbiosis was determined by dark green plants and the appearance of cylindrical pink nodules.

2.17.3. Extraction of bacteria from nodules

To confirm that the *S. meliloti* mutants were not reverting during the symbiosis and to determine the numbers of parent strain and mutant bacteria for the competition assays individual nodules were sampled and bacteria were extracted as follows: Using a 96 well plate each nodule was surface sterilized for 50 seconds in 50% v/v bleach and then crushed in 200 µl of LBMC supplemented with 0.3 M glucose. The released bacteria were next serially diluted and 10 µl aliquots from each dilution spotted in triplicate onto LBMC agar plates with and without the appropriate antibiotics to select for the mutants.

2.17.4. Medium for plant growth

To make Jensen's medium the following ingredients were placed into a 2 litre flask containing 500 ml of distilled water and a stir bar:

1 g $CaHPO_4$

0.1 g $FeCl_3.6H_2O$

100 µl 10M NaOH

1 ml of trace minerals (see below)

0.2 g of $MgSO_4.7H_2O$

0.2 g of K_2HPO_4

0.2 g of NaCl

The final volume was then made up to 1 litre with distilled water and the solution was left to stir overnight. The next day 15 g of Bacto agar was added to the media, prior to autoclaving (performed with the stir bar in the flask). After autoclaving the Jensen's agar was mixed for 20 mins and then the plates were poured immediately, each containing 40 ml of Jensen's agar.

To make 500 ml of trace minerals for the Jensen's agar, the following ingredients were added to 200 ml of distilled water:

0.5 g H_3BO_3

0.5 g $ZnSO_4.7H_2O$

0.25 g $CuSO_4.5H_2O$

0.25 g $MnCl_2.4H_2O$

0.5 g $NaMoO_4.2H_2O$

The solution was then mixed and made up to 500 ml with distilled water. The trace minerals were stored long term at 4ºC and mixed well before use.

2.18. Detection of plant polyphenolics

2.18.1. Histochemical staining

This method is a modification from a previously published protocol (Vasse *et al.*, 1994). The buffer and stains were made up fresh on the day of use, each time the procedure was performed. 10 mM PIPES buffer was made as follows: 0.3 g of PIPES sodium salt purchased from Sigma was dissolved in a final volume of 100 ml of distilled water and then the pH was adjusted to 7.2. The 0.04% potassium permanganate solution was made as follows: A 4% stock solution was made by dissolving 2 g of potassium permanganate powder, purchased from Sigma into a final volume of 50 ml of distilled water and 1/100 dilution was then made to give a 0.04 % (w/v) solution. Finally, a 0.01% solution of methylene blue was made as follows:

0.5 g of methylene blue powder, purchased from Sigma was dissolved in 50 ml of distilled water, and then diluted 1/100 to give a 0.01% (w/v) solution.

Prior to staining, individual nodules were removed and sliced longitudinally using a scalpel. After slicing the nodules were fixed in 2.5 % (w/v) glutaraldehyde and 10 mM PIPES (pH 7.2) for 1 hour. The nodule slices were then immersed in 0.04 % (w/v) potassium permanganate for 1 hour, rinsed in 10 mM PIPES and then stained with 0.01 % (w/v) methylene blue for 2 minutes. The nodule slices were then immersed in 50 % (v/v) bleach for 3 mins and visualised using bright field optics (10 x magnification, Zeiss Axioskop microscope). Images were obtained by photography with a Zeiss Axiocan camera and processed using Axio Vision software.

2.18.2. Fluorescence microscopy

Prior to fluorescence microscopy individual nodules were sampled, sliced and fixed as described for the polyphenolic staining. The nodule slices were then cleared by soaking in 50% (v/v) bleach for 3 mins, followed by 3 washes in sterile distilled water and then visualised for fluorescent polyphenolics by microscopy (10 x magnification, Zeiss Axioskop microscope) using UV excitation. Images were obtained and processed as described for the histochemical staining.

2.19. Transmission electron microscopy of nodules

Nodules were sampled from the alfalfa plants at the defined time points, the nodules were then halved and fixed in 2.5% (w/v) glutaraldehyde in 0.1 M sodium cacodylate (pH 7.0) overnight at 4ºC. The fixed nodules were then sent to Euan James (University of Dundee) who processed the samples as follows: The fixed nodules were either dehydrated in an ethanol series and embedded in LR White acrylic resin (Agar Scientific) for light microscopy or post-fixed in 1% OsO_4 (w/v), dehydrated in an ethanol series, and then embedded in Durcupan epoxy resin (Sigma) for conventional TEM. Semi-thin sections (1 mm) and ultrathin sections (70 nm) were taken from the resinembedded samples (LR White and Durcupan) using a Reichert Ultracut E ultramicrotome. The semi-thin sections were collected on glass slides and stained with 1% (w/v) toluidine blue in sodium borate and viewed and photographed

using a Zeiss Axioskop optical microscope fitted with an AxioCamdigital camera (Carl Zeiss Imaging). The ultrathin sections for conventional TEM were collected on pioloform-coated copper grids and stained with uranyl acetate (10 min) and lead citrate (5 min) before being viewed with a JEOL 1200 EX transmission electron microscope.

2.20. Statistical analysis

Where shown, the significance of differences among bacterial strains was assessed by using the students unpaired *t*-test using Microsoft Excel 2003. Values of $P < 0.05$ were considered statistically significant.

Chapter 3: The Effect of the Glycopeptide Bleomycin on *S. meliloti*

3.1. Introduction

As previously discussed, although the exact function of the BacA protein is still unknown, loss of the protein in *S. meliloti* results in a 50% reduction in the amount of lipid A species modified with a VLCFA, relative to the parent, in which all the lipid A molecules are modified with a VLCFA (Ferguson *et al.*, 2002; Ferguson *et al.*, 2004). However, in addition to affecting the lipid A, loss of BacA in *S. meliloti* results in a number of altered sensitivities to different stresses and agents in the free-living state, relative to the parent strain (Ferguson *et al.*, 2002; Glazebrook *et al.*, 1993; Ichige & Walker, 1997), one of which is an increased resistance to the glycopeptide bleomycin. Hence, the work in this chapter set out to investigate how the BacA protein sensitizes *S. meliloti* towards bleomycin.

The glycopeptide antibiotic bleomycin was initially isolated from *Streptomyces verticillus* (Umezawa *et al.*, 1966) and was subsequently found to be an important anti-tumour agent, owing mainly to its ability to damage DNA (Blum *et al.*, 1973; Burger *et al.*, 1981; Stubbe & Kozarich, 1987; Umezawa, 1974). The structure of bleomycin is characterized by metal- and DNA-binding domains, a carbohydrate moiety and an R-group, which varies depending upon the form of bleomycin used (Fig. 3-1). Unlike the other domains, very little is known about the carbohydrate moiety, except that bleomycin lacking this region exerts a much less powerful genotoxic effect (Tounekti *et al.*, 2001). Bleomycin binds to Fe II and in the presence of oxygen, forms a free radical reactive complex that attacks both DNA and RNA (Burger *et al.*, 1979). Bleomycin induced DNA damage can result in both single and double strand breaks (Burger, 1998; Giloni *et al.*, 1981; Worth *et al.*, 1993).

In prokaryotic cells, much work has been performed looking at the effects of bleomycin on *E. coli* cells, where it has been shown to inhibit both DNA and RNA synthesis (Suzuki *et al.*, 1968) and induce DNA damage Bleomycin sensitivity of *E. coli* is increased by either *lexA* or *recA* mutations (Yamamoto & Hutchinson, 1979). Since the RecA protein and the LexA repressor are key regulators in the SOS response, which is induced in *E. coli* upon DNA damage (Brent & Ptashne, 1981; Little *et al.*, 1980; Little *et al.*, 1981), this would suggest in *E. coli* the SOS response is an important mechanism bacterial cells use to inhibit the toxic effects of the drug

(Yamamoto & Hutchinson, 1984).

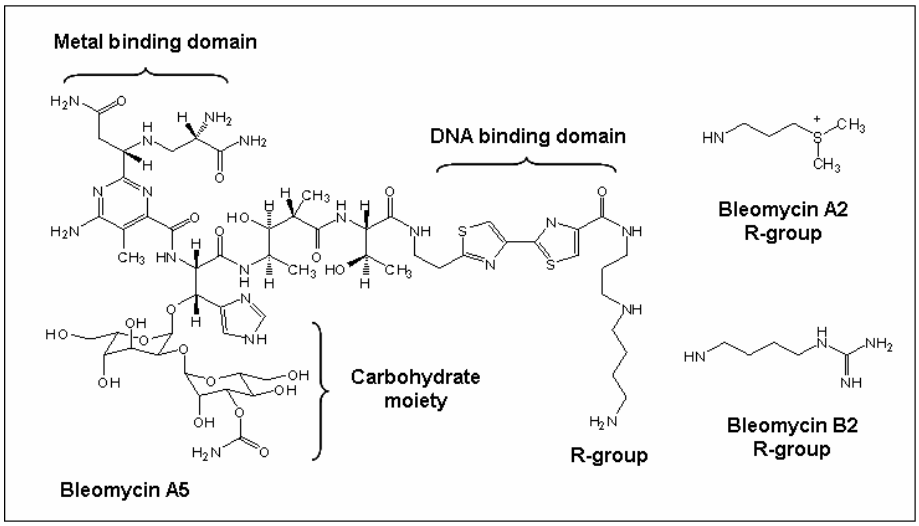

Figure 3-1. The structure of bleomycin A_5 and the R-groups of the A_2 and B_2 forms.
Depiction of the structure of bleomycin A_5. The R-groups of bleomycin A_2 and B_2 are also shown. All structures were prepared by Hazel Phillips using ChemDraw Std 10.0.

In *E. coli*, a mutant lacking the BacA homologue, SbmA, is also resistant to bleomycin and microcin antibiotics (Ichige & Walker, 1997; Salomon & Farias, 1995). However, the *E. coli sbmA* mutant was found to be as sensitive to internally-synthesized microcins as the parent strain. Therefore, a model was proposed whereby SbmA could be involved in the uptake of certain classes of peptides (Lavina *et al.*, 1986; Salomon & Farias, 1995). Subsequently it was determined that when expressed in *E .coli,* the *S. meliloti bacA* gene suppressed all known defects of the *E. coli sbmA* mutants, namely the increased resistance to bleomycin and microcin antibiotics. Additionally, when placed under the control of the *S. meliloti bacA* gene promoter, the *E. coli sbmA* gene suppressed all the *S. meliloti bacA* mutant phenotypes (Ichige & Walker, 1997). Combined these findings demonstrated a functional similarity between the two proteins and it was proposed that like SbmA, BacA may also be involved in uptake of bleomycin (Fig. 3-2), either directly (Fig. 3-2A) or indirectly (Fig. 3-2B). There also remained the possibility that the VLCFA reduction in the Rm1021 Δ*bacA* mutant could be linked to the bleomycin phenotype. However, this seemed unlikely since the same phenotype is observed in the *E. coli sbmA* mutant (Ichige & Walker, 1997; Salomon & Farias, 1995) despite

the fact that the lipid A of *E. coli* is not modified with VLCFA (Raetz & Whitfield, 2002). In a more recent study transposon mutagenesis was performed under saturating conditions to select for *S. meliloti* bleomycin resistant mutants. In this selection only *S. meliloti* mutants with disruptions in their *bacA* gene were isolated (Ferguson *et al.*, 2006). Therefore, these findings are consistent with a model whereby the BacA protein may be involved in bleomycin uptake into *S. meliloti*. They also suggest that either an additional bleomycin uptake system does not exist or that it is essential for growth or functionally redundant. Interestingly, recent work has been able to show that SbmA is involved in the intracellular accumulation of a proline-rich peptide, Bac7 in *E. coli* (Mattiuzzo *et al.*, 2007).

Thus, in order to examine a possible role for the *S. meliloti* BacA protein in the uptake of bleomycin the aims of this chapter were to further investigate the effect of bleomycin on *S. meliloti*, investigate the intracellular accumulation of fluorescently labelled bleomycin and to characterise the bleomycin resistance determinants of *S. meliloti.*

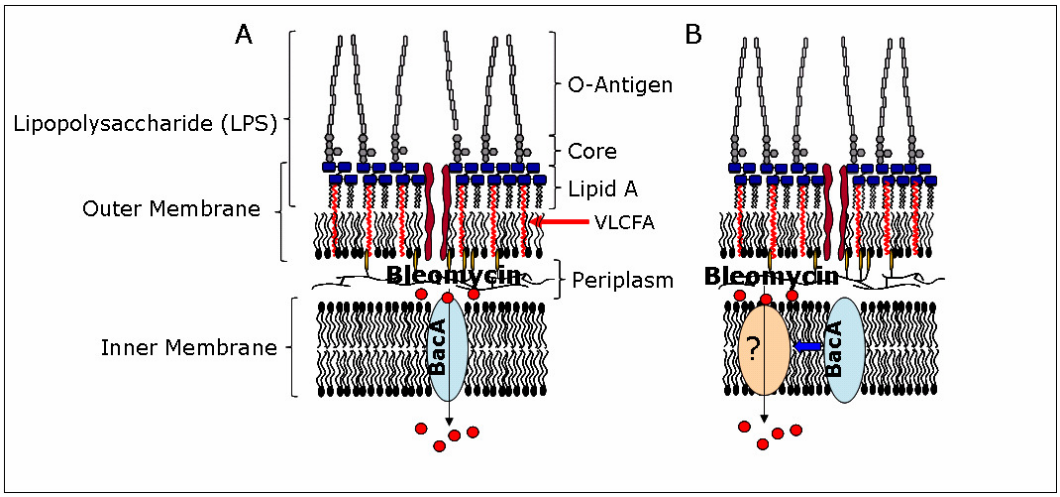

Figure 3-2. Proposed model for BacA function. Once the glycopeptide bleomycin has reached the periplasmic space it could then be directly transported across the cytoplasmic membrane by the BacA protein (A) or alternatively BacA may be having an effect on an unknown protein involved in bleomycin uptake (B).

3.2. Results

3.2.1. BacA mediated bleomycin sensitivity is independent of the VLCFA modification

The first question to address was to determine if the increased resistance to bleomycin observed in an *S. meliloti* Δ*bacA*mutant was linked to the reduction in the amount of lipid A modified with a VLCFA, relative to the parent strain. Recent work had revealed that the lipid A species produced by the *S. meliloti acpXL* and *lpxXL* insertional mutants in LB medium supplemented with 2.5mM $MgSO_4$ and 2.5mM $CaCl_2$ completely lacked the lipid A VLCFA modifications (Ferguson *et al.*, 2005). The *acpXL* and *lpxXL* gene products encode a VLCFA-acyl carrier protein (Brozek *et al.*, 1996) and an acyl transferase protein (Basu *et al.*, 2002), respectively. Therefore, these mutants provided a means to investigate how the complete absence of the lipid A modification in *S. meliloti* influences bleomycin sensitivity. Bleomycin A_2 filter disc assays were performed to assess the sensitivity of the Rm1021 Δ*bacA, acpXL*, and *lpxXL* single mutants to bleomycin, relative to the parent strain. However, in contrast to the Rm1021 Δ*bacA* mutant which exhibits resistance to bleomycin, both the Rm1021 *acpXL* and Rm1021 *lpxXL* insertional mutants display an increased sensitivity towards bleomycin A_2 relative to the Rm1021 parent strain (Fig. 3-3A) (Ferguson *et al.*, 2006). The *lpxXL* mutant was shown to be more sensitive to bleomycin than the *acpXL* mutant, since despite lacking the VLCFA modification, the *acpXL* mutant is still able to transfer a shorter chain C16:0 or C18:0 unhydroxylated fatty acid not normally found in the *S. meliloti* LPS, onto a portion of its lipid A molecules (Ferguson *et al.*, 2005). Therefore, the *acpXL* mutant is still able to produce a significant portion of its lipid A molecules in the pentaacylated state (Ferguson *et al.*, 2005), which must confer some protection against bleomycin. Thus these data show that in the complete absence of the VLCFA modification, *S. meliloti* is more sensitive to bleomycin A_2 and the bleomycin phenotype of the *S. meliloti* Δ*bacA* mutant is unlikely to be due to a reduction in the lipid A VLCFA modification content.

To provide further evidence that the bleomycin resistance phenotype occurs independently of the lipid A modification in the Rm1021 Δ*bacA* mutant, the

bleomycin sensitivity of the Rm1021 *acpXL/ΔbacA* and Rm1021 *lpxXL/ΔbacA* double mutants were compared to their respective single mutants (Fig. 3-3B & C, respectively) (Ferguson *et al.*, 2006). Previous work has demonstrated the *acpXL/bacA* and *lpxXL/bacA* double mutants have identical lipid A profiles to that of their *acpXL* and *lpxXL* single mutants (Ferguson *et al.*, 2005), and that their lipid A molecules completely lack the VLCFA modification. However, it was observed that deletion of *bacA* in an *S. meliloti acpXL* and *lpxXL* mutant background still confers resistance against bleomycin compared to the respective *acpXL* and *lpxXL* single mutants, despite the fact that the single and double mutants have identical lipid A profiles. Therefore these data show that the BacA mediated sensitivity to bleomycin is independent of the VLCFA modification.

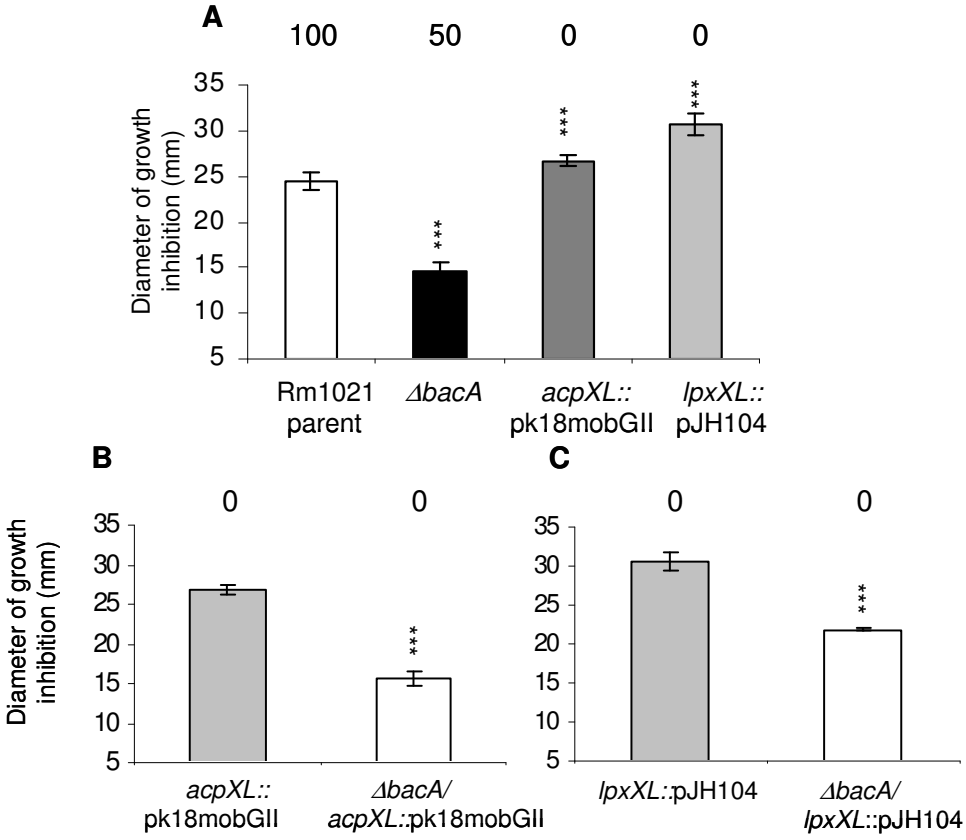

Figure 3-3. **Sensitivity of the Rm1021 *acpXL* and *lpxXL* mutants to bleomycin A_2 in the presence and absence of the *bacA* gene.** (A) The defined *S. meliloti* strains were exposed to bleomycin A_2 (5 µl of a 5 $mg.ml^{-1}$ aqueous stock solution) on LBMC. The significant values (***P<0.001) shown, represent comparisons of the Δ*bacA* mutant to the Rm1021 parent strain and the *acpXL*::pk18mobGII and *lpxXL*::pJH104 mutants to the Δ*bacA* mutant. (B) As (A), except the significant values (***P<0.001) shown, represent comparisons of the *ΔbacA/acpXL*::pk18mobGII double mutant to the *acpXL*::pk18mobGII single mutant. (C) As (A), except the significant values (***P<0.001) shown, represent comparisons of the Δ*bacA/lpxXL*::pJH104 mutant to the *lpxXL*::pJH104 mutant. For each dataset the numbers above the bars indicate the percentage of the lipid A modified with VLCFAs in each strain (Ferguson *et al*., 2005). Each dataset shown is representative of the trends observed in two independent experiments and in each case the error bars represent the standard deviation from the mean (n=3) for one experiment. (Ferguson *et al*., 2006).

3.2.2. The BacA protein confers sensitivity of *S. meliloti* towards different forms of bleomycin independent of the nature of the R-group

Different forms of bleomycin vary in the nature of their R-group (Fig. 3-1). Previous studies investigating the role of the *S. meliloti* BacA protein in conferring bleomycin sensitivity had used the A_2 form of the drug (Ferguson *et al.*, 2006; Ichige & Walker, 1997; LeVier & Walker, 2001). Therefore, the sensitivity of the Rm1021 parent and the Rm1021 Δ*bacA* mutant towards different forms of bleomycin (Fig. 3-1) was assessed using a disc diffusion assay (Fig. 3-4A). For this assay, cells were exposed to bleomycin A_5, A_2, B_2 and the sulphate form. Bleomycin sulphate is a mixture of glycopeptides, the two principle components of the mixture are A_2 (55-70%) and B_2 (25-32%). From these data it can be determined that the presence of the BacA protein confers an increased sensitivity to *S. meliloti* against all forms of bleomycin, irrespective of the nature of the bleomycin R-group (Fig. 3-4 A). Additionally, cell viability was assessed after bleomycin addition, where it was also found that both bleomycin sulphate and bleomycin A_5 reduce the viability of *S. meliloti*. As observed for the filter disc assays, the presence of the BacA protein conferred an increased sensitivity towards these drugs (Fig. 3-4B & C, respectively). Combined, these findings would suggest that if the BacA protein is involved in bleomycin uptake, then it is involved in the uptake of all forms of bleomycin since BacA-mediated increased sensitivity of *S. meliloti* towards bleomycin appears to be independent of the nature of the bleomycin R-group.

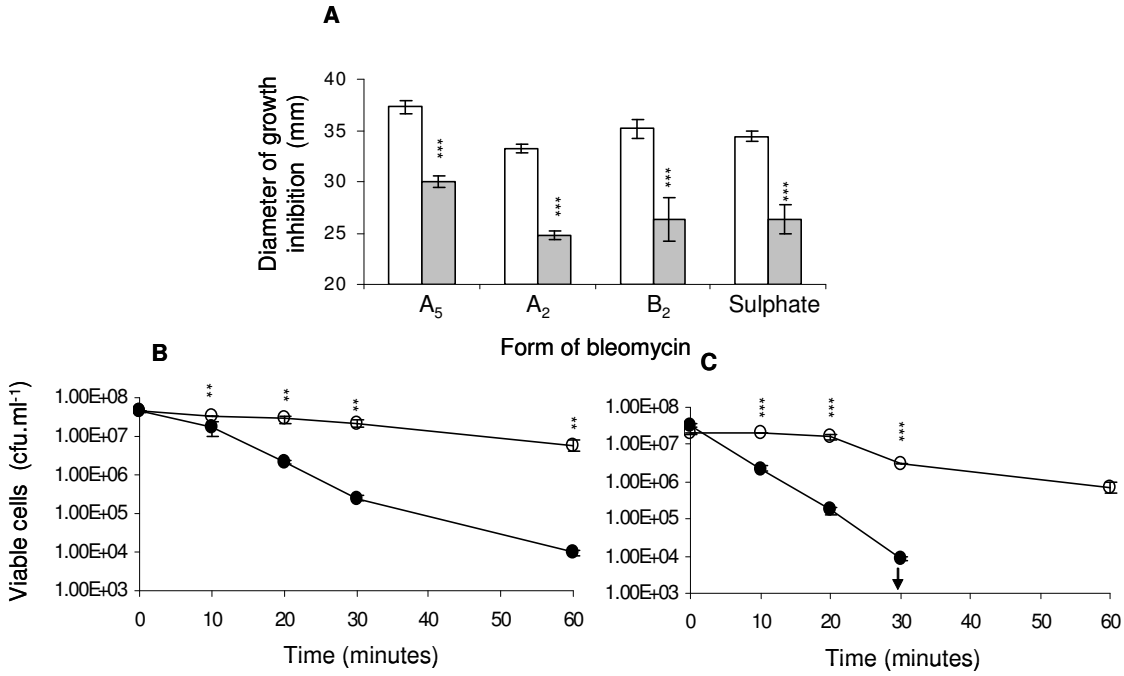

Figure 3-4. **Growth inhibition and viability of the Rm1021 parent strain and Rm1021 *ΔbacA* mutant after exposure to different forms of bleomycin.** (A) Growth inhibition for the *S. meliloti* Rm1021 parent strain (open bars) and Rm1021 Δ*bacA* mutant (shaded bars) exposed to the defined forms of bleomycin (For forms A_2, B_2 and sulphate 5 µl of a 5 mg.ml^{-1} aqueous stock solution was used and for A_5 5 µl of a 2 mg.ml^{-1} aqueous stock solution was used). (B) The Rm1021 parent strain (closed circles) and the Δ*bacA* mutant (open circles) were treated with bleomycin sulphate (15 µg.ml^{-1}) and viability was determined at the defined times. (C) As in (B) except cells were treated with bleomycin A_5 (3 µg.ml^{-1}), the arrow represents complete loss of viability. The significant values shown (*P <0.05; **P<0.01; ***P<0.001) represent comparisons of the Rm1021 Δ*bacA* mutant and the Rm1021 parent. All datasets shown are representative of trends observed in two independent experiments and in each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

3.2.3. Presence of the BacA protein results in an increased level of bleomycin induced DNA degradation in *S. meliloti*

Bleomycin treatment is known to cause DNA damage in bacterial cells (Yamamoto & Hutchinson, 1984). Since labelled bleomycin was unavailable commercially, bleomycin A_5 induced DNA degradation was next assessed as a means to quantify bleomycin uptake into *S. meliloti*. To assess DNA damage mid-exponential phase *S. meliloti* cells were exposed to bleomycin A_5 over 2 hours, then the genomic DNA

was extracted and analysed by agarose gel electrophoresis, relative to genomic DNA extracted from untreated cells. These data clearly show that in the Rm1021 parent strain, after bleomycin treatment, the genomic DNA appears degraded (Fig. 3-5A), relative to untreated cells (Fig. 3-5B). This degradation is shown by the disappearance of the DNA. Contrastingly, in the absence of the BacA protein there appears to be minimal DNA degradation (Fig. 3-5A), relative to the DNA from the untreated control culture (Fig. 3-5B). Additionally, it was observed that presence of the pJG51A plasmid (containing the *S. meliloti bacA* gene) in the Rm1021 *ΔbacA* mutant, resulted in complete degradation of the DNA (Fig. 3-5C), relative to the untreated cells (Fig. 3-5D). Contrastingly, in Rm1021 Δ*bacA* mutant cells carrying the pRK404 control plasmid, minimal degradation was observed (Fig. 3-5C). Despite the fact that little DNA degradation is observed in strains lacking the BacA protein, these cells did lose viability, with less than 1% of the Rm1021 Δ*bacA* and Rm1021 Δ*bacA* & pRK404 viable cells remaining after treatment. Overall these data show that in *S. meliloti* there is more bleomycin induced DNA damage in the presence of the BacA protein which is consistent with a role for BacA in bleomycin uptake.

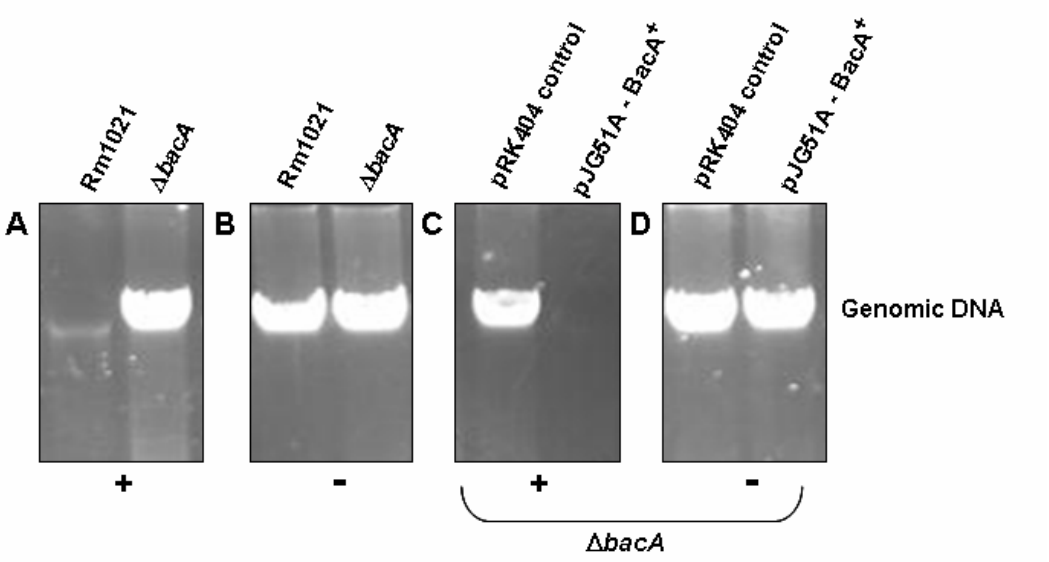

Figure 3-5. **Agarose gel electrophoresis of *S. meliloti* genomic DNA extracted from bleomycin treated and untreated cells.** Cultures of the defined strains in LB were treated with (A and C) and without (B and D) bleomycin A_5 (20 µg.ml^{-1}) in LB. The genomic DNA was then extracted and 2 µg analysed on a 1% (w/v) agarose gel. The symbols + and – refer to bleomycin treated and untreated DNA, respectively.

To rule out the possibility that bleomycin treatment may have caused changes to the genomic DNA, which may have resulted in its loss during the isolation procedure, a method to assess DNA degradation was used which did not rely on the isolation of the DNA. For this purpose, 4',6'-diamidino-2-phenylindole 2HCl (DAPI) was used. DAPI is a specific dye that forms a fluorescent complex upon binding DNA, with the advantage that it can be used to measure DNA in fixed bacterial cells (Ferguson *et al.*, 2000). Again cells were treated with bleomycin exactly as for the genomic DNA extraction. However, following treatment the cell pellets were re-suspended in toluene to permeabilise the cells. Subsequently the cells were incubated with DAPI overnight and the fluorescence of the treated cells was assessed, relative to the untreated control (Fig. 3-6). It could be observed that in the Rm1021 parent strain and the Rm1021 Δ*bacA* strain with pJG51A (encoding the *S. meliloti bacA* gene in pRK404), there was a substantial decrease in DAPI fluorescence, relative to the untreated control. Contrastingly the decrease in DAPI fluorescence in the Rm1021 Δ*bacA* with and without the pRK404 control plasmid was much smaller. So these data would also suggest that in *S. meliloti* there is more bleomycin induced DNA damage in the presence of the BacA protein. However, since there was a decrease in DAPI fluorescence in the Rm1021 Δ*bacA* strains with and without the pRK404 vector, indicative of genomic DNA loss, this would suggest there must be a BacA independent route of bleomycin entry into *S. meliloti.*

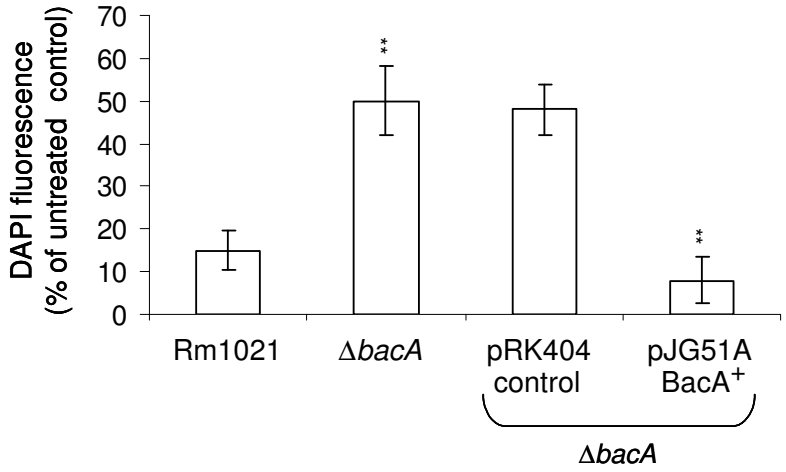

Figure 3-6. DAPI staining of fixed *S. meliloti* cells after bleomycin treatment, relative to untreated cells. Cultures of the defined strains were treated with and without bleomycin A_5 as in figure 3-5, and then the DNA was stained with 4',6-diamidino-2-phenylindole.2HCl (DAPI) and the fluorescence intensity of each strain was compared to that of untreated cells. The fluorescence intensity for each bleomycin treated strain is shown as a percentage relative to that of the untreated control. The significant values (**P<0.01) represent comparisons of the Rm1021 Δ*bacA* mutant with the parent strain and the Rm1021Δ*bacA* mutant carrying pJG51A with the Rm1021 Δ*bacA* mutant carrying pRK404. The error bars represent the standard deviation from the mean (n=3) for one experiment. The dataset shows preliminary data.

3.2.4. Transmission electron microscopy of *S. meliloti* cells treated with bleomycin reveals no observable signs of membrane damage or lysis

In eukaryotic cells bleomycin is able to cause cell damage independent from its effect on DNA by inducing lipid peroxidation (Hay *et al.*, 1991). Transmission electron microscopy (TEM) analysis of *S. cerevisiae* following bleomycin treatment revealed extensive lesions in the yeast cell wall (Moore *et al.*, 1992). To rule out that the loss of genomic DNA observed after bleomycin treatment of *S. meliloti* (Figs 3-5 & 3-6) was due to membrane damage and/or cell lysis, cultures of the Rm1021 parent strain and the *bacA* mutant were treated with and without bleomycin A_5 and then analysed by TEM. Here, the bleomycin treatment was performed using the exact same conditions as for the genomic DNA extraction and the DAPI experiment (Figs 3-5 & 3-6, respectively). Additionally, the viability of the samples was also assessed and it was observed that there was complete loss of viability for the

Rm1021 parent strain and less then 1% of the Rm1021 Δ*bacA* cells remained viable. Under these conditions no visible signs of bleomycin-induced lesions were detected in the cell envelope of either the Rm1021 parent (Fig. 3-7A &B) or the Rm1021 Δ*bacA* mutant (Fig. 3-7C & D), relative to the untreated control cultures (Fig 3-7E, F, G & H). Additionally, it was observed that bleomycin treatment of *S. meliloti* strains with and without the BacA protein, under the same conditions, did not lead to cell lysis since the OD_{600} of the cultures remained constant throughout the time course of the experiment (Fig. 3-8). Taken together these results would suggest that bleomycin A_5 treatment does not lead to cell envelope damage in *S. meliloti.*

However, during the TEM analysis it could be observed that cells of the *S. meliloti* Rm1021 Δ*bacA* mutant contained increased amounts of white granules after bleomycin treatment relative to the Rm1021 parent strain (Fig. 3-7CD and AB, respectively). These granules were absent in the untreated control cells (Fig. 3-7E, F, G and H). The chemical composition of these white granules will require further investigation.

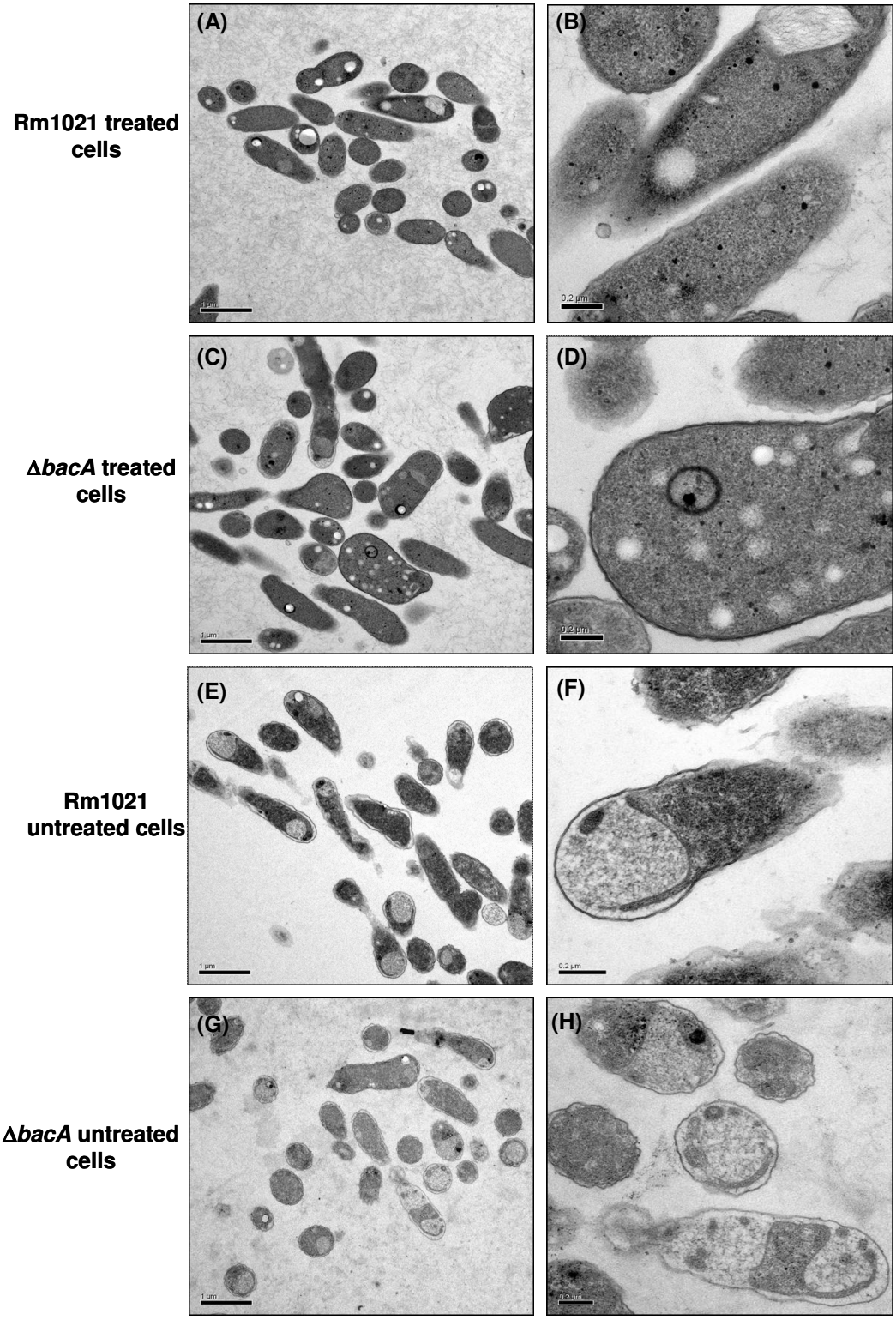

Figure 3-7. TEM micrographs of *S. meliloti* cells treated with and without Bleomycin A_5. The defined strains were treated with (A, B, C and D) and without (E, F, G and H) bleomycin A_5 (20 µg.ml^{-1}) in LB medium for 2 hours. Bars: 1 µm (A, C, E and G) and 0.2 µM (B, D, F and H).

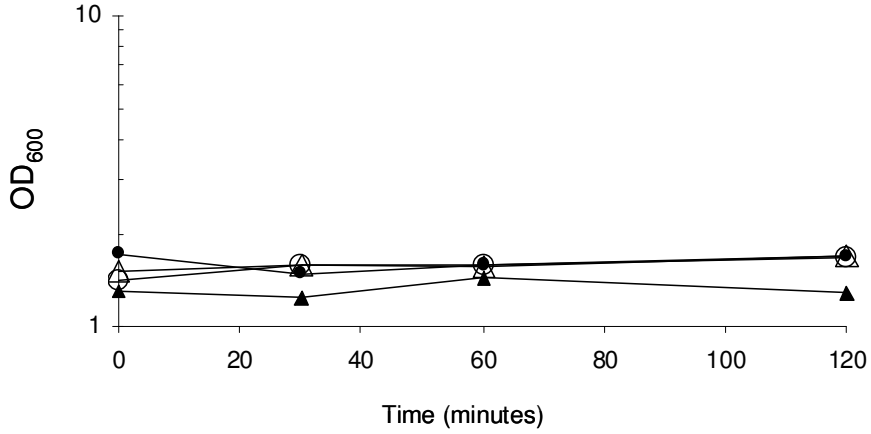

Figure 3-8. Optical density of *S. meliloti* strains exposed to Bleomycin A_5. Mid-exponential phase cultures of the Rm1021 parent (open circles), the Δ*bacA* mutant (filled circles, *ΔbacA* & pRK404 (open triangles) and *ΔbacA* & pJG51A (filled triangles) were exposed to bleomycin A_5 (20 $\mu g.ml^{-1}$) for 2 hours and the optical density of the cultures was measured at the defined time points.

3.2.5. The BacA protein does not increase the sensitivity of *S. meliloti* towards other DNA damaging agents

Although the data presented so far are consistent with a role for BacA in bleomycin uptake, it may be possible that the BacA protein could also be affecting *S. meliloti* DNA resulting in an increased sensitivity towards bleomycin A_5-induced DNA damage. Hence, the sensitivity of the Rm1021 parent strain and the Rm1021 Δ*bacA* mutant towards three other DNA damaging agents, methylglyoxal (Ferguson *et al.*, 2000), mitomycin C (Otsuji & Murayama, 1972) and methyl methane sulfonate (MMS) (Grzesiuk & Janion, 1996), previously shown to induce DNA damage in *E. coli*, was next assessed. The Rm1021 parent cells and the Rm1021 Δ*bacA* mutant were exposed to these agents by filter disc assay (Fig. 3-9) and growth inhibition was assessed. However, it was observed that presence of the BacA protein did not sensitize *S. meliloti* cells to any of the agents tested. In fact loss of the BacA protein appeared to slightly sensitize *S. meliloti* cells to mitomycin C induced damage (Fig. 3-9B).

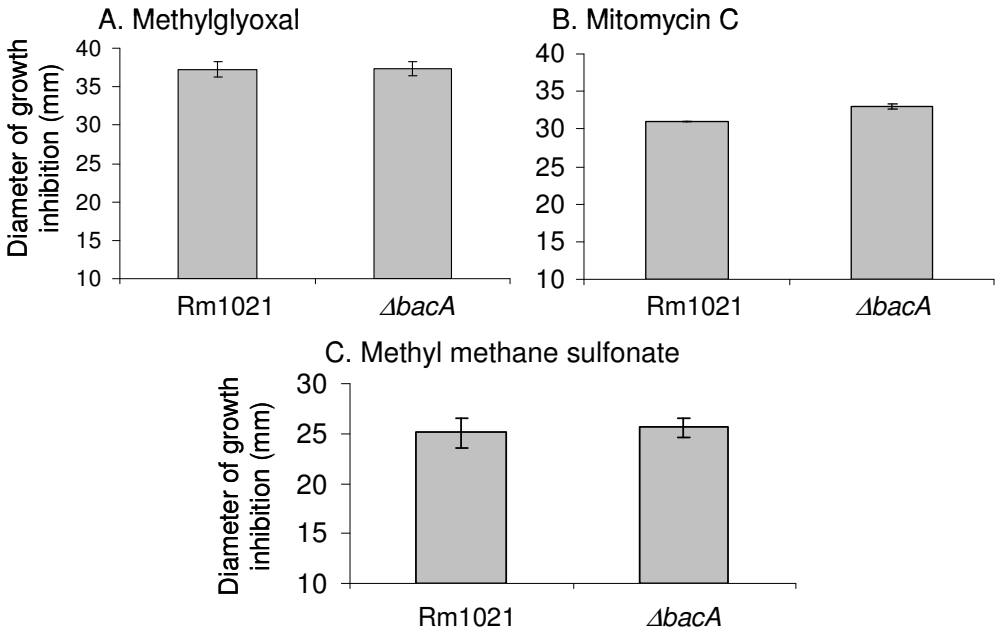

Figure 3-9. Sensitivity of the Rm1021 parent and Rm1021 *ΔbacA* mutant exposed DNA damaging agents by disc diffusion assay. The defined strains were exposed to methylglyoxal (5 µl of a 40% w/v aqueous solution) (A), mitomycin C (5 µl of a 0.3 mg.ml^{-1} aqueous stock solution) (B) and methyl methane sulfonate (5 µl of a 50 % v/v solution) (C) on LB agar. The error bars represent the standard deviation from the mean (n=3) for one experiment. For (A) and (B) the datasets shown are representative of the trends seen in two independent experiments and (C) shows preliminary data.

Additionally, the genomic DNA content of *S. meliloti* strains with and without the BacA protein, after exposure to the DNA damaging agent MMS, was next examined. Mid-exponential phase cells were exposed to MMS and the genomic DNA was extracted and analysed as described previously. Upon exposure of the Rm1021 parent and Rm1021 Δ*bacA* mutant to 1% (v/v) MMS for 2 hours (Fig. 3-10A), complete degradation of the genomic DNA was observed for both strains, relative to the untreated control (Fig. 3-10C), with no differences observed in the presence and absence of the BacA protein. Next, cells were exposed a lower dose of 0.25% (v/v) MMS for 30 minutes (Fig. 3-10B), DNA degradation was observed in the Rm1021 parent strain (Fig. 3-10B), relative to the untreated cells (Fig. 3-10C) with slightly less degradation occurring in the Rm1021 Δ*bacA* mutant. The genomic DNA of the Rm1021 Δ*bacA* mutant carrying the pRK404 control plasmid and pJG51A (encoding the *S. meliloti bacA* gene in pRK404) was next assessed. Upon exposure to 1% (v/v)

MMS for 2 hours, complete genomic DNA degradation was observed for both strains (Fig. 3-10D), relative to the untreated cells (Fig. 3-10F). However, when the cells were exposed to a lower dose of 0.25% (v/v) MMS for 30 minutes (Fig. 3-10E), more genomic DNA degradation was apparent in the Rm1021 Δ*bacA* mutant carrying the pRK404 vector than in the Rm1021Δ*bacA* mutant carrying pJG51A. Therefore, taken together these data would suggest that MMS induced DNA degradation is not BacA dependent.

Furthermore the viability of the strains was assessed after exposure to 0.5% (v/v) MMS for 2 hours. In this case, for all four strains no viable cells remained at the end of the treatment. Viability was also assessed after treatment of the cells with 0.3% MMS (v/v) for 2 hours. In this case, no differences were observed in the presence and absence of the BacA gene with 0.03-0.05% of viable cells remaining after the treatment, for all strains. Therefore, taken together these data provide evidence that the BacA protein specifically sensitizes *S. meliloti* DNA towards bleomycin A_5-induced damage and are consistent with the BacA protein playing a role in uptake of bleomycin into cells, rather than exerting its affect at the level of the DNA.

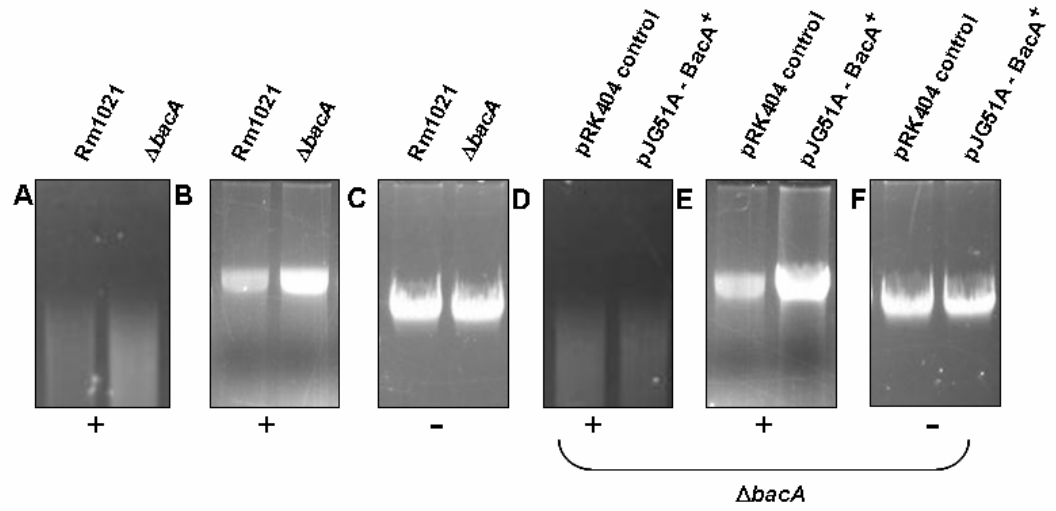

Figure 3-10. Agarose gel electrophoresis of *S. meliloti* genomic DNA extracted from MMS treated and untreated cells. Mid-exponential phase cells of the defined strains were exposed to 1% MMS (v/v) (A) and (D) for 2 hours, 0.25% MMS (v/v) (B) and (E) for 30 minutes or grown in LB for 2 hours (C) and (F). After treatment, genomic DNA was extracted and 2 µg was resolved on a 1% agarose gel. The symbols + and – refer to MMS treated and untreated DNA respectively. The dataset shows preliminary data.

3.2.6. The RecA protein protects *S. meliloti* against bleomycin damage but loss of *bacA* still confers protection in the absence of the RecA protein

As previously discussed the RecA protein is a key regulator in the SOS response, which is induced in *E. coli* upon DNA damage (Brent & Ptashne, 1981; Little *et al.*, 1980; Little *et al.*, 1981). In *E. coli* it has been demonstrated that the RecA protein is involved in the repair process of bleomycin induced DNA damage (Yamamoto *et al.*, 1984). The sensitivity of *S. meliloti* cells lacking the RecA protein to bleomycin was next investigated. It was found that the *S. meliloti* Rm1021 *recA*::Tn*5-233* mutant did display an increased level of growth inhibition when exposed to both bleomycin A_5 and A_2, relative to the Rm1021 parent strain (Fig. 3-11A & B, respectively). Therefore, these data provide further evidence that bleomycin can enter into *S. meliloti* causing DNA damage which would be repaired by RecA. To investigate if the RecA protein is essential for the increased level of bleomycin resistance observed in the Rm1021 Δ*bacA* mutant, the *recA*::Tn*5-233* insertion was next transduced into the Rm1021 Δ*bacA* mutant background using M12 phage (Finan *et al.*, 1984). It was observed that the *S. meliloti* Rm1021 Δ*bacA/recA*::Tn*5-233* double mutant displayed an decreased level of growth inhibition, relative to the Rm1021 *recA*::Tn*5-233* single mutant (Fig. 3-11A & B). Therefore, this would suggest that the BacA protein is not increasing bleomycin-induced DNA damage through an effect on the RecA protein. However, since the *S. meliloti* Rm1021 Δ*bacA/recA*::Tn*5-233* double mutant is more sensitive to bleomycin than the *S. meliloti* Rm1021 Δ*bacA* single mutant, this suggests that some bleomycin can still enter *S. meliloti* in the absence of BacA and cause damage, which would normally be repaired by RecA.

In order to confirm these findings, the viability of the same strains was assessed over 60 minutes, upon exposure to bleomycin A_5 (Fig. 3-11C) in liquid culture. Here, the strains showed the same levels of sensitivity as for the filter disc assays, in that overall the Rm1021 Δ*bacA* mutant displayed an increased resistance to bleomycin relative to the Rm1021 parent strain, and the Rm1021 *recA*::Tn*5-233* mutant exhibits the greatest sensitivity, which is reduced by loss of the BacA protein.

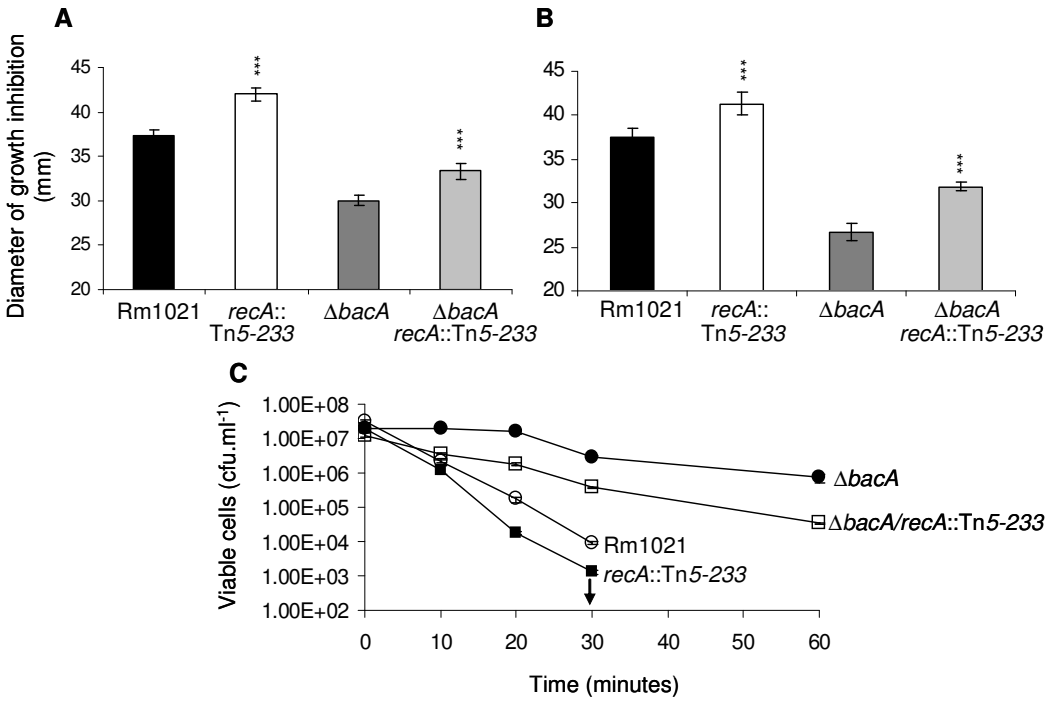

Figure 3-11. Sensitivity of the *S. meliloti* Rm1021 *recA*::Tn*5-233* mutant to bleomycin in the presence and the absence of the BacA protein. Growth inhibition for the defined strains exposed to bleomycin A_5 (5 µl of a 2 mg.ml^{-1} stock) (A) and bleomycin A_2 (5 µl of a 5 mg.ml^{-1} stock) (B). The defined strains were exposed to bleomycin A_5 (3 µg.ml^{-1}) over 60 minutes and viability assessed at the defined times (C). All the datasets shown are representative of the trends seen in two independent experiments. In each case the error bars represent the standard deviation from the mean (n=3) for one experiment. In (A) and (B) the significant values (***P<0.001) represent comparisons of the Rm1021 *recA*::Tn*5-233* mutant to the Rm1021 parent strain and comparisons of the Rm1021 Δ*bacA*/*recA*::Tn*5-233* double mutant to the Rm1021 *recA*::Tn*5-233* mutant. In (C) upon comparison of the Rm1021 *recA*::Tn5-233 mutant to the Rm1021 parent strain, a significant value of at least (*P<0.05) was observed for each time point and for comparison of the Rm1021 Δ*bacA*/*recA*::Tn*5-233* double mutant to the Rm1021 *recA*::Tn*5-233* mutant a significant value was at least (***P<0.001) was observed for each time point. In data set (C) the arrow represents complete loss of viability.

3.2.7. Glutathione protects *S. meliloti* against bleomycin but protection is independent of the BacA protein

The data presented so far in this chapter, would suggest the BacA protein is involved but not essential for bleomycin uptake. However, it may be possible that less bleomycin accumulates in the Rm1021 Δ*bacA* mutant, resulting in less DNA damage due to an increase in bleomycin detoxification, relative to the parent strain. Hence it was important to rule out the possibility that the BacA protein was affecting a detoxification process. Several studies have implicated a role for the tri-peptide glutathione in detoxification of bleomycin in eukaryotic cells. The hypersensitivity of Chinese hamster ovary cells to bleomycin was found to be due to a lack of glutathione *S*-transferase activity (Giaccia *et al.*, 1991) and cellular glutathione levels were also found to be up-regulated by bleomycin in bovine pulmonary endothelial cells (Day *et al.*, 2002). In a previous study intracellular glutathione has been shown to be necessary for a successful symbiosis of *S. meliloti* with alfalfa (Harrison *et al.*, 2005). Glutathione is the most abundant non-protein thiol found in many organisms (Fahey *et al.*, 1978; Fahey & Sundquist, 1991; Penninckx & Elskens, 1993) (refer to section 5.1 for more details). Hence, it was next investigated if the presence of glutathione was important for protection in *S. meliloti* from bleomycin toxicity.

The *S. meliloti gshA* mutant (defective in the γ-glutamyl cysteine synthetase enzyme) which lacks intracellular glutathione (Harrison *et al.*, 2005) was found to display an increased level of sensitivity to bleomycin A_5. This would suggest a role for glutathione in detoxification of bleomycin in *S. meliloti* (Fig. 3-12). Next, to determine if glutathione was important for the increased resistance observed in the Rm1021 Δ*bacA* mutant the *gshA* insertion was transduced into the Rm1021 Δ*bacA* mutant using M12 phage (Finan *et al.*, 1984). However, since the *S. meliloti* Δ*bacA/gshA* double mutant displayed an increased level of resistance, relative to the Rm1021 *gshA* single mutant (Fig. 3-12), these data would suggest that the presence of glutathione is not essential for the increased resistance towards bleomycin in the absence of the BacA protein. Yet, it does appear the loss of BacA confers less protection in *S. meliloti* in the absence of glutathione. However, these data do provide further evidence that the reduced level of bleomycin induced DNA

degradation observed in the absence of the BacA protein is due to a reduced level of bleomycin uptake, relative to the Rm1021 parent strain.

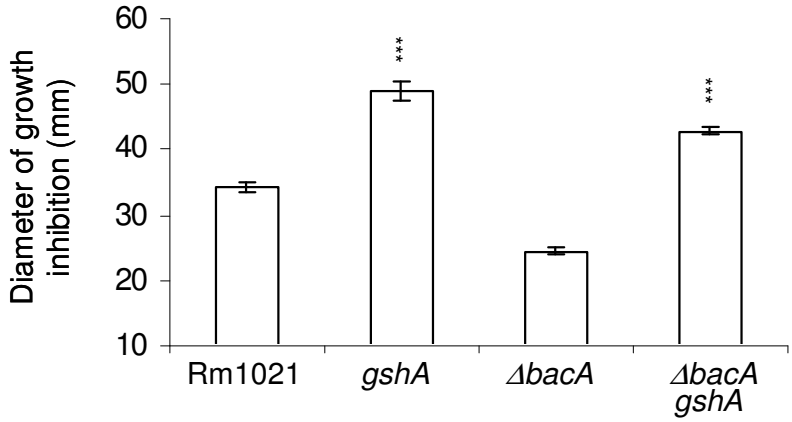

Figure 3-12. Sensitivity of the *S. meliloti* glutathione mutant to bleomycin A_5. Growth inhibition of the defined strains exposed to bleomycin A_5 (2 µl of a 2 $mg.ml^{-1}$ stock) on LB agar. The significant values (***P<0.001) shown, represent comparisons between the Rm1021*gshA* mutant and the Rm1021 parent strain and the Rm1021 Δ*bacA*/*gshA* double mutant was compared to the Rm1021 *gshA* single mutant. In each case the error bars represent the standard deviation from the mean (n=3) for one experiment. The dataset shown is representative of the trends seen in two independent experiments.

3.2.8. The BacA protein plays a role but is not essential for the uptake of fluorescently labelled bleomycin

To investigate further the possible role of the BacA protein in bleomycin uptake, fluorescently labelled bleomycin A_5 (F-BLM) was prepared by conjugation of bleomycin A_5 and the fluorescent molecule 5-(and-6)-carboxyfluorescein, succinimidyl ester [5(6)-FAM, SE] (chapter 2, section 2.9). A viability assay was initially performed to determine if the presence of the BacA protein also conferred an increased sensitivity of *S. meliloti* towards F-BLM. The number of viable cells for the Rm1021 parent strain and the Rm1021 *ΔbacA* mutant were determined after exposure to 1.5 $\mu g.ml^{-1}$ of F-BLM (Fig. 3-13 A) and a higher dose of F-BLM (Fig. 3-13 B) over one hour. These data show that F-BLM reduces the viability of *S. meliloti* and that the presence of the BacA protein confers an increased sensitivity

towards this labelled form of the drug, thus confirming that F-BLM would be suitable to use as an indicator of bleomycin entry into *S. meliloti* cells.

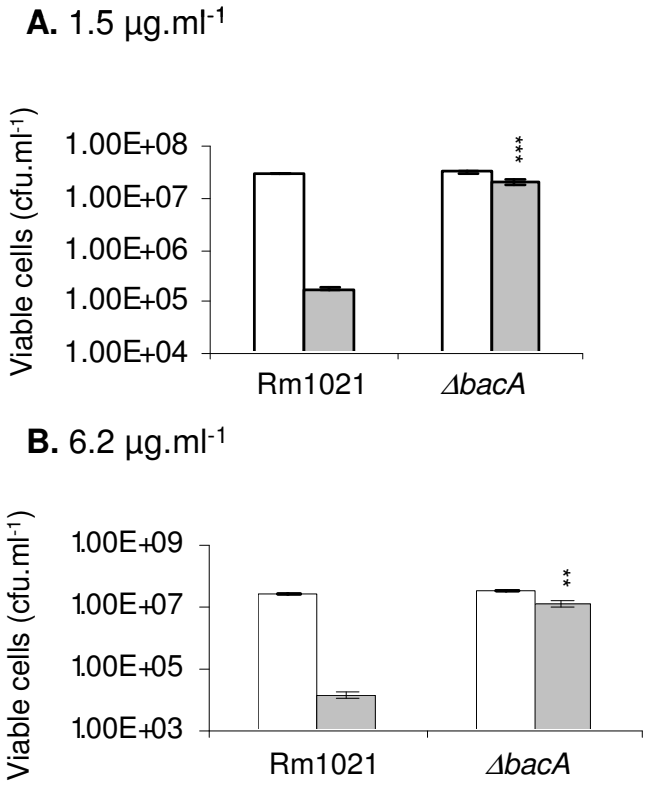

Figure 3-13. Cell viability of the Rm1021 parent strain and *ΔbacA* mutant after exposure to F-BLM. For (A) and (B) mid-exponential phase cultures of the defined strains were exposed to F-BLM as defined and cell viability was determined before (open bars) and 60 mins (shaded bars) after addition. The significant values shown (**P<0.01; ***P<0.001) represent comparisons of the Rm1021 Δ*bacA* mutant and the Rm1021 parent strain. Dataset (A) is representative of the trends observed in two independent experiments. Dataset (B) shows preliminary data. In each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

A previous study investigating bleomycin uptake in the yeast *Saccharomyces cerevisiae* assessed F-BLM uptake using a fluorometer (Aouida *et al.*, 2004). Thus, in this work initial F-BLM uptake experiments were attempted using a fluorometer, to measure intracellular fluorescence. However, this methodology did not prove to be sensitive enough to accurately detect intracellular F-BLM in *S. meliloti*. Instead, it was decided to examine accumulation of F-BLM in *S. meliloti* by flow cytometry analysis, since this method had been shown to be sensitive enough to accurately

assess uptake of a fluorescent labelled form of the proline rich peptide Bac7 in *E. coli* cells (Mattiuzzo *et al.*, 2007).

For the flow cytometry experiments, mid-exponential phase cells were exposed to F-BLM, after which, the cells were washed in LB medium, finally re-suspended in sodium phosphate buffer and analysed immediately. An initial flow cytometry experiment determined that following treatment for 15 minutes with F-BLM, Rm1021 parent cells showed detectable cell-associated fluorescence, relative to untreated cells (Fig. 3-14A). Additionally treatment of Rm1021 cells with the 5(6)-FAM, SE label alone did not increase the fluorescence above the untreated control (Fig. 3-14B), thus providing evidence that an increase in fluorescence in the F-BLM treated cells was not due to unspecific interaction of 5(6)-FAM, SE with the cells. These data would therefore suggest that F-BLM can associate with *S. meliloti* cells. To examine if the BacA protein is involved in the association of F-BLM with *S. meliloti*, flow cytometry analysis was next performed with the Rm1021 Δ*bacA* mutant cells after exposure to F-BLM for 15 minutes (Fig. 3-14C). These data show that in the absence of the BacA protein, a smaller number of cells increase their fluorescence after F-BLM exposure (Fig. 3-14C) compared to cells of the Rm1021 parent strain (Fig. 3-14B). Thus, these data show that F-BLM can associate with *S. meliloti* cells, even in the absence of the BacA protein.

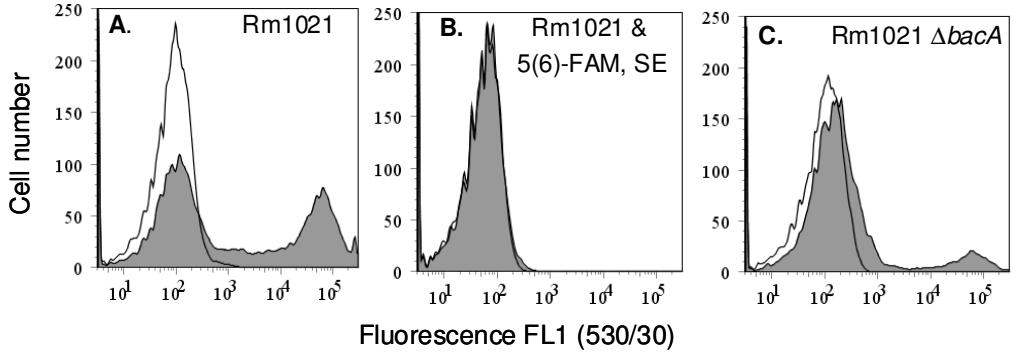

Figure 3-14. Flow cytometry analysis of *S. meliloti* cells exposed to F-BLM and 5 (6)-FAM, SE for 15 minutes. Mid-exponential phase cells of the defined strains were treated with (shaded histograms) or without (empty histograms) 1.5 μg.ml^{-1} of F-BLM (A) and (C) or 3 μg.ml^{-1} of 5(6)-FAM, SE (B). Treatment in all cases was for 15 minutes. All datasets are representative of the trends observed in two independent experiments.

Next the Rm1021 parent strain and Rm1021 Δ*bacA* mutant were incubated with F-BLM for a longer time period of 60 minutes. From these data it can also be observed that both the Rm1021 parent strain and the Rm1021 Δ*bacA* mutant show cell associated fluorescence, relative to the untreated control (Fig. 3-15A &B, respectively). Again it can be observed the Rm1021 Δ*bacA* mutant has a slightly lower number of cells showing cell associated fluorescence relative to the Rm1021 parent strain (Fig. 3-15A & B, respectively).

However, it can also be noted that as observed for the 15 minute time point (Fig. 3-14A &C) in both the Rm1021 parent and Rm1021 Δ*bacA* mutant, not all the cell population appears to become associated with F-BLM (Fig. 3-15A & B). Interestingly, a study looking at the uptake of radio-labelled bleomycin A_2, in *S. cerevisiae* also found that not all cells took up bleomycin, when a cell population was assessed (Moore *et al.*, 1992).

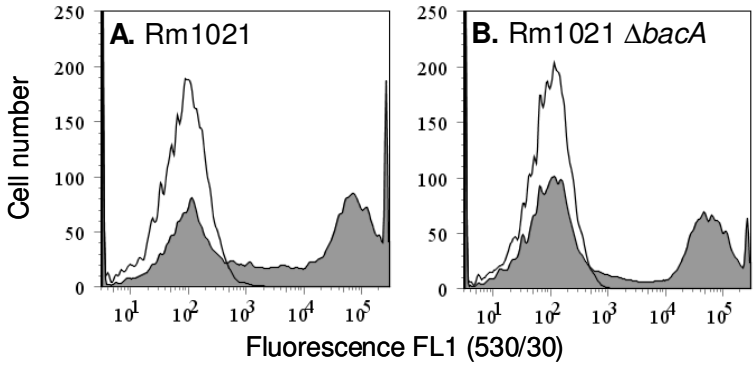

Figure 3-15. Flow cytometry analysis of *S. meliloti* cells exposed to F-BLM for 1 hour. Mid-exponential phase cultures of the defined strains (A) and (B) were treated with (shaded histograms) or without (empty histograms) F-BLM (1.5 μg.ml^{-1}) for 1 hour. The datasets are representative of the trends observed in three independent experiments.

The data presented so far provide evidence that F-BLM can associate with *S. meliloti* cells and that this association is not dependent upon the presence of the BacA protein. However, the data does not differentiate between intracellular and extracellular accumulation of F-BLM. Although the *S. meliloti* cells were washed prior to flow cytometry analysis to remove any surface-attached F-BLM, cells were next pre-treated with the extracellular quencher Trypan Blue (TB). A recent study had established and optimized the use of TB as an extracellular fluorescence

quencher in bacterial cells (Mattiuzzo *et al*, 2007). By using fluorescently labelled polymyxin B, a antibiotic peptide known to interact with cells through binding to LPS on the outer membrane, it was possible to show that TB specifically quenched fluorescence that originated from the outer surfaces of *E. coli* cells (Mattiuzzo *et al*, 2007).

Treatment of both the Rm1021 parent and Rm1021 Δ*bacA* mutant cells with TB prior to analysis, did result in a significant reduction in fluorescence of the cells (Fig. 3-16A & C, respectively), when compared to the Rm1021 parent and Rm1021 Δ*bacA* mutant cells without TB treatment (Fig. 3-16B &D, respectively). Hence this would suggest a large amount of the fluorescence observed was due to extracellular binding. However, following TB treatment, it can still be observed that less Rm1021 Δ*bacA* mutant cells appear to accumulate F-BLM, relative to the Rm1021 parent (Fig. 3-16A & C). These data therefore suggest that the BacA protein plays some role in the uptake of F-BLM, but it does not appear to be essential for uptake.

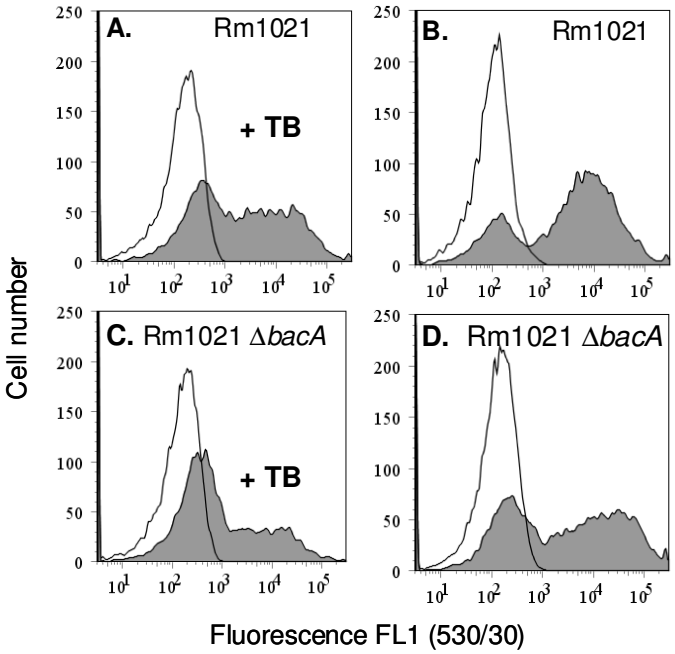

Figure 3-16. Flow cytometry analysis of *S. meliloti* cells exposed to F-BLM for 1 hour. The defined strains were treated with (shaded histograms) or without (empty histograms) 6.2 μg.ml^{-1} of F-BLM for 60 minutes and analysed by flow cytometry with (A & C) and without (B & D) trypan blue (TB) (1 mg.ml^{-1}) treatment for 10 minutes. The datasets show preliminary data.

3.2.9. The polyamine spermine protects *S. meliloti* against bleomycin independently of the nature of the R-group and the BacA protein

As discussed previously, different forms of bleomycin vary in the nature of their R-group (Section 3.1 and fig. 3-1). In the yeast *S. cerevisiae,* the bleomycin A_5 R-group has been implicated to be important for bleomycin uptake (Aouida *et al.*, 2004). The bleomycin A_5 R-group has a structure similar to polyamines (Fig. 3-1 & 3-17). The same study found that deletion of a protein kinase *ptk2* in *S. cerevisiae* which positively regulates polyamine transport prevented uptake of fluorescently labelled bleomycin A_5 (Aouida *et al.*, 2004). So in yeast it was hypothesized bleomycin A_5 may enter yeast cells through a polyamine transport system. Additionally, the study found that a 16 hour pre-incubation of the cells with both the polyamines spermine and spermidine reduced the uptake of fluorescently labelled bleomycin-A_5 in a concentration dependent manner. Thus, it was next investigated if spermine protects *S. meliloti* against bleomycin damage.

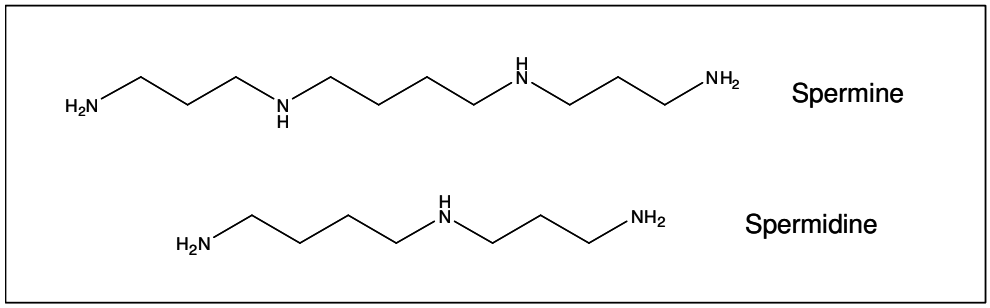

Figure 3-17. Depiction of the polyamines spermine and spermidine. Structures were prepared by Hazel Phillips using ChemDraw Std 10.0.

Sensitivity of the Rm1021 parent strain to bleomycin A_5 was assessed in liquid culture in the presence of 1 mM spermine and indeed it could be observed that presence of this polyamine did confer protection from bleomycin A_5 killing, relative to cells without spermine (Fig. 3-18).

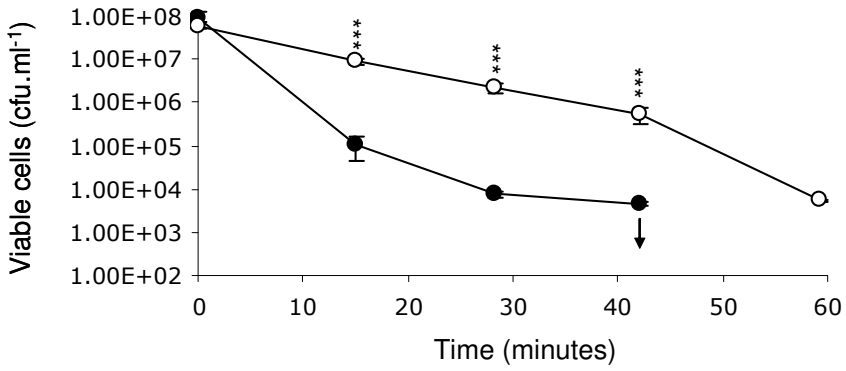

Figure 3-18. Sensitivity of the Rm1021 parent exposed to bleomycin A_5 in liquid culture in the presence of 1 mM spermine. The Rm1021 parent was exposed to bleomycin A_5 (6 µg.ml^{-1}) in LB alone (closed circles), and LB supplemented with 1mM spermine (open circles) and viability was assessed at the defined times**.** In each case the error bars represent the standard deviation from the mean (n=3) for one experiment. The dataset shows preliminary data. The significant values (***P<0.001) represent comparisons of the Rm1021 parent in LB and in LB plus 1 mM spermine.

Having demonstrated that the presence of spermine increases the resistance of the *S. meliloti* Rm1021 parent strain to bleomycin, it was next important to determine if there was any interplay between the protection by the polyamine spermine and protection in the absence of the BacA protein. The sensitivity of the Rm1021 Δ*bacA* mutant to bleomycin in the presence of spermine was next assessed. Like the Rm1021 parent (Fig. 3-19A), the Rm1021 Δ*bacA* mutant (Fig. 3-19B) also exhibits an increased resistance to bleomycin in the presence of spermine, providing evidence spermine protects *S. meliloti* even in the absence of the *bacA* gene. Thus, these data would suggest that protection by spermine is independent of the BacA protein. Additionally, for both the Rm1021 parent (Fig. 3-19A) and the *bacA* mutant (Fig. 3-19B), it was possible to demonstrate that the presence of spermine was able to protect against different forms of bleomycin, showing protection by spermine occurs independently of the nature of the R-group.

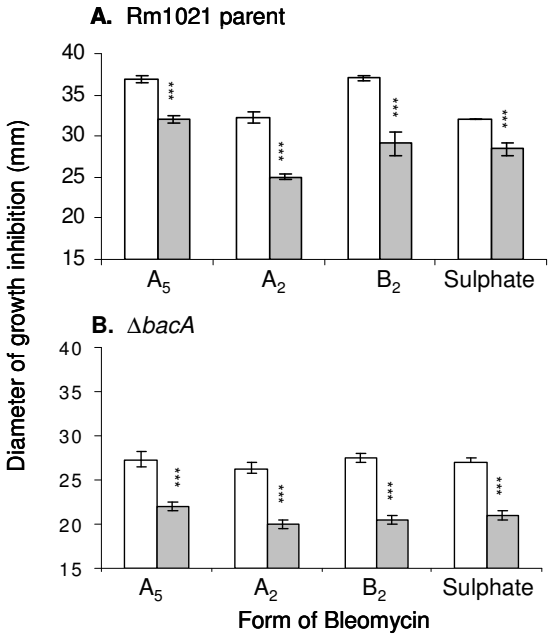

Figure 3-19. Sensitivity of the Rm1021 parent and the Rm1021 *ΔbacA* mutant to bleomycin in the presence of spermine. (A) Growth inhibition for the *S. meliloti* Rm1021 parent strain exposed to bleomycin on LB (empty bars), and LB supplemented with 1 mM spermine (filled bars). For bleomycin forms A_2, B_2 and sulphate, cells were exposed to 5 µl of a 5 mg.ml^{-1} aqueous stock solution and for bleomycin A_5 cells were exposed to 5 µl of a 2 mg.ml^{-1} aqueous stock solution. (B) As in (A) but data for the Δ*bacA* mutant is shown. In each case the error bars represent the standard deviation from the mean (n=3) for one experiment. The significant values (***P<0.001) represent comparisons of sensitivity on LB and LB plus spermine. For the B_2 form of bleomycin the datasets shown are preliminary.

Thus, combined these data show that polyamines can protect *S. meliloti* when present in the growth media and that protection occurs independently of the BacA protein and of the bleomycin R-group.

3.2.10. Preliminary investigation into a putative polyamine ABC transport system in *S. meliloti*

As discussed, a common uptake system has been implicated for both bleomycin and polyamine uptake in yeast (Aouida *et al.*, 2004). These data presented thus far have demonstrated that in *S. meliloti* spermine protects against bleomycin damage independently of the BacA protein. Additionally, it has been shown that in the

absence of the BacA protein bleomycin is still able to enter *S. meliloti.* Thus, it was next investigated if disruption of a putative polyamine uptake system in *S. meliloti* would affect sensitivity to bleomycin. In *E. coli* the rate of polyamine uptake is in the order: putrescine> spermidine> spermine (Kashiwagi *et al.*, 1990). Uptake is mainly catalysed by two polyamine uptake systems, the spermidine-preferential *potABCD* operon, (Furuchi *et al.*, 1991; Kashiwagi *et al.*, 1990), and the putrescine-specific system *potFGHI* (Kashiwagi *et al.*, 1990; Pistocchi *et al.*, 1993). Both of these operons encode ATP binding cassette (ABC) polyamine uptake systems (Fig. 3-20).

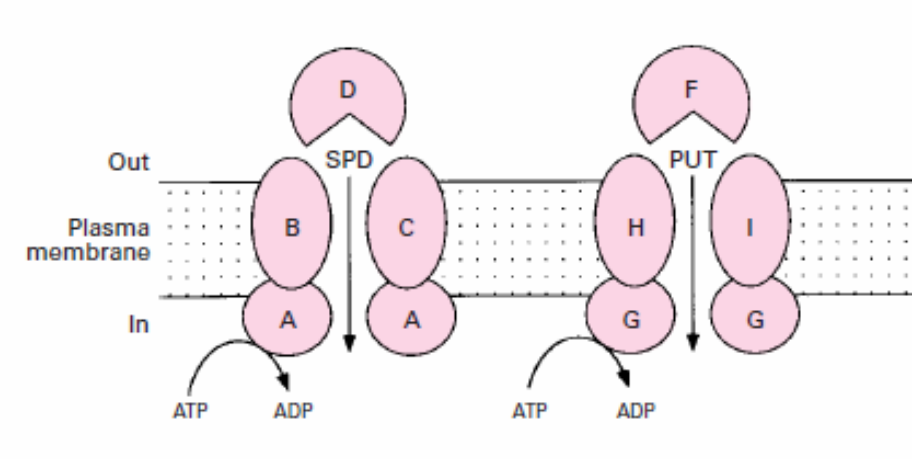

Figure 3-20. Polyamine transport systems in *E. coli*. (Igarashi & Kashiwagi, 1999) SPD=Spermidine. PUT=Putrescine.

At the time when the experiments were performed for this chapter, the *S. meliloti* genome (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi) had four genes *smb21273*, *smb21274*, *smb21275* and *smb21276* annotated as *potD, potB, potC* and *potA* respectively. However, in June 2008 due to new resources and data made available there was an annotation update in the *S. meliloti* genome (Becker *et al.*, 2008), resulting in changes in annotation and some genes being removed. Subsequently, the *smb21276* gene annotation was removed and the *smb21273* gene was re-annotated as encoding an ABC transporter periplasmic solute binding protein and *smb21274* and *smb21275* genes were re-annotated as encoding ABC transporter permease components (Fig. 3-21). Collectively these three genes are still annotated as an ABC transport system, and are located downstream of a putative transcriptional

regulator (Fig. 3-21), with the *smb21273* gene (formerly annotated *potD*) still thought to encode a solute binding protein and the *smb21275* gene (formerly annotated *potC*) still thought to encode a permease component. When protein BLAST searches (http://blast.ncbi.nlm. nih.gov/Blast.cgi) were performed with the *smb21273* and *smb21275* sequences against either the *S. meliloti* protein database or against all bacterial protein databases, proteins with a high sequence similarity and identity were annotated as polyamine transporters.

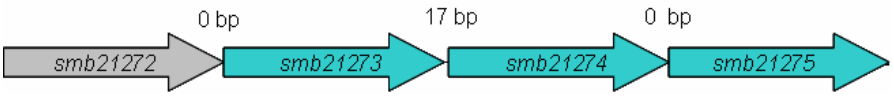

Figure 3-21. Diagrammatic representation of the *smb21273*, *smb21274* and *smb21275* gene operon. The genes within this region are *smb21272* (annotated as encoding a putative LacI family transcriptional regulator), *smb21273* (annotated as encoding an ABC transporter periplasmic solute binding protein) and *smb21274* and *smb21275* (annotated as encoding ABC transporter permease components). The numbers shown above in base pairs are the distances between each gene. (iant.toulouse.inra.fr/bacteria/ annotation/cgi/rhime.cgi).

With the view of making mutants in the putative *S. meliloti pot* operon, the plasmid integration mutant library (http://www.cebitec.uni Bielefeld .de /transcriptomics/sm-genome/sm-mutagenesis.html), purchased from the University of Bielefeld (chapter 2, section 2.5) was used. In this case two *E. coli* S17-1 clones, one containing a 334 bp internal fragment of the *smb21273* gene and the second containing a 309 bp internal fragment of *smb21275* gene in the pK19mob2ΩHMB mobilizable suicide vector were available in the library and these were mobilized into Rm1021 via conjugation. The recombinant clones were then selected by purification onto the appropriate antibiotics.

Due to time constraints it was not possible to confirm disruption of the *smb21275* and *smb21273* genes by PCR. It was observed that neither the putative *smb21275* mutant nor the putative *smb21273* mutant appeared to have an increased resistance to bleomycin A_5 (Fig. 3-22). However, the mutants did appear to have an increased sensitivity to bleomycin, relative to the Rm1021 parent. When exogenous spermine was added to the LB agar it was shown to still confer protection in the putative mutants against bleomycin (Fig. 3-22). It should be noted that there are multiple predicted polyamine uptake systems encoded in the genome of *S. meliloti*

(http://iant.toulouse.inra.fr/ bacteria/ annotation /cgi/ rhime.cgi). However, these preliminary data have shown that these putative mutants display an increased sensitivity to bleomycin, relative to the Rm1021 parent strain. Therefore, this may suggest this ABC transport system may be involved in uptake of a particular solute(s) able to protect against bleomycin induced damage. However, these two putative mutants require confirmation by PCR, before further speculation.

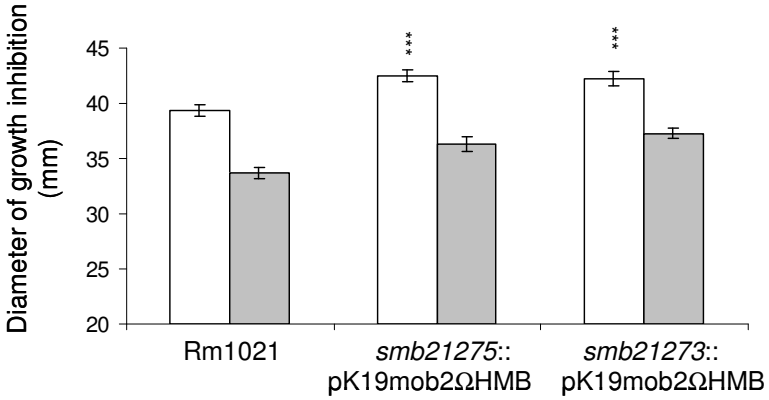

Figure 3-22. Bleomycin sensitivity of mutants which carry disruptions in the predicted ABC transport operon, in the presence and absence of 1mM spermine. Growth inhibition of the defined strains exposed to bleomycin A_5 (5 µl of a 2 mg.ml^{-1}stock) on LB plates (empty bars), and LB plates supplemented with 1 mM spermine (filled bars). In each case the error bars represent the standard deviation from the mean (n=3) for one experiment. The dataset shows preliminary data. The significant values (***P<0.001) represent comparisons of the *smb21275* and *smb21273* mutants to the Rm1021 parent strain

3.2.11. Loss of the putative glyoxalase/bleomycin resistance protein (Smc04266) in *S. meliloti* does not result in an increased sensitivity to bleomycin

Interestingly in the *S. meliloti* genome, there is a gene, *smc04266*, located in the same region as the *lpxXL* and *acpXL* genes (Fig. 3-23), annotated as encoding a putative glyoxalase /bleomycin resistance protein. It was next investigated if this protein plays any role in *S. meliloti* in protection against bleomycin. Initially, BLAST searches (http://blast.ncbi.nlm. nih.gov/Blast.cgi) were performed using the Smc04266 protein sequence against all bacterial protein sequence databases. It was found that proteins with a significant identity and similarity to Smc04266 are also

annotated as putative glyoxalase/bleomycin resistance proteins. The protein sequence was then compared to the *E. coli* bleomycin resistance protein (Tn*5 ble*), the *Streptococcus aureus* bleomycin resistance protein (Sh *ble*) and the *S. meliloti* and *E. coli* glyoxalase I proteins (Table 3-1). The protein with the greatest similarity/identity to Smc04266 was the *S. aureus* bleomycin resistance protein (Berg *et al.*, 1998), with 36% identity and 40% similarity.

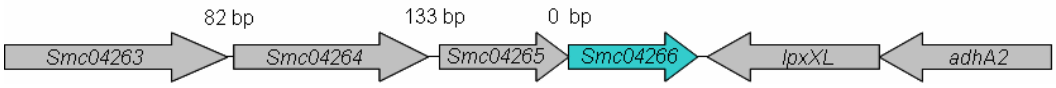

Figure 3-23. Diagrammatic representation of the genomic region surrounding the *smc04266* gene. Genes surrounding *smc04266* are *smc04263* (encoding a putative sodium:alanine symporter family protein), *smc04264* (encoding a putative transcriptional regulator), *Smc04265* (encoding a conserved hypothetical protein). Genes on the opposite stand are *lpxXL* (encoding a lipid A biosynthesis C28-acyltransferase) and *adhA2* (encoding a probable alcohol dehydrogenase). Another nearby gene not shown includes *acpXL* (encoding an acyl carrier protein, involved in the transfer of long hydroxylated fatty acids to lipid A). (iant.toulouse.inra.fr/bacteria/ annotation/cgi/rhime.cgi)

Table 3.1 Identity and similarity of the *S. meliloti* Smc04266 protein sequence to the sequences of the defined proteins.

Protein	Identity (%)	Similarity (%)
E. coli bleomycin resistance protein (Tn*5* ble) (Yamamoto, 2006)	33	40
S. aureus bleomycin resistance protein (Sh *ble*) (Berg *et al.*, 1998)	36	40
S .meliloti glyoxalase I (*gloA*) (Galibert *et al.*, 2001)	9	21
E.coli glyoxalase I (*gloA*) (Blattner *et al.*, 1997)	16	30

To determine if the Smc04266 protein played any role in sensitivity of *S. meliloti* to bleomycin, the gene was disrupted in the Rm1021 parent strain using a 267 bp internal gene fragment of the *smc04266* gene, cloned into the pJH104 suicide vector (chapter 2, section 2.3.6). Additionally the *smc04266* disruption was transduced into the Rm1021 Δ*bacA* background using M12 phage (Finan *et al.*, 1984), since if a role for the Smc04266 protein in bleomycin resistance was found, it would be important

to determine if there was any interplay with BacA. However, it was observed that disruption of *smc04266* did not result in an increased sensitivity to bleomycin A_5 (Fig 3-24A), relative to the Rm1021 parent strain, suggesting that the Smc04266 protein did not play a role in bleomycin resistance in *S. meliloti*. Loss of the Smc04266 protein in the Rm1021 Δ*bacA* background resulted in the same level of resistance as seen in the Rm1021 Δ*bacA* single mutant (Fig 3.24A). Since Smc04266 is also annotated as a putative glyoxalase and glyoxalase enzymes are involved the detoxification of methylglyoxal (Inoue & Kimura, 1995), sensitivity of the mutant to methylglyoxal was next assessed. It was observed that disruption of the gene did not result in an increased resistance to methglyoxal. Taken together these data would suggest that despite being annotated as a putative glyoxalase/bleomycin resistance protein, the Smc04266 protein does not appear to play a role in bleomycin resistance or methylglyoxal detoxification in *S. meliloti*.

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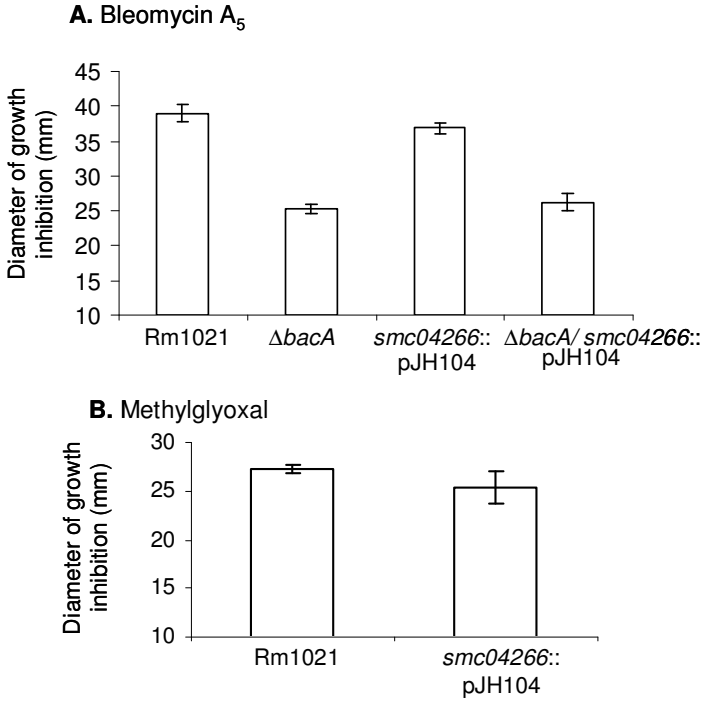


Figure 3-24. Sensitivity of the *smc04266* mutant to bleomycin A_5 and methylglyoxal by filter disc assay. Growth inhibition of the defined strains exposed to bleomycin A_5 (5 µl of a 2 $mg.ml^{-1}$stock) on LB plates (A) and growth inhibition of the defined strains to methylglyoxal (40 % aqueous stock solution) on LB plates (B). In each case the error bars represent the standard deviation from the mean (n=3) for one experiment. The dataset shows preliminary data.

To determine if the Smc04266 protein was important for the *S. meliloti* legume symbiosis, the mutant was inoculated into alfalfa plants and plant growth and nodule characteristics were recorded after a four week period (Table 3-2 and Fig. 3-25). It was determined that *S. meliloti* cells lacking the Smc04266 protein could form a successful symbiosis, in fact it appeared that alfalfa plants inoculated with the Rm1021 *smc04266* mutant appeared to have enhanced growth and often the plants appeared darker green in colour (Table 3.2 and Fig. 3-25), relative to the Rm1021 parent strain. However, unfortunately there were only 7 Rm1021 parent strain inoculated plants available for assessment, relative to 34 Rm1021 *smc04266* mutant inoculated plants, since several Rm1021 plants had been used for nodule sampling prior to week 4. Thus, to confirm this preliminary data the Rm1021 *smc04266* mutant will need to be assessed in the plant relative to a larger number of Rm1021 parent strain inoculated plants.

Table 3-2. Effect of loss of the Smc04266 protein on the alfalfa symbiosis

Bacterial strain	Symbiosis	Plant height (cm)	Plant colour	Mean no. of pink nodules per plant
Rm1021 parent[a]	Yes	11.2 ± 1.2	Dark green	12.3 ± 3.3
Rm1021 *smc04266*[b]	Yes	14.1 ± 2.0	Dark green	12.0 ± 5.0

± shows the standard derivation from the mean

[a] 7 plants were analysed containing the Rm1021 parent strain

[b] 34 plants were analysed containing the Rm1021 *smc04266* mutant

Figure 3-25. Alfalfa seedlings after inoculation with the Rm1021 parent and the *S. meliloti smc04266* mutant. Alfalfa seedlings were inoculated with either the Rm1021 parent strain (A) or the Rm1021 *smc04266* mutant (B). Plant growth was photographed 4 weeks post infection.

3.2.12. The *S. meliloti* Δ*bacA* mutant displays an increased resistance to the glycopeptide vancomycin on solid media

It was next investigated if the BacA protein sensitized *S. meliloti* to another glycopeptide. Vancomycin (Fig. 3-26) is produced by the bacterium *Amycolatopsis orientalis* (Griffith, 1984) and became available for use over 50 years ago and is active against most gram positive bacteria. In more recent years due to the spread of methicillin-resistant *Staphylococcus aureus and* penicillin resistant *Streptococcus pneumoniae* (Peacock *et al.*, 1980), there has been a resurgence in vancomycin use. In gram positive bacteria the toxic action of vancomycin resides in its ability to bind to the C-terminal D-Ala–D-Ala peptides of the polymeric lipid-PP-disaccharide-pentapeptides. Consequently, this interferes with the cross-linking of these chains in the growing peptidoglycan cell wall (Barna & Williams, 1984; Reynolds, 1989) resulting in a weak point in the resulting cell wall which makes the bacterial cell susceptible to lysis.

Most gram negative bacteria are resistant to the action of vancomycin, when exposed to clinically relevant doses, typically 30 µg infiltrated vancomycin discs are used (Woodford *et al.*, 1995). However, growth inhibition was observed in *S. meliloti* when a higher dose was used in the disc diffusion assay (Fig. 3-27A). Additionally, it was determined that disruption of *bacA* in *S. meliloti* did result in an increased level of resistance to vancomycin, relative to the Rm1021 parent strain (Fig. 3-27A). However, when viability of the Rm1021 parent and Rm1021 Δ*bacA* mutant were assessed in liquid media (Fig. 3-27B), no difference in sensitivity was observed. Therefore, these preliminary data would suggest that the disruption of *bacA* in *S. meliloti* cells results in an increased resistance to the glycopeptide vancomycin on solid media.

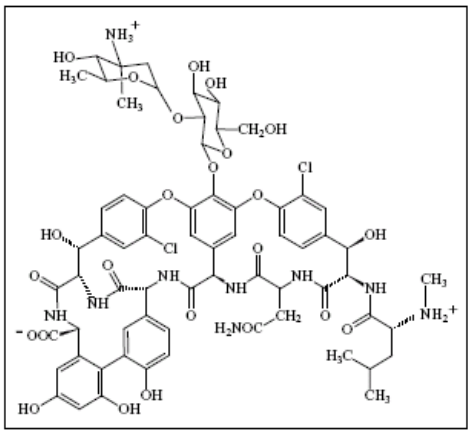


Figure 3-26. Structure of the glycopeptide vancomycin
(Schafer *et al.*, 1996)

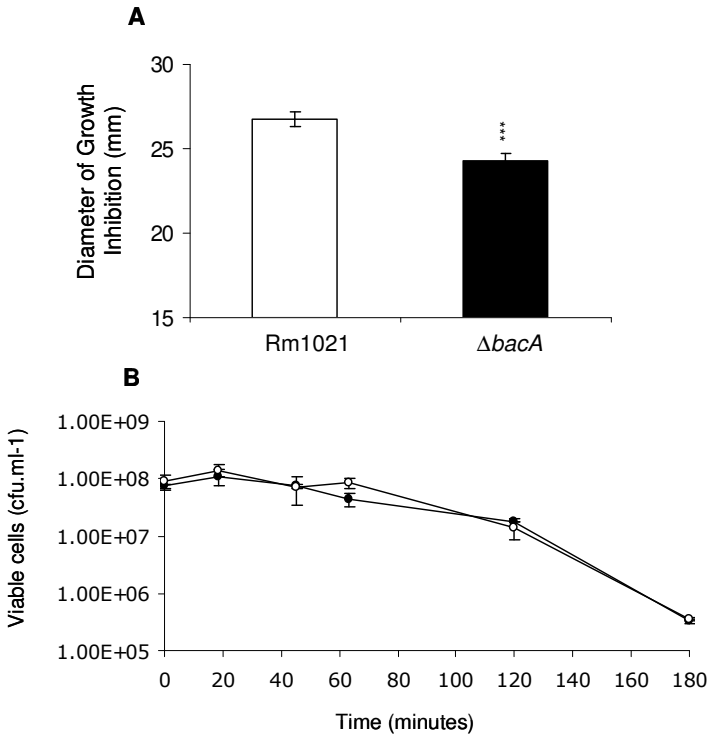


Figure 3-27. Sensitivity of the Rm1021 parent strain and the Rm1021 Δ*bacA* mutant to the glycopeptide vancomycin on solid and in liquid media. The defined strains were exposed to vancomycin (5 µl of 20 mg.ml-1 stock solution) by filter disc assay and growth inhibition was assessed (A). The Rm1021 parent (closed circles) and the Rm1021 Δ*bacA* mutant (open circles) were exposed to vancomycin (300 µg.ml $^{-1}$) and cell viability was assessed at the defined times (B). In (A) the significant value (***P<0.001) represents a comparison of the Rm1021 Δ*bacA* strain and the Rm1021 parent strain. In each case the error bars represent the standard deviation from the mean (n=3) for one experiment. Both datasets show preliminary data.

3.3. Discussion

Work in this chapter has shown that the presence of the *S. meliloti* BacA protein dramatically increased the amount of bleomycin induced DNA degradation. Contrastingly BacA did not sensitize *S. meliloti* towards other DNA damaging agents, suggesting the BacA induced sensitization to DNA damage is specific for bleomycin. It was also observed that the presence of both the RecA protein and intracellular glutathione protected *S. meliloti* against bleomycin damage. However, it was determined that the increased resistance to bleomycin observed in the absence of the BacA protein is independent of RecA mediated DNA repair. It was also shown the presence of glutathione was not essential for the increased resistance to bleomycin observed in the absence of BacA. Furthermore, it was possible to show that loss of the BacA protein reduced the amount of F-BLM taken up by *S. meliloti* cells. These data are all consistent with a role for the BacA protein in bleomycin uptake. However, since F-BLM still accumulated in *S. meliloti* in the absence of the BacA protein, these data also show there must be a BacA independent mode of bleomycin uptake into *S. meliloti* cells (Fig. 3-28).

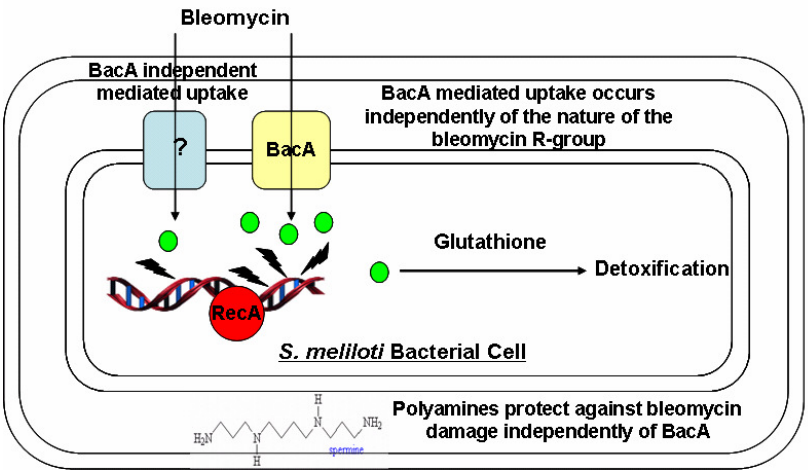


Figure 3-28. Illustration of the *S. meliloti* cell summarising the major findings of this chapter. The presence of the BacA protein increases the amount of bleomycin induced DNA degradation. However, in the absence of BacA, bleomycin is still able to enter *S. meliloti*. Both the RecA protein and intracellular glutathione protect *S. meliloti* against bleomycin damage. However, glutathione and RecA mediated DNA repair are not essential for the increased resistance to bleomycin observed in the absence of the BacA protein. Presence of the BacA protein was shown to increase the amount of F-BLM taken up by *S. meliloti* cells.

3.3.1. The increased resistance to bleomycin in the absence of the BacA protein is independent of the altered VLCFA

Although previous work has shown that loss of the BacA protein in both *S. meliloti* and *B. abortus* results in a reduction in the VLCFA content of the lipid A (Ferguson *et al.*, 2004). Work in this chapter was able to show the increased resistance to bleomycin in the absence of BacA was not as a consequence of the altered lipid A. It was shown that the Rm1021 *acpXL* and *lpxXL* mutants, which completely lack the VLCFA modification (Ferguson *et al.*, 2005), actually display an increased level of sensitivity to bleomycin. It has previously been shown that the Rm1021 *acpXL*/Δ*bacA* and *lpxXL*/Δ*bacA* double mutants have identical lipid A profiles to their respective *acpXL* and *lpxXL* single mutants. However, it was observed that loss of the BacA protein in the *acpXL* and *lpxXL* mutant backgrounds still conferred resistance against bleomycin. Thus, these findings confirmed that the increased resistance to bleomycin in the absence of the BacA protein is independent of the altered lipid A. Additionally, unlike *S. meliloti* the lipid A of *E. coli* is not modified with VLCFAs, yet loss of the BacA homolog SbmA has also been shown to cause an increased resistance to bleomycin. Moreover, recent work has found that loss of a BacA related protein (Rv1819c) in *M. tuberculosis,* as well as compromising chronic infection also results in an increased resistance to bleomycin (Domenech *et al.*, 2008). Although the cell envelope of *M. tuberculosis* does not contain LPS, very long chain fatty acids are present (C_{60}-C_{90}), known as the mycolic acids (Brennan & Nikaido, 1995). However it was observed that loss of the BacA like protein in *M. tuberculosis* did not result in any detectable changes in the abundance or structure of the mycolic acids (Domenech *et al.*, 2008).

3.3.2. The increased resistance of *S. meliloti* to bleomycin is independent of the nature of the R-group

In the yeast *S. cerevisiae* it has been proposed that the bleomycin R-group may be important for uptake (Aouida *et al.*, 2004). To date, this is the only study implicating a particular domain of the bleomycin molecule as being important in uptake, since to date relatively little is known concerning bleomycin uptake in both eukaryotic and

prokaryotic cells. However, in this work it was found that the BacA protein conferred sensitivity to all forms of bleomycin, suggesting that the R-group is not important for BacA-dependent uptake. It may be possible that another bleomycin domain is important or even crucial for BacA induced uptake. Deglyco-bleomycin A_2 is a bleomycin derivative, which lacks the carbohydrate moiety (Oppenheimer *et al.*, 1982). Research has shown that although loss of the carbohydrate moiety does not seem to affect the ability of bleomycin A_2 to recognise sequences on the DNA and produce breaks (Bailly *et al.*, 1995), when exposed to Chinese hamster fibroblasts, it did induce significantly less toxicity (Tounekti *et al.*, 2001). It was hoped to obtain some deglyco-bleomycin $A_{2,}$ to determine if the carbohydrate moiety was important for BacA mediated uptake, however it was unfortunate that a source was not available during the course of this work.

3.3.3. BacA sensitizes *S. meliloti* to bleomycin induced DNA damage

Extraction and analysis of *S. meliloti* genomic DNA by agarose gel electrophoresis following bleomycin treatment clearly demonstrated that in the presence of the BacA protein there was a substantial loss of genomic DNA. Contrastingly, there appeared to be no significant loss of genomic DNA in the Rm1021 Δ*bacA* mutant. However, *in situ* staining of the genomic DNA with DAPI following bleomycin treatment under the same conditions did reveal some DNA degradation in the Rm1021 Δ*bacA* mutant, albeit to a smaller extent than the parent. This would suggest that the DAPI method was a more sensitive means of analysis. Since genomic DNA damage was used as a means to assess intracellular bleomycin, taken together these data are consistent with a role for BacA in uptake. However, since DNA degradation also occurred in the absence of the Rm1021 BacA protein the data also suggests bleomycin can enter the cells in a BacA independent manner. The observation that the BacA protein did not sensitize *S. meliloti* cells to three other DNA damaging agents (methylglyoxal, mitomycin C and MMS), or enhance MMS induced DNA degradation ruled out the possibility that BacA could be affecting *S. meliloti* genomic DNA, leading to an increased susceptibility towards DNA damaging agents, thus confirming that BacA-mediated sensitivity was specific for bleomycin.

However, since bleomycin requires a reduced transition metal, either Fe^{2+} or Cu^{2+} for activity (Chen & Stubbe, 2005), it is possible that BacA instead of having a role in bleomycin transport, could be necessary for the transport of one of these metals. Unlike bleomycin, metal co-factors do not appear to be necessary for methylglyoxal, mitomycin C and MMS induced DNA damage, although, an *in vitro* study with methylglyoxal demonstrated that when plasmid DNA was incubated with methylglyoxal and lysine, Cu^{2+} enhanced DNA stand breakage induced by the glycation reaction of methylglyoxal and lysine (Kang, 2003). Additionally, an *in vitro* study performed some years ago with mitomycin C found that iron chelators reduced the degradation of DNA, under low oxygen conditions (Gutteridge *et al.*, 1984). However, the proposal that BacA could be involved in iron transport has recently been investigated in *M. tuberculosis* (Domenech *et al.*, 2008). As discussed, this study found that loss of the BacA like protein in *M. tuberculosis* results in an increased resistance to bleomycin. Also in this study $^{55}FeCl_3$ accumulation assays were performed using the parental and BacA deficient strains. However, it was observed that both strains had the same rate of $^{55}FeCl_3$ accumulation, therefore showing that in *M. tuberculosis* iron uptake was independent of BacA. Moreover, work investigating the increased bleomycin resistance of the *B. abortus bacA* mutant, has also shown that iron uptake is independent of BacA (R.M. Roop, unpublished data). Therefore, taken together these data do not support a role for BacA in the uptake of iron.

3.3.4. Bleomycin treatment did not appear to induce membrane lysis in *S. meliloti*

The observation that no membrane damage or cell lysis occurred in *S. meliloti*, following bleomycin treatment was able to show that the DNA degradation observed was not as a result of cell envelope damage. Moreover, since no membrane damage or cell lysis was apparent, this would also suggest that BacA independent uptake of bleomycin, is unlikely to occur in a non specific manner, suggesting another transport system is likely to be involved. It was also interesting to note the appearance of white granules in the Rm1021 Δ*bacA* cells treated cells, relative to the Rm1021 parent treated cells and these granules were absent in the untreated cells.

Poly-3-hydroxybutyrate (PHB) granules, whose accumulation, degradation and utilization have been shown to be increased under stress conditions in bacteria (Okon *et al*., 1992), appear as white granules. PHB granules have been well characterised in *S. meliloti*, where they are used to store excess carbon (Tombolini *et al.*, 1995). However, future work looking at a *S. meliloti* mutant in PHB biosynthesis could determine if these granules are in fact PHB granules. Additionally, further work involving elemental TEM may allow more to be learnt regarding the composition of these granules. Yet at this point it is interesting to speculate that the appearance of these white granules in the absence of the BacA protein may be linked to the altered bleomycin sensitivity observed, relative to the Rm1021 parent strain.

3.3.5. Glutathione appears to protect against bleomycin induced damage and this protection is independent of the BacA protein

The observation that glutathione protects *S. meliloti* against bleomycin, suggests that *S. meliloti* has a glutathione-dependent bleomycin detoxification system. The hypersensitivity of Chinese hamster ovary cells to bleomycin was found to be due to a lack of glutathione *S*-transferase activity (Giaccia *et al.*, 1991). Glutathione-*S*-transferases catalyse the nucleophilic conjugation of both xenobiotic and endogenous electrophiles with glutathione, thereby decreasing their reactivity (Armstrong, 1997). *S. meliloti* has several glutathione *S*-tranferases annotated in the genome (http://iant.toulouse.inra. fr/bacteria/annotation /cgi/rhime.cgi). Thus, it may be possible that detoxification of bleomycin in *S. meliloti* is catalysed by glutathione *S*-transferases. However, since deletion of *bacA* still conferred protection of an *S. meliloti* mutant lacking glutathione against bleomycin, this provided evidence that the reduced amount of bleomycin-induced DNA degradation observed in the absence of BacA is not due to an increase in glutathione-dependent bleomycin detoxification. Furthermore, the fact that the Rm1021 Δ*bacA/gshA* double mutant displayed an increased level of sensitivity, relative to the Rm1021 Δ*bacA* mutant provides further evidence that bleomycin is able to enter *S. meliloti* in the absence of BacA protein.

3.3.6. In the absence of the BacA protein *S. meliloti* appears to accumulate less F-BLM

Whilst investigating the uptake of F-BLM, use of the extracellular quencher TB did reveal that a proportion of the F-BLM associated with *S. meliloti* cells was membrane bound. However, a proportion of the cells did still maintain cell associated fluorescence, following TB treatment. Therefore, these data suggest that F-BLM is able to enter *S. meliloti* cells. It was hoped to confirm these findings by fluorescence microscopy to visualise F-BLM within the cells, additionally use of a fluorescent membrane dye would enable confirmation that F-BLM had entered the cell cytoplasm and not just the cell envelope. Unfortunately, in this case fluorescence microscopy did not prove a sensitive enough means to detect F-BLM in *S. meliloti*. However, following TB incubation it was still observed that in the presence of the BacA protein more F-BLM appeared to accumulate in *S. meliloti*, consistent with a role for BacA in uptake.

The viability experiments with F-BLM also showed that the presence of the BacA protein conferred increased sensitivity to F-BLM. However, there still remains the possibility that labelling of the bleomycin A_5, with the 5(6)-FAM, SE fluorophore may have affected the toxicity or mechanism of action of the bleomycin A_5. Unfortunately at the time of this work, the F-BLM produced had not been analysed further following purification, and upon the advice of another research group, also working with F-BLM, a one to one ratio of 5(6)-FAM, SE to bleomycin was assumed (M. Aouida, personal communication). However, more recently electrospray ionisation-mass spectrometry analysis determined that F-BLM contains a 2:1 ratio of 5(6)-FAM, SE (M. Scocchi and G.P.Ferguson, unpublished data). This analysis also determined the molecular weight of F-BLM. Subsequently, work is currently under way in the laboratory using equal molar concentrations of F-BLM and bleomycin A_5 to assess sensitivity of the Rm1021 parent and Rm1021 Δ*bacA* and to determine if the labelling has affected the toxicity. Additionally DNA degradation is currently being assessed in the Rm1021 parent and Rm1021 Δ*bacA* mutant, following treatment with F-BLM, under the same conditions as used for the flow cytometry experiments. However, the data obtained thus far assessing F-BLM uptake are consistent with a role for BacA in bleomycin uptake.

3.3.7. The Role of the BacA protein in bleomycin uptake

Although only *S. meliloti bacA* transposon mutants were isolated after screening of a *S. meliloti* transposon library for bleomycin resistant mutants (Ferguson *et al.*, 2006), there may be still other bleomycin uptake systems, that are either essential for *S. meliloti* growth, or may have functional redundancy. In the yeast *S. cerevisiae*, a study found that deletion of a protein kinase *ptk2* which positively regulates polyamine transport prevented uptake of F-BLM (Aouida *et al.*, 2004). So in yeast it was hypothesized that bleomycin A_5 may enter yeast cells through a polyamine transport system. This finding combined with the structural similarity of the bleomycin A_5 R-group to polyamines, led the authors to propose that the R-group of bleomycin may be important in uptake (Aouida *et al.*, 2004). However, it should be noted there are no data stating if *S. cerevisiae* was sensitive to other forms of bleomycin, where the polyamine like R-group would be absent. Consistent with the work in yeast it was also found that the polyamine spermine protected against bleomycin A_5 damage in *S. meliloti*. This protection was shown to be independent of the nature of the bleomycin R-group. Since spermine protected against bleomycin in *S. meliloti* and there appeared to be a BacA independent mode of uptake, it was hoped to investigate if disruption of a polyamine uptake system in *S. meliloti* would result in an increased resistance to bleomycin. Disruptions were made in what was thought to be the *S. meliloti potABCD* operon, whose role in polyamine uptake is very well characterised in *E. coli* (Furuchi *et al.*, 1991; Kashiwagi *et al.*, 1990). However, re-annotation of the *S. meliloti* genome has meant this operon is now annotated as encoding a ABC transporter only. Interestingly a previous study in *S. meliloti*, using transcriptional fusions found that this operon is induced by glucose-6-phosphate and glycerol-3-phosphate (Mauchline *et al.*, 2006), perhaps suggesting it plays a role in the uptake of sugar phosphates. It was observed that the two putative mutants *smc21272* and *smc21275* did not display an increased resistance to bleomycin, therefore once these disruptions are verified, this will enable the assumption to be made that this transport system is not involved in bleomycin uptake. It was unfortunate that the mutants could not be verified in the course of this work. However, the increased level of sensitivity to bleomycin observed in the putative mutants may suggest this operon is involved in the uptake of a solute(s)

involved in protection against bleomycin induced damage. The genome of *S. meliloti* encodes multiple polyamine uptake systems (iant.toulouse.inra.fr/bacteria/ annotation/cgi/rhime.cgi), further work will be necessary to determine if one or more of these systems is involved in bleomycin uptake. However, since polyamine induced protection still occurred in the absence of BacA, this would suggest polyamines are not protecting *S. meliloti* by reducing the amount of BacA mediated uptake. However, it is quite possible polyamines may be inducing their protective effect by some other means independent of uptake. Indeed polyamines have multiple physiological functions in bacteria (Shah & Swiatlo, 2008) and have been shown to protect DNA against the formation of radiation-induced double stranded breaks (Oh & Kim, 1998) so a role for spermine in protection against bleomycin induced DNA damage in *S. meliloti* cannot be ruled out.

3.3.8 Disruption of the putative glyoxalase/bleomycin resistance protein did not result in an increased sensitivity to bleomycin

The discovery of the *smc04266* gene encoding the putative glyoxalase/bleomycin resistance protein came about since it is located in the same genome region as the *acpXL* and *lxpXL* genes, (iant.toulouse.inra. fr/ bacteria/ annotation/cgi/rhime.cgi), which have been studied in the laboratory for several years. Performing BLAST analysis revealed a low level of protein sequence similarity to other well characterised bleomycin resistance proteins or glyoxalase enzymes. Upon re-annotation of the *S. meliloti* genome in June 2008, the annotation of *smc04266* was updated, to encode a putative glyoxalase/bleomycin resistance/dioxygenase protein. Further research revealed there is a superfamily of glyoxalase/bleomycin resistance/ dioxygenase proteins (http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html), which are grouped together based on domain similarity. Several bacterial species are thought to encode similar proteins belonging to this family. The *S. meliloti* Smc04266 protein is included in this superfamily, in fact the *S. meliloti* genome is thought to encode 23 proteins belonging to this superfamily. Further work would be required to determine if any of these proteins are involved in bleomycin resistance. However, in this work it was found the Smc04266 protein was not important for bleomycin resistance or methglyoxal detoxification.

Interestingly, preliminary data suggested loss of this protein may confer an advantage to *S. meliloti* in the alfalfa host interaction. It was found alfalfa plants inoculated with the Rm1021 *smc04266* mutant appeared to have enhanced growth relative to the Rm1021 parent strain. Work is currently underway in the laboratory to see if the phenotype is reproducible and if so to investigate this further.

3.3.9. The *S. meliloti* Δ*bacA* mutant displays an increased resistance to the glycopeptide vancomycin on solid media

It is well documented that the glycopeptide vancomycin is unable to enter most gram negative bacteria, as due to its size it is thought to be unable to pass though the porins of the gram negative outer membrane (Rida *et al.*, 1996). However, by using a high dose of vancomycin it was possible to observe both growth inhibition on solid media and cell death in *S. meliloti* when exposed in liquid culture. It may be possible that at higher doses vancomycin may have a different effect on bacterial cells which results in growth inhibition and cell death. However, to date all the literature detailing the mechanism of vancomycin damage only describes the drug's interference with the cross-linking of peptide chains in the growing peptidoglycan cell wall (Barna & Williams, 1984; Reynolds, 1989). It was interesting to observe that the *S. meliloti* Δ*bacA* mutant displayed a reduced level of growth inhibition to vancomycin, relative to the parent strain, although at the concentration tested no differences were observed in the actual cell death between the *S. meliloti* Δ*bacA* mutant and the Rm1021 parent. It may be possible that only an increased resistance to growth inhibition, not cell death is observed in the *S. meliloti* Δ*bacA* mutant, relative to the Rm1021 parent strain. However, further experiments using different concentrations of vancomycin in the liquid viability experiments would be necessary to confirm this.

Before one is able to speculate as to why the *S. meliloti* Δ*bacA* mutant displays decreased growth inhibition, relative to the parent strain when exposed to vancomycin, it will be essential to determine if this is dependent or independent of the VLCFA alteration.

3.3.10. Could uptake of a molecule similar to bleomycin be important for host persistence?

Bleomycin was initially isolated from the soil bacterium *Streptomyces verticillus* (Umezawa *et al.*, 1966). Since *S. meliloti* can also be found free-living in the soil, these findings may suggest that BacA will increase the susceptibility of *S. meliloti* towards bleomycin and other peptide antibiotics found within the soil. It was previously hypothesized the critical role of the BacA protein may be linked to its role in the uptake of a peptide with structural similarities to bleomycin, essential for persistence within the host (Ichige & Walker, 1997). However, since this work has shown there is a BacA independent route of bleomycin uptake, this seems unlikely. Peptides with a similar structure to bleomycin have to date not been identified in nodules. Interestingly, transcriptome analysis of another *S. meliloti* host, *Medicago truncatula* has revealed the presence of hundreds of cysteine-rich peptides within the nodules (Alunni *et al.*, 2007; Mergaert *et al.*, 2003; Mergaert *et al.*, 2006). Although the function of these peptides is currently unknown, it has been proposed they may play a role in *S. meliloti* bacteroid development (Mergaert *et al.*, 2006). The BacA homolog in *E. coli* SbmA has been proposed for several years to be involved in the uptake of bleomycin and microcin antibiotics (Ichige & Walker, 1997; Salomon & Farias, 1995). However, more recently SbmA has been found to be involved in the uptake of a proline rich peptide Bac7 (Mattiuzzo *et al.*, 2007). Since this suggests that SbmA may be involved in the uptake of structurally diverse peptides, it may be possible within the host that BacA may play a role in the uptake of one of these cysteine-rich peptides, whose uptake is essential for successful bacteroid development.

Future studies will be necessary to determine the precise function of BacA in peptide uptake and the contribution of this process to the chronic infection process. However, since both *B. abortus* and *M. tuberculosis* lacking the BacA protein also display an increased resistance to bleomycin and since antimicrobial peptides are known to be present in mammalian cells, these findings further support a model whereby BacA may be essential for the uptake of a host derived peptide, which may play an important role in the outcome of an infection.

Chapter 4: Investigation into the Role of the BacA Protein in the Uptake of the Proline Rich Peptide Bac7(1-16)

4.1. Introduction

The BacA protein has been proposed for some time to be involved in the uptake of an essential peptide and it is hypothesized that this may be linked to its crucial role in host persistence (Glazebrook *et al.*, 1993; Ichige & Walker, 1997). The presence of the BacA protein sensitizes both *S. meliloti* and *B. abortus* to the glycopeptide bleomycin (Ferguson *et al.*, 2002; Ferguson *et al.*, 2006; Ichige & Walker, 1997; LeVier *et al.*, 2000; LeVier & Walker, 2001) and *E. coli* mutants lacking the BacA homologue, SbmA, are also resistant to bleomycin (Ichige & Walker, 1997). Thus, in chapter 3 the role of the *S. meliloti* BacA protein in the uptake of bleomycin was investigated. However, it was determined that BacA was not essential for the uptake of a fluorescently labelled form of bleomycin in *S. meliloti*, since uptake still occurred in the absence of the BacA protein, although it was found that in the absence of the BacA protein, fewer cells appeared to accumulate fluorescently labelled bleomycin, suggesting BacA may play some role in uptake. However, since the BacA protein does not appear to be the direct route of bleomycin uptake in *S. meliloti*, this would make it highly unlikely that the uptake of a peptide with structural similarities to bleomycin is essential for host persistence.

Interestingly, recent work has shown the *E. coli* SbmA protein is involved in the intracellular accumulation of a proline-rich, fluorescently labelled peptide, Bac7(1-35) in *E. coli* (Mattiuzzo *et al.*, 2007). Full length Bac7 is a linear antimicrobial peptide (AMP), characterised by a high content of proline and arginine residues. The full length Bac7 peptide was originally isolated from bovine neutrophils as a 60 amino acid peptide (Frank *et al.*, 1990). However, truncated forms of this peptide, Bac7(1-35) and (1-16), still maintain full antimicrobial activity against *E. coli* (Benincasa *et al.*, 2004). The Bac7 peptide is predominantly active against gram-negative bacterial species (Gennaro *et al.*, 2002; Otvos, 2002) and has been shown to exert its cytotoxic effect by a non-lytic mechanism, that depends on the penetration of the peptide into bacterial cells (Podda *et al.*, 2006). Research is currently underway to define the nature of this putative intracellular target in *E. coli* (Scocchi *et al.*, 2008). This mechanism of action is unusual for AMPs, as most exert

their cytotoxic effects through perturbing and permeabilizing membranes of both gram negative and gram positive bacterial species (Andreu & Rivas, 1998; Brogden, 2005).

The SbmA protein can functionally compensate for the absence of *S. meliloti* BacA (Ichige & Walker, 1997) and the *S. meliloti* BacA protein is 79% similar (64% identical) to the *E. coli* SbmA protein. Hence, it may be possible that like SbmA, the BacA protein may also play a role in the uptake of the Bac7 peptide (Fig. 4-1). Interestingly, two gene families (glycine rich proteins and cysteine rich peptides), which encode secreted AMPs, have been identified to have nodule-specific expression in *M. truncatula,* a legume host of *S. meliloti* (Alunni *et al.*, 2007; Kevei *et al.*, 2002; Mergaert *et al.*, 2003; Mergaert *et al.*, 2006). It has been suggested previously that these peptides could play a role in initiating bacteroid development (Mergaert *et al.*, 2003; Mergaert *et al.*, 2006). Additionally, as the full length Bac7 peptide was originally isolated from bovine neutrophils (Frank *et al.*, 1990), it is plausible that *B. abortus* may encounter proline rich peptides within its mammalian host. Thus, the uptake of a peptide could also be important for signalling the transition from the acute to chronic state of *B. abortus* infection. Since the *S. meliloti* and *B. abortus* BacA proteins are 68% identical and the *B. abortus bacA* mutant also displays an increased resistance to bleomycin, (LeVier *et al.*, 2000) it is highly likely that the *B. abortus* BacA protein may also be involved in peptide uptake.

Therefore, in this chapter the hypothesis that the *S. meliloti* BacA protein may play a role in Bac7(1-16) uptake (Fig. 4-1) and that the uptake of a peptide may be linked to its essential role in chronic infection was investigated.

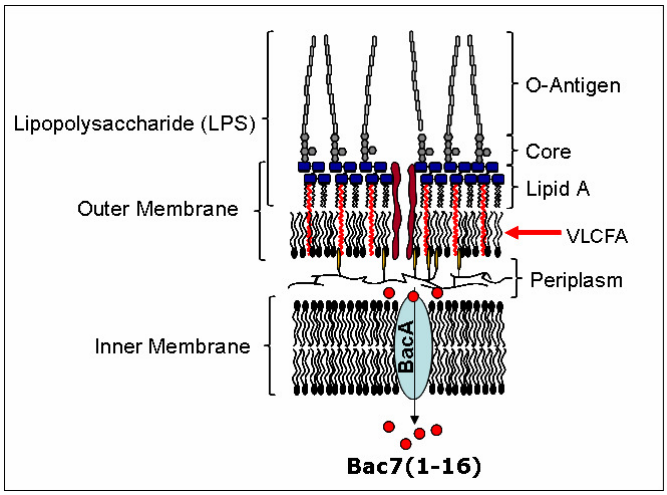


Figure 4-1. Proposed model for BacA function. The *S. meliloti* cell envelope is shown. Once the proline-rich peptide Bac7 has reached the periplasmic space, it could then be transported across the cytoplasmic membrane by the BacA protein.

4.2. Results

4.2.1. The *S. meliloti* BacA or the *E. coli* SbmA proteins sensitize *S. meliloti* towards Bac7(1-16)

A previous study demonstrated that the BacA homolog, SbmA, increases the sensitivity of *E. coli* towards truncated forms of the proline-rich peptide, Bac7 (Mattiuzzo *et al.*, 2007). Thus, it was first investigated whether the BacA protein was also involved in sensitizing *S. meliloti* towards the truncated Bac7 peptide, Bac7 (1-16; RRIRPRPPRLPRPRPR). Viability of *S. meliloti* cells, after treatment with the Bac7(1-16) peptide was initially assessed. It was determined that treatment of the Rm1021 parent cells with 0.25 µM Bac7(1-16) for 1 hour resulted in loss of cell viability (Fig. 4-2A). Contrastingly, when the viability of Rm1021 Δ*bacA* mutant cells were assessed under the same treatment conditions, no loss in cell viability occurred (Fig. 4-2A). Additionally, when cells were exposed to 1 µM Bac7(1-16), the Rm1021 Δ*bacA* mutant was still completely resistant to the killing effects of the peptide (Fig. 4-2B) and the Rm1021 parent strain showed even more sensitivity to this higher dose of the peptide (Fig. 4-2B). Thus, these data suggest that the *S. meliloti* BacA protein sensitizes *S. meliloti* to Bac7(1-16).

To confirm the role of the BacA protein, sensitivity of the Rm1021 Δ*bacA* mutant with pJG51A (pRK404 plasmid carrying the *S. meliloti* wild-type *bacA* gene), was assessed after treatment with 0.25 and 1 µM Bac7(1-16) (Fig. 4-2A & B, respectively). Indeed, it could be seen that presence of the pJG5IA plasmid increased the sensitivity of the Rm1021 Δ*bacA* mutant to Bac7(1-16). However, the Rm1021 Δ*bacA* mutant carrying the pRK404 control plasmid remained resistant to the killing effects of Bac7(1-16) (Fig. 4-2A & B). Furthermore, it was also determined that the Rm1021 Δ*bacA* mutant with pAI351 (pRK404 plasmid carrying the *E. coli sbmA* gene), also displayed an increased sensitivity to Bac7(1-16). Overall, these data show that either the presence of the BacA protein or the *E. coli* SbmA protein are essential for *S. meliloti* to be sensitive to killing by the Bac7(1-16) peptide.

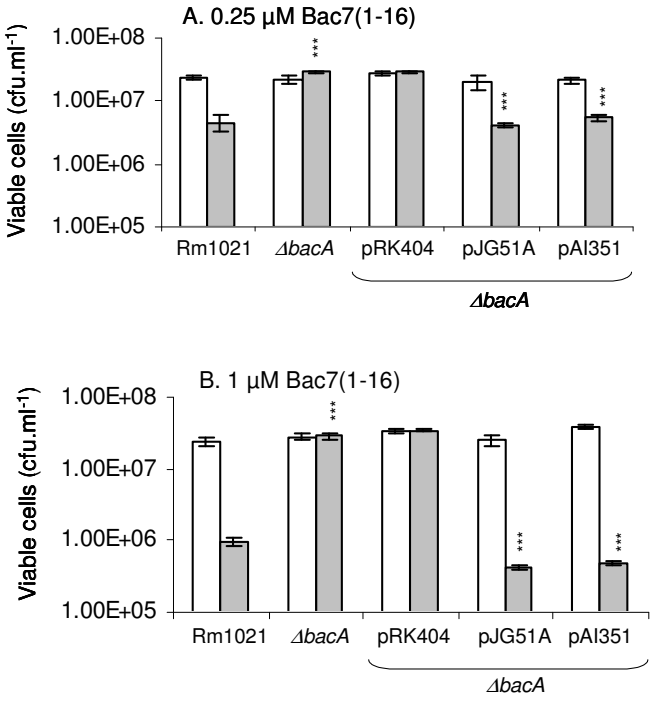


Figure 4-2. Sensitivity of *S. meliloti* strains with and without either BacA or SbmA proteins to Bac7(1-16). Cultures of the defined strains were exposed to either 0.25 µM (A) or 1 µM Bac7(1-16) (B) and cell viability determined before (open bars) and 1 hour (shaded bars) after addition. The significant values (***P<0.001) represent comparisons of the Rm1021 Δ*bacA* mutant with the Rm1021 parent strain and the Rm1021 Δ*bacA* mutant with either pJG5IA (*S. meliloti bacA* gene cloned into pRK404) or pAI351 (*E. coli sbmA* gene cloned in pRK404) to pRK404 (control plasmid with no insert). The datasets shown are representative of the trends observed in two independent experiments and in each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

4.2.2. BacA-mediated sensitivity of *S. meliloti* towards Bac7(1-16) is independent of the VLCFA modification

It had previously been shown that the Rm1021 Δ*bacA* mutant has a 50% reduction in the amount of lipid A species modified with a VLCFA, relative to the Rm1021 parent strain (Ferguson *et al.*, 2004). Hence, it was essential to investigate if this lipid A alteration was involved in the increased resistance of the Rm1021 Δ*bacA* mutant to Bac7(1-16). A previous study had revealed that the lipid A species produced by the *S. meliloti* Rm1021 *acpXL* mutant in LB medium, under standard growth conditions, completely lacks the lipid A VLCFA modification (Ferguson *et al.*, 2005). The AcpXL protein is an acyl carrier protein, which plays an essential role in the biosynthesis of the lipid A VLCFA in free-living *S. meliloti* (Brozek *et al.*, 1996). This mutant therefore provided a means to investigate how the absence of the lipid A modification in *S. meliloti* influences Bac7(1-16) sensitivity. In contrast to the Rm1021 Δ*bacA* mutant it was found that the Rm1021 *acpXL* mutant did not display an increased resistance to Bac7(1-16) (Fig. 4-3), with the mutant displaying the same level of sensitivity as the Rm1021 parent. These data therefore provide evidence that complete loss of VLCFA modification does not affect the sensitivity of *S. meliloti* cells to the peptide, suggesting that the VLCFA alteration in the Rm1021 Δ*bacA* mutant was not accounting for the increased resistance to Bac7(1-16). Previous work has demonstrated that the Rm1021 *acpXL* mutant and Rm1021 *ΔbacA/acpXL* double mutant have identical lipid A profiles and that their lipid A molecules completely lack the VLCFA modification. However, it was observed that deletion of *bacA* in an Rm1021 *acpXL* background conferred resistance to Bac7(1-16) (Fig. 4-3). Therefore, the data provided so far would support the hypothesis that the BacA protein is involved in the uptake of Bac7(1-16) in *S. meliloti.*

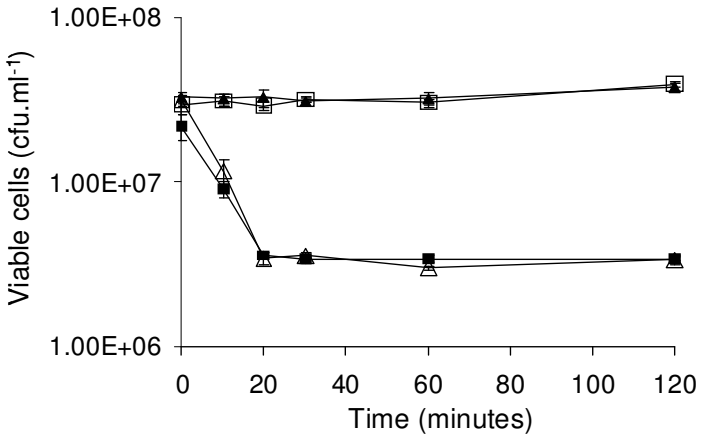


Figure 4-3. Sensitivity of the Rm1021 *acpXL* mutant to Bac7(1-16) in the presence and absence of the *bacA* gene. Mid-exponential phase cultures of the Rm1021 Δ*bacA* (open squares), Rm1021 *acpXL*::pk18mobGII (open triangles), Rm1021 Δ*bacA* /*acpXL*::pk18mobGII (filled triangles) mutant strains and parent strain, Rm1021 (filled squares) were treated with 0.25 µM Bac7(1-16) and cell viability determined at the defined times. The dataset shown is representative of the trends observed in two independent experiments and in each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

4.2.3. The BacA protein is essential for the uptake of fluorescently labelled $Bac7_{1-16}$-BODIPY

To determine if the BacA protein is involved in the uptake of the Bac7 peptide, a fluorescent derivative of Bac7(1–16) was used, which was labelled at a C-terminal cysteine residue with the fluorophore BODIPY ($Bac7_{1-16}$ –BY) (Scocchi *et al.*, 2008). As was the case for unlabelled Bac7(1-16) (Fig. 4-2) it could be observed that *S. meliloti* possessing either the *S. meliloti bacA* or *E. coli sbmA* gene were highly sensitive to killing by $Bac7_{1-16}$-BY, whereas *S. meliloti* lacking either BacA or SbmA were resistant (Fig. 4-4). If the viability data is compared for exposure of the cells to 0.25 µM unlabeled Bac7 (Fig. 4-2A) and the labelled form (Fig. 4-4), it appears that the labelled form is more toxic to the cells, under these conditions. However, since the fluorescent labelling of Bac7(1-16) did not interfere with BacA-mediated sensitivity towards this peptide in *S. meliloti*, it was next used to monitor Bac7(1-16) uptake.

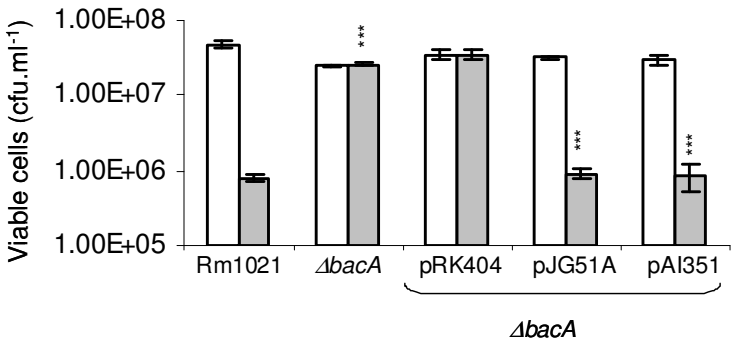


Figure 4-4. Effect of $Bac7_{1-16}$-BY on the viability of Rm1021 strains with and without the BacA protein. Cultures of the defined strains were exposed to 0.25 µM $Bac7_{1-16}$-BY and cell viability determined before (open bars) and 1 hour (shaded bars) after addition. The significant values (***P<0.001) represent comparisons of the Rm1021Δ*bacA* mutant with the Rm1021 parent strain and the Rm1021Δ*bacA* mutant with either pJG5IA (*S. meliloti bacA* gene cloned into pRK404) or pAI351 (*E. coli sbmA* gene cloned in pRK404) to pRK404 (control plasmid with no insert). The datasets shown are representative of the trends observed in two independent experiments and in each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

Using flow cytometry analysis, uptake of $Bac7_{1-16}$-BY by *S. meliloti* cells was next investigated. It could be clearly observed that incubation of Rm1021 parent cells with $Bac7_{1-16}$-BY resulted in all of the cell population displaying increased cell associated fluorescence, relative to the untreated control cells (Fig. 4-5A). Although the cells had been washed after $Bac7_{1-16}$-BY treatment, to discriminate between membrane bound and internalised peptide the extracellular quencher trypan blue (TB) was employed. Pre-treatment of *E. coli* bacterial cells with this dye has been successfully shown to quench extracellular fluorescence in a recent study (Mattiuzzo *et al.*, 2007) . It could be observed that treatment of the Rm1021 parent cells with TB prior to analysis had only a slight effect on the fluorescence of the cells (Fig. 4-5B), thus confirming that the fluorescence profile observed is due to the intracellular accumulation of $Bac7_{1-16}$-BY in the Rm1021 parent cells.

Next flow cytometry analysis was performed with the Rm1021 Δ*bacA* mutant cells treated under the same conditions (Fig. 4-5C &D). In contrast to the Rm1021 parent strain (Fig. 4-5A), there was only a small increase in the cell associated

fluorescence of the Rm1021 Δ*bacA* mutant cells after treatment (Fig. 4-5C). However, after pre-treatment of the Rm1021 Δ*bacA* cells with TB this increased fluorescence was abolished (Fig. 4-5D), suggesting this was due to extracellular binding of the peptide.

As discussed, it has been hypothesized in *S. meliloti* that the BacA protein may be essential for the uptake of a peptide involved in the differentiation of the cells into nitrogen fixing bacteroids (Mergaert *et al.*, 2003; Mergaert *et al.*, 2006). Since upon differentiation into bacteroids *S. meliloti* increase in cell size and DNA content (Mergaert *et al.*, 2006) it was next logical to determine if exposure to the Bac7 peptide resulted in an increase in cell size. Thus, the dot plot profiles of untreated (Fig. 4-6A& B) and treated cells (Fig. 4-6C& D) of the Rm1021 parent (Fig. 4-6A& C) and Rm1021 Δ*bacA* (Fig. 4-6B& D) mutant are presented. These dot plots show forward scatter (FSC-A), which relates to cell size, versus side scatter (SSC-A), which relates to granularity of the cells. Hence, these profiles show the distribution of cells based upon size. However, it can be seen that under these conditions, for both the Rm1021 parent (Fig. 4-6A& C) and the Rm1021 Δ*bacA* mutant (Fig. 4-6B& D) treatment with $Bac7_{1\text{-}16}$-BY does not appear to result in any major change in cell size.

Overall, these data show that under these conditions, the presence of the *S. meliloti* BacA protein appears to be essential for the intracellular accumulation of the truncated form of $Bac7_{1\text{-}16}$-BY.

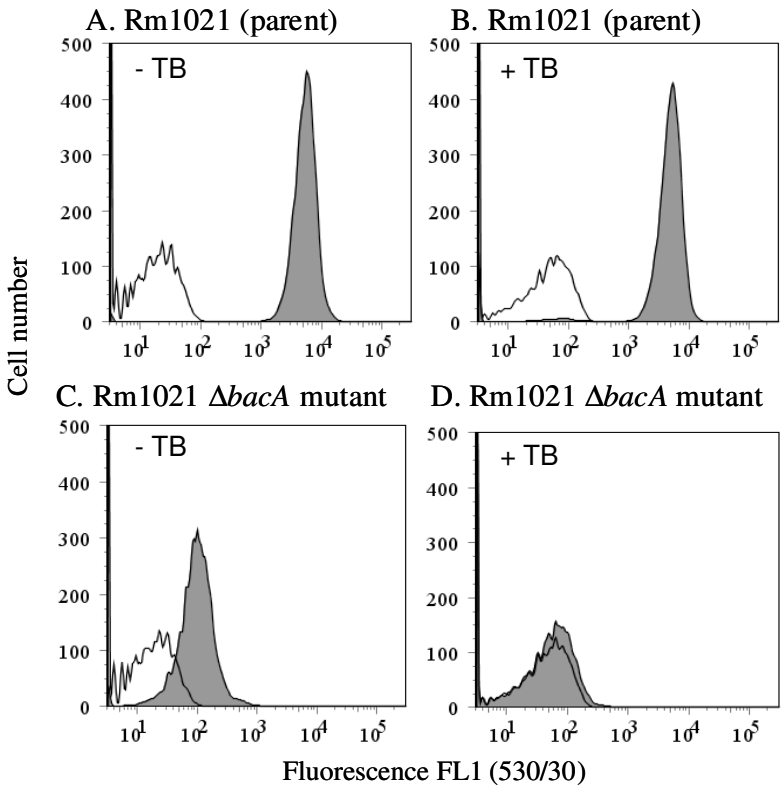


Figure 4-5. Flow cytometry analysis of Rm1021 parent cells and Rm1021 *ΔbacA* mutant cells exposed to Bac7$_{1-16}$-BY. Mid-exponential phase cells of the defined strains were treated with (shaded histograms) or without (empty histograms) 0.25 µM of the Bac7$_{1-16}$-BY for 1 hour and analysed by flow cytometry with (B & D) and without (A & C) trypan blue (TB) pre-incubation as indicated. The datasets shown are representative of the trends observed in two independent experiments.

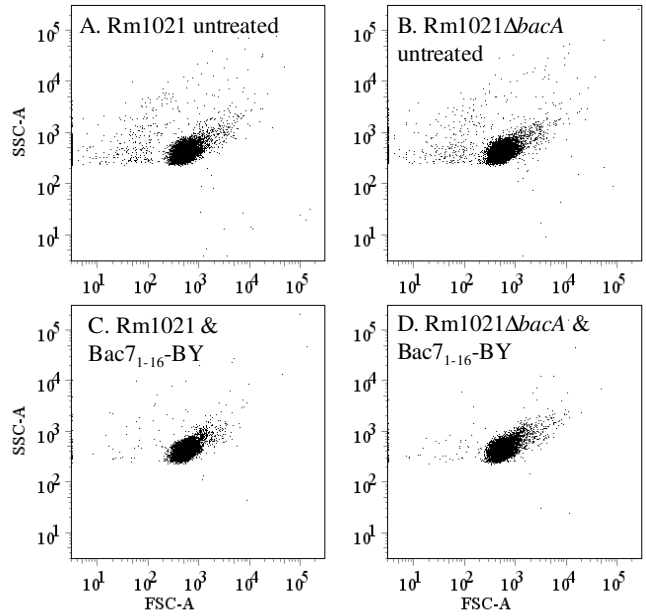


Figure 4-6. Flow cytometry analysis of Rm1021 parent cells and Rm1021 *ΔbacA* mutant cells exposed to Bac7$_{1-16}$-BY. Dot plot profiles showing forward scatter (FSC-A), which is an indicator of cell size, against side scatter (SSC-A), which is an indicator of granularity of the cells. The Rm1021 parent (A & C) and Rm1021 *ΔbacA* mutant (B & D) untreated and treated respectively are shown. The datasets shown are representative of the trends observed in two independent experiments.

4.2.4. BacA or *E. coli* SbmA complement the Bac7$_{1-16}$-BY uptake defect of the *S. meliloti* Δ*bacA* mutant

To confirm that the differences observed by flow cytometry (Fig. 4-5) were due to the BacA protein, uptake of Bac7$_{1-16}$-BY into the Rm1021 Δ*bacA* mutant with either a control plasmid (pRK404) (Fig. 4-7A & B) or pJG51A (containing the *S. meliloti* wild-type *bacA* gene) (Fig. 4-7C & D) was next assessed. As seen for the Rm1021 Δ*bacA* mutant (Fig. 4-5C), an increased fluorescence of Rm1021 Δ*bacA* with the pRK404 control plasmid after Bac7$_{1-16}$-BY addition was observed (Fig. 4-7A) but this was eliminated by TB incubation (Fig. 4-7B), suggesting it was extracellular binding. In contrast, a dramatic increase in the fluorescence of the Rm1021 Δ*bacA* mutant with pJG51A after incubation with Bac7$_{1-16}$-BY was observed both with and without subsequent incubation with TB (Fig. 4-7C & D respectively).

Since it had previously been determined that the *E. coli* SbmA protein could compensate for the absence of BacA in sensitizing *S. meliloti* towards Bac7(1-16) and $Bac7_{1-16}$-BY it was next assessed whether the *E. coli* SbmA protein could also compensate for the role of BacA in $Bac7_{1-16}$-BY uptake (Fig. 4-8E & F). It could be observed that the *E. coli* SbmA protein could restore the ability of *S. meliloti* lacking BacA to accumulate $Bac7_{1-16}$-BY. Thus, these data show that presence of either the *S. meliloti bacA* gene or the *E. coli sbmA* gene restores the ability of a *S. meliloti* strain lacking BacA to accumulate Bac7(1-16).

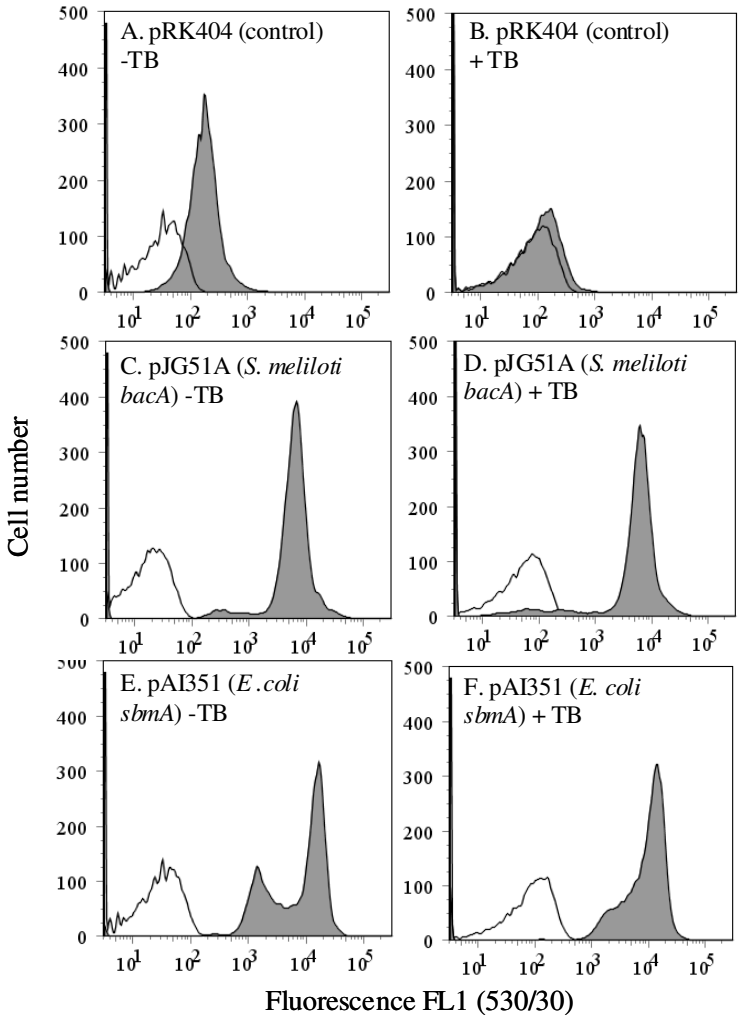


Figure 4-7. Flow cytometry analysis of Rm1021 *ΔbacA* mutant cells with and without the BacA protein exposed to $Bac7_{1-16}$-BY. Mid-exponential phase cultures of the Rm1021 *ΔbacA* mutant cells with the defined plasmid were treated with (shaded histograms) or without (empty histograms) 0.25 μM of the $Bac7_{1-16}$-BY for 1 hour and analysed by flow cytometry with and without trypan blue (TB) pre-incubation as indicated. The datasets shown are representative of the trends observed in two independent experiments.

4.2.5. Site-directed mutations in the *bacA* gene affect the sensitivity of *S. meliloti* towards Bac7(1-16) and the uptake of Bac7

As already mentioned it has been shown previously that antimicrobial peptides can be found in the root nodules of legumes and mammalian cells (Alunni *et al.*, 2007; Frank *et al.*, 1990; Kevei *et al.*, 2002; Mergaert *et al.*, 2003). Hence, to investigate whether the ability of the *S. meliloti* BacA protein to take up a host-derived peptide(s) is linked to its essential role in host persistence, a set of plasmid-borne site-directed mutants (SDMs) were next assessed. These SDMs had been previously constructed (LeVier & Walker, 2001) in the Rm8002 parent background and characterized in terms of their symbiotic ability with alfalfa (LeVier & Walker, 2001). Thus to be consistent with previously published data (LeVier & Walker, 2001) the Rm8002 background was also used in this work. In these SDMs conserved residues had been mutated to glycines. Interestingly, four of the amino acids changed in this mutant set (Q193G, D198G, R284G and R389G) were found to be conserved within the human adrenoleukodystrophy protein (Ferguson *et al.*, 2004). It has also previously been shown that these four site-directed mutants were symbiotically defective in the alfalfa host (LeVier & Walker, 2001) and have a reduction in their lipid A VLCFA content relative to the Rm1021 parent strain (Ferguson *et al.*, 2004). The sensitivity of the nine SDMs which had previously been shown to be symbiotically defective in alfalfa (LeVier & Walker, 2001), to Bac7(1-16) was assessed, relative to the Rm8002 Δ*bacA* mutant with the pRK404 control vector and Rm8002 Δ*bacA* with pJG51A (containing the *S. meliloti bacA* gene) (Fig. 4-8A). It was found that 7 out of the 9 site-directed mutants, including the D198G and R284G mutants, were highly resistant to the killing effects of Bac7(1-16), as no reduction in viability was observed (Fig. 4-8A). However, the *bacA* site-directed mutants Q193G and R389G were sensitive to the toxic effects of the peptide (Fig. 4-8A). To confirm that sensitivity of these SDMs to Bac7(1-16) was linked to uptake, $Bac7_{1\text{-}16}$-BY intracellular accumulation in Q193G, D198G, R284G and R389G was next assessed, relative to Rm8002 Δ*bacA* with the pRK404 control plasmid and Rm8002 *bacA* with pJG51A (Fig. 4-8 B). Here, prior to flow cytometry, all cells were pre-treated with TB. Consistent with the viability data (Fig. 4-8 A), D198G and R284G were completely defective in $Bac7_{1\text{-}16}$-BY uptake.

However, Q193G and R389G were able to accumulate $Bac7_{1-16}$-BY (Fig. 4-8B). Hence, since the symbiotically defective mutants Q193G and R389G can still take up Bac7(1-16), this suggests that the essential role of BacA in peptide uptake is unlikely to fully account for its essential role in the intracellular persistence of *S. meliloti*. Since it was shown previously that the Q193G and R389G mutants have reductions in their VLCFA contents (Ferguson *et al.*, 2004), these data suggest that the loss of BacA function necessary for lipid A VLCFA modification may account for their symbiotic defects.

However, when the remaining site-directed mutants, which had previously been shown to be symbiotically competent in alfalfa (LeVier & Walker, 2001) were next assessed for their sensitivity to Bac7(1-16) (Fig. 4-9A), all mutants were found to be sensitive to Bac7(1-16) induced killing (Fig. 4-9A). Additionally, when a selection of these mutants were analysed by flow cytometry they were all found to accumulate $Bac7_{1-16}$-BY (Fig. 4-9B). So taken together, all these data would support a model whereby both the role of the BacA protein in peptide uptake and in affecting the VLCFA modification could account for its crucial role in chronic infection.

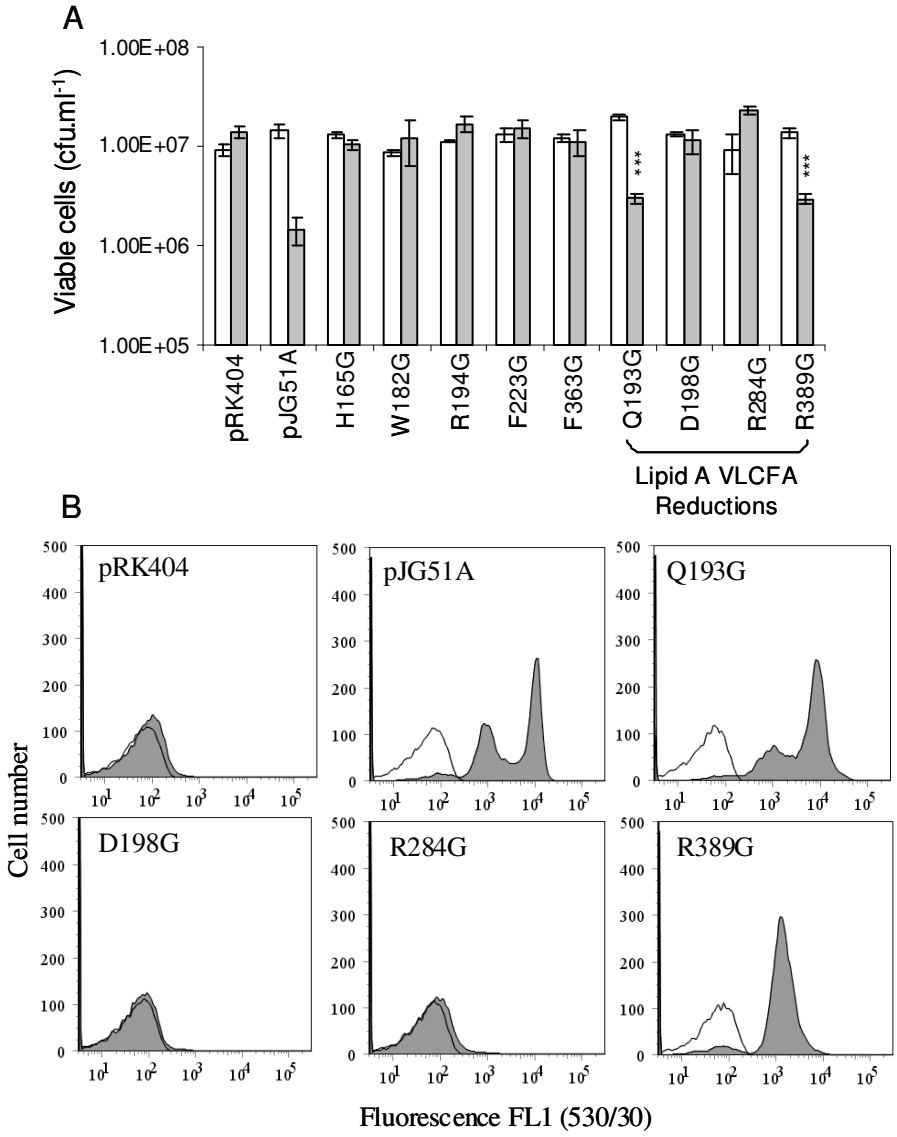


Figure 4-8. Symbiotically defective site-directed mutants were assessed for sensitivity to Bac7(1-16) and ability to accumulate Bac7$_{1-16}$-BY. (A) Cultures of the Rm8002 Δ*bacA* mutant with pRK404 (control vector), Rm8002 Δ*bacA* with pJG51A (containing the *S. meliloti bacA* gene) or the defined symbiotically defective *bacA* site directed mutants, were exposed to 1 µM Bac7(1-16) and the cell viability was determined before (open bars) and 1 hour (shaded bars) after addition. The significant values shown represent comparisons of the Rm8002 Δ*bacA* mutant strain with either Q193G or R389G compared with pRK404 (control vector). Mutants which have been previously shown to have a reduction in their lipid A VLCFA content (Ferguson *et al.*, 2004) are highlighted under the graph. (B) As in A, except cultures were treated with (shaded profiles) and without (open profiles) 0.25 µM Bac7$_{1-16}$-BY for 1 hour and analysed by flow cytometry. In all profiles shown cells had been pre-incubated with TB prior to analysis. Dataset A is representative of the trends observed in two independent experiments and dataset B shows preliminary data.

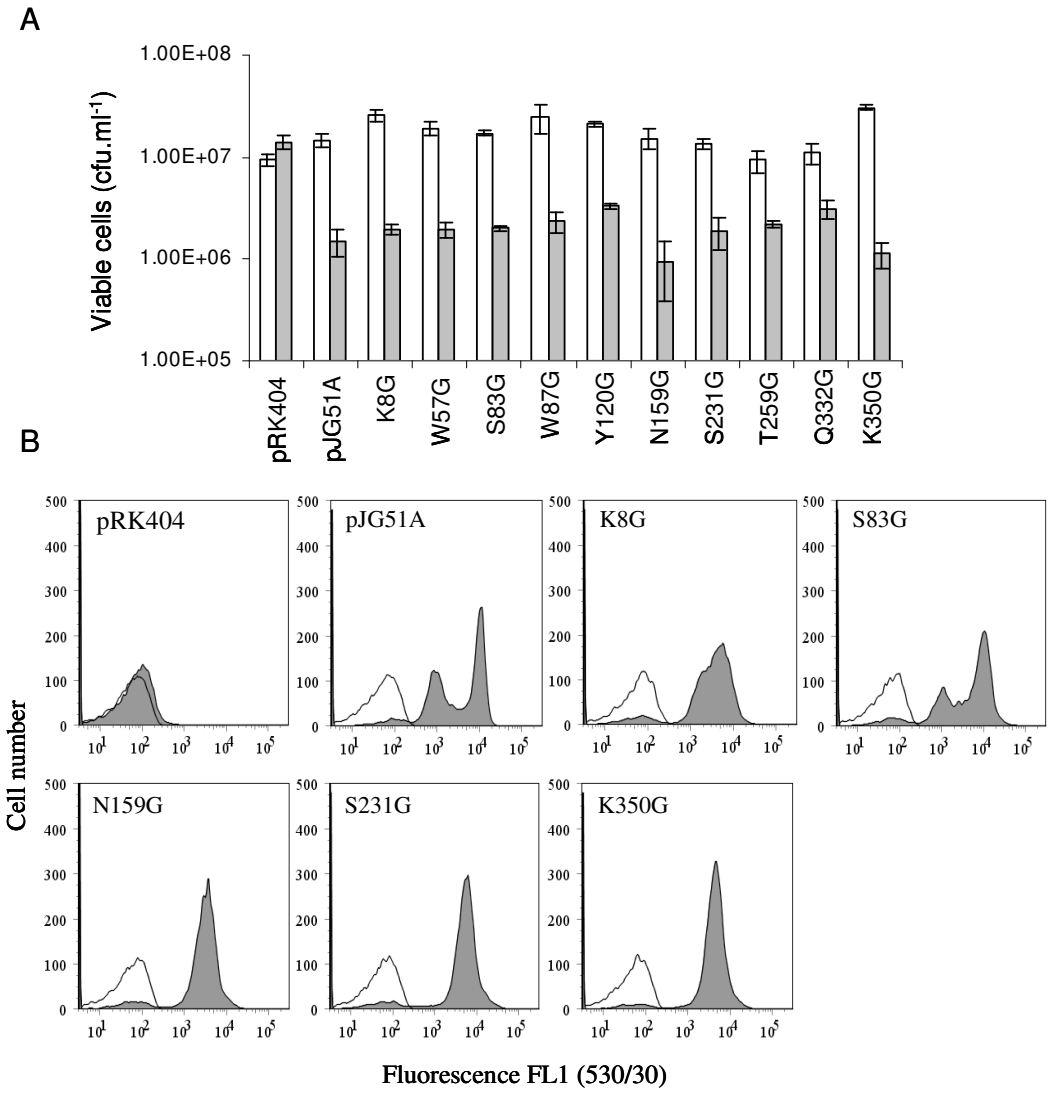


Figure 4-9. Symbiotically competent site-directed mutants were assessed for sensitivity to Bac7(1-16) and ability to accumulate Bac7$_{1-16}$-BY. (A) Cultures of the Rm8002 Δ*bacA* mutant with pRK404 (control vector), Rm8002 Δ*bacA* mutant with pJG51A (pRK404 vector with the *S. meliloti bacA* gene) or the defined symbiotically competent *bacA* site directed mutants, were exposed to 1 µM Bac7 (1-16) and the cell viability was determined before (open bars) and 1 hour (shaded bars) after addition. (B) As in A, except cultures were treated with (shaded profiles) and without (open profiles) 0.25 µM Bac7$_{1-16}$-BY for 1 hour and analysed by flow cytometry. In all profiles shown cells had been pre-incubated with TB prior to analysis. Dataset A is representative of the trends observed in two independent experiments and dataset B shows preliminary data.

4.2.6. Expression of the *Mycobacterium tuberculosis* BacA homolog in the *E. coli* RYC1001 *sbmA* mutant did not result in an increased intracellular accumulation of $Bac7_{1-16}$-BY

A recent study found that like the *S. meliloti* and *B. abortus* BacA proteins, the BacA like protein in *M. tuberculosis* plays an essential role in maintaining chronic infection (Domenech *et al.*, 2008). Likewise, disruption of the *M. tuberculosis bacA* gene also resulted in an increased resistance to the glycopeptide bleomycin. Additionally, it has been found that expression of the *M. tuberculosis* BacA homolog in the *E. coli* RYC1001 *sbmA* mutant sensitised the *sbmA* mutant to Bac7(1-16). Interestingly, the *M. tuberculosis* BacA like protein is predicted to be an ABC transporter (Domenech *et al.*, 2008). Hence, these findings would suggest that like the *S. meliloti* BacA and *E. coli* SbmA proteins, the *M. tuberculosis* BacA protein may play a role in peptide uptake.

To investigate the hypothesis that the *M. tuberculosis* BacA protein may play a role in Bac7(1-16) uptake, sensitivity of cells to Bac7(1-16) over 1 hour (Fig. 4-10) was firstly determined. The strains assessed were the *E. coli* RYC1001 parent strain, the RYC1001 *sbmA* (spontaneous) mutant, the RYC1001 *sbmA* (spontaneous) mutant carrying the pWSK29 control plasmid and the *sbmA* (spontaneous) mutant carrying pWSK-Mt*bacA* (expressing *M. tuberculosis* BacA). In the case of the latter two strains, the cells were induced with 0.4 mM IPTG for 2 hours prior to the assay and additionally during exposure to the peptide. The presence of IPTG should induce transcription of the M. *tuberculosis* BacA protein, which was under control of the *lac* promoter (Domenech *et al.*, 2008). However, it can be seen that upon exposure of the cells to 10 µM Bac7(1-16) under these conditions, no killing was observed in any of the four strains (Fig. 4-10). Thus, this data would suggest that under these conditions *E. coli* appears more resistant (Fig. 4-10) to Bac7(1-16), than *S. meliloti* (Fig. 4-2A & B). Unfortunately due to a limited stock of the Bac7(1-16) peptides, at the time of the assay, it was not possible to repeat the assay with a higher concentration of Bac7(1-16). Since it had not been determined if the RYC1001 *sbmA* mutant displayed an increased resistance to Bac7(1-16), relative to the RYC1001 parent, it was not possible to investigate if the presence of the *M. tuberculosis bacA* gene sensitized cells to the Bac7 peptide, under these conditions.

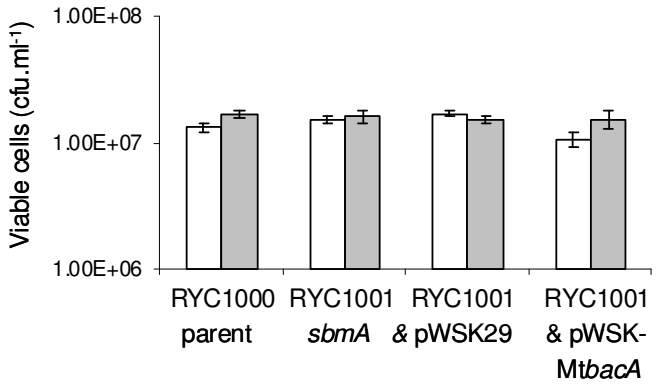


Figure 4-10. Effect of Bac7(1-16) on the viability of *E. coli* strains with and without the SbmA protein. Cultures of the defined strains were exposed to 10 µM Bac7(1-16) and cell viability determined before (open bars) and 1 hour (shaded bars) after addition. The dataset shows preliminary data. The error bars represent the standard deviation from the mean (n=3) for one experiment.

It had previously been observed in *E. coli* that a higher dose of Bac7(1-16) was required to induce killing of the cells (Podda *et al.*, 2006), relative to the concentration used for *S. meliloti* in this work. Yet, in *E .coli* uptake of Bac7 was still observed at concentrations which did not induce cell death (Mattiuzzo *et al.*, 2007). Hence, despite the fact no killing had been observed (Fig. 4-10) uptake of $Bac7_{1-16}$-BY was next assessed in the *E. coli* strains. In this case all cells were pre-treated with TB, prior to analysis (Fig. 4-11). It was observed that like the RYC1001 parent strain (Fig. 4-11A), the RYC1001 *sbmA* mutant cells (Fig. 4-11B) showed an increased fluorescence, relative to the untreated control cells, suggesting that the *E. coli* cells took up $Bac7_{1-16}$-BY, even in the absence of SbmA. Although the histrogram profiles of the RYC1001 plus control vector (Fig. 4-11C) and RYC1001 plus *M. tuberculosis bacA* gene (Fig. 4-11D) are shown, since the RYC1001 *sbmA* mutant cells did not display any phenotype, it could not be investigated if the presence of the *M. tuberculosis bacA* gene resulted in an increased level of Bac7(1-16) uptake.

Thus, unfortunately it was not possible under these conditions to assess the role of the *M. tuberculosis bacA* gene in uptake of the Bac7(1-16) peptide.

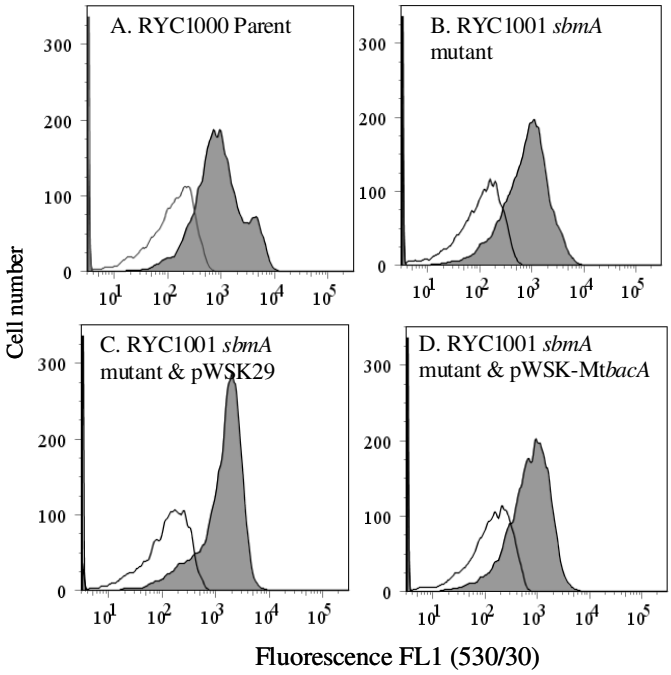


Figure 4-11. Flow cytometry analysis of *E. coli* cells with and without the SbmA and BacA proteins exposed to Bac7$_{1-16}$-BY. Mid-exponential phase cultures of the defined strains were treated with (shaded histograms) or without (empty histograms) 0.25 µM of the Bac7$_{1-16}$-BY for 1 hour and analysed by flow cytometry followed by TB pre-incubation. The datasets shown are preliminary data.

4.3. Discussion

The work in this chapter has clearly shown that the *S. meliloti* BacA protein is essential for uptake of the truncated eukaryotic peptide Bac7(1-16). This is a significant finding, since for several years BacA has been implicated in the uptake of the glycopeptide bleomycin (Ichige & Walker, 1997). The essential role found for BacA in the uptake of this truncated eukaryotic peptide helps to rationalise the crucial role for the BacA protein in host persistence. Additionally, the finding that two symbiotically defective *bacA* site directed mutants (Q193G and R389G) which had previously been shown to have VLCFA reductions (Ferguson *et al.*, 2004), could take up the peptide, suggest that the BacA function that leads to the VLCFA modification may also play an essential role in the chronic infection.

4.3.1. The BacA protein is essential for the uptake of Bac7(1-16)

A previous study with *E. coli* found that the BacA homolog SbmA, also plays a role in the uptake of a truncated form of Bac7, $Bac7_{1-35}$-BY (Mattiuzzo *et al.*, 2007). However, some sensitivity to the peptide and lower level of uptake was observed in the absence of SbmA, suggesting possible involvement of another transport system. Yet, at concentrations of the Bac7(1-16) peptide tested in this work, no killing was observed and no uptake was seen in the absence of the *S. meliloti* BacA protein. Additionally, recent work has shown the *S. meliloti* Δ*bacA* mutant is also resistant to the killing effects of the longer truncated form of the drug Bac7(1-35), even though exposure resulted in a total loss in cell viability in the Rm1021 parent strain (A.F.Haag and G.P.Ferguson, unpublished data). Since the *S. meliloti* Δ*bacA* mutant is unable to take up the peptide and is completely resistant to the killing effects of the peptide, this is also consistent with the cytotoxic effects of Bac7 being due to interaction with a cellular target as proposed previously (Scocchi *et al.*, 2008). However, the exact molecular targets of the Bac7 peptide are currently unknown. The proline-rich AMPs pyrrhocoricin and drosocin, which were isolated from insects, are better characterised and have been shown to kill bacterial cells by inhibition of the chaperone protein DnaK (Kragol *et al.*, 2001; Kragol *et al.*, 2002). Additionally, the proline rich AMP PR-39, isolated from pigs is known to inhibit protein and DNA synthesis in bacteria (Boman *et al.*, 1993).

4.3.2. Sensitivity to Bac7(1-16) is independent of the VLCFA modification

Although loss of the BacA protein in *S. meliloti* and *B. abortus* results in a reduction in the VLCFA content of the lipid A (Ferguson *et al.*, 2004), this work was able to show that resistance to Bac7(1-16) was not as a consequence of this altered VLCFA. Firstly, it was shown that the Rm1021 *acpXL* mutant, which completely lacks the VLCFA modification (Ferguson *et al.*, 2005), displayed the same level of sensitivity to Bac7(1-16) as the Rm1021 parent strain. Additionally, the Rm1021 *acpXL* and Rm1021 *acpXL*/Δ*bacA* mutants have previously been shown to have the exact same lipid A profile (Ferguson *et al.*, 2005), yet deletion of *bacA* in a *acpXL* mutant

background resulted in an increased resistance to Bac7(1-16). Additionally, unlike *S. meliloti* the lipid A of *E. coli* does not contain the VLCFA modification, yet the *E. coli* SbmA protein has been shown to have a role in Bac7 uptake in both *E. coli* and *S. meliloti*. Moreover, the finding that two symbiotically defective mutants (Q193G and R389G mutations), previously shown to be to have a reduced VLCFA content, relative to the parent were sensitive to the effects of the peptide further confirmed that the increased resistance of the Rm1021 Δ*bacA* mutant is not an indirect result of the lipid A VLCFA reduction.

4.3.3. The role of the VLCFA modification in the symbiosis

When Bac7(1-16) uptake was assessed in the Q193G and R389G SDMs, it was confirmed that despite being symbiotically defective, these mutants were able to take up the peptide. Therefore, this would suggest that the role of BacA in peptide uptake cannot solely account for the essential role of this protein in the symbiosis. Instead, it would suggest that the function of BacA that affects the lipid A VLCFA modification also plays an essential role in the symbiosis. Previous studies characterising the *in planta* phenotypes of the *S. meliloti acpXL* and *lpxXL* mutants which completely lack the VLCFA modification, have shown that although they form a symbiosis, they are less competitive in the host, relative to the parent strain (Ferguson *et al.*, 2005; Sharypova *et al.*, 2003). These data suggest that, although not essential, the VLCFA modification does play an important role in the symbiosis.

Additionally, in the closely related bacterium *R. leguminosarum*, an *acpXL* mutant which also completely lacks the VLCFA in the free-living state was found to have the VLCFA modification partially restored after passage though the plant (Vedam *et al.*, 2006). This suggests that in an *R. leguminosarum* mutant lacking AcpXL there may be host induced lipid A modifications occurring. Taken together, these data highlight the importance of the VLCFA modification, suggesting that the reduction in the Rm1021 Δ*bacA* mutant VLCFA content may affect the symbiosis. However, the importance of the VLCFA modification in the symbiosis is investigated in chapter 6 where the in planta phenotypes of the *S. meliloti acpXL* and *lpxXL* mutants are further characterised.

4.3.4. Root nodule peptides have been hypothesized to play an important role in bacteroid development

It has been proposed that peptides produced in root nodules may play an important role in bacteroid development (Mergaert *et al.*, 2003). Work investigating the differentiation of bacteroids in the *S. meliloti*-legume symbiosis (Mergaert *et al.*, 2006) found that repeated DNA replication occurs leading to amplification of the *S. meliloti* genome and elongation of the cell (Mergaert *et al.*, 2006). However, it was observed that the *S. meliloti* bacteroids lacking the BacA protein were not elongated and did not amplify their genome (Mergaert *et al.*, 2006). This finding is consistent with the BacA protein been involved in the uptake of a peptide that may be essential for bacteroid differentiation and ultimate survival within the host.

Although in this study, the truncated peptide Bac7(1-16) results in cell death in the legume host, the BacA protein could be important for uptake of a related but non-lethal eukaryotic peptide essential for bacteroid differentiation. Transcriptome analysis of *Medicago truncatula* nodules identified a family of genes called nodule-specific cysteine rich (NCR), with over 300 genes in the family (Alunni *et al.*, 2007; Mergaert *et al.*, 2003). The encoded polypeptides are typically 60-90 amino acids long and reveal extensive sequence divergence, aside from a conserved cysteine motif. Expression of the peptides is mainly restricted to developing and mature nodules (Mergaert *et al.*, 2003), which is consistent with a role for these peptides in the symbiosis.

In the legume *Medicago truncatula* RNA interference studies revealed a role for two host genes ENOD40-1 and ENOD40-2 in bacteroid development (Wan *et al.*, 2007). Interestingly, these ENOD40 genes are thought to encode peptides, one of the open reading frames of the ENOD40-1 gene has been shown to encode a 13 amino acid peptide (MKLLCWEKSIHGS). Therefore, peptides produced by the ENDO40 genes could be potential candidates for BacA mediated uptake leading to bacteroid differentiation.

Although to date proline rich peptides have not been identified in legume nodules, several nodule specific genes have been shown to encode proline rich proteins (Nap & Bisseling, 1990). In *Medicago truncatula* one member of the proline rich gene family MtPRP4 has been characterised (Wilson *et al.*, 1994). The

mature protein encoded by MtPRP4 consists of a repetitive pentapeptide domain rich in proline, lysine and tyrosine. Proline rich proteins are thought to be components of the host cell wall and it is hypothesized that they may play a role in remodelling of the extracellular matrix upon infection by *S. meliloti*. Since these proline rich proteins have been shown to be expressed early in nodule development it is tempting to speculate that the turn-over or cleavage of these proteins may be a source of proline rich peptides for *S. meliloti* at the beginning of the symbiosis.

4.3.5. The full length Bac7 peptide was isolated from bovine neutrophils

Although in this work a truncated form of the eukaryotic peptide Bac7(1-16) was used, the full length 60 amino acid Bac7 was isolated from bovine neutrophils (Frank *et al.*, 1990). Since *B. abortus* is a pathogen of cattle, the peptide may have a biological relevance. Although the truncated Bac7(1-16) was toxic to *S. meliloti* under the conditions tested, perhaps in bovine host cells the *B. abortus* BacA protein may be essential for uptake of the full length peptide where it may be important in signalling the transition from the acute to chronic state of infection. Despite the fact the *B. abortus* BacA mutant is attenuated in infection in BALB/c mice (LeVier *et al.*, 2000), in another mouse host strain C57BL/6, infection with the *B. abortus bacA* mutant was actually found to be more pathogenic (Parent *et al.*, 2007), relative to the parent strain. Since these two mice strains are known to have differences in their immune responses (Parent *et al.*, 2007), they may also have differences in the types of peptides produced. Therefore, BacA mediated peptide uptake may be essential for the chronic infection of *B. abortus* in BALB/c but not C57BL/6 mice.

4.3.6. Role of the BacA protein in peptide uptake

Although this work has shown that presence of the BacA protein is essential for Bac7 uptake, it is not known whether BacA is directly or indirectly involved in peptide uptake. As discussed in the case of *E. coli*, some Bac7 uptake did still occur in the absence of the SbmA protein (Mattiuzzo *et al.*, 2007), suggesting another transport system may be involved. The distant sequence relationship between BacA and a family of eukaryotic peroxisomal membrane proteins including the

adrenoleukodystrophy protein (Ferguson *et al.*, 2004), led to the proposal whereby BacA could be directly involved in the transport of activated VLCFAs out of the cytoplasm across the inner membrane where they are used to modify the lipid A. If this model is true and BacA also directly transports Bac7(1-16), then the BacA protein would need to be capable of transporting two completely different molecules in opposite directions (Fig. 4-12A). Alternatively, the BacA protein may affect the VLCFA modification indirectly by action of another protein (Fig. 4-12B). In addition, there also remains the possibility that BacA may affect uptake of a peptide by some action on another inner membrane protein (Fig. 4-12B).

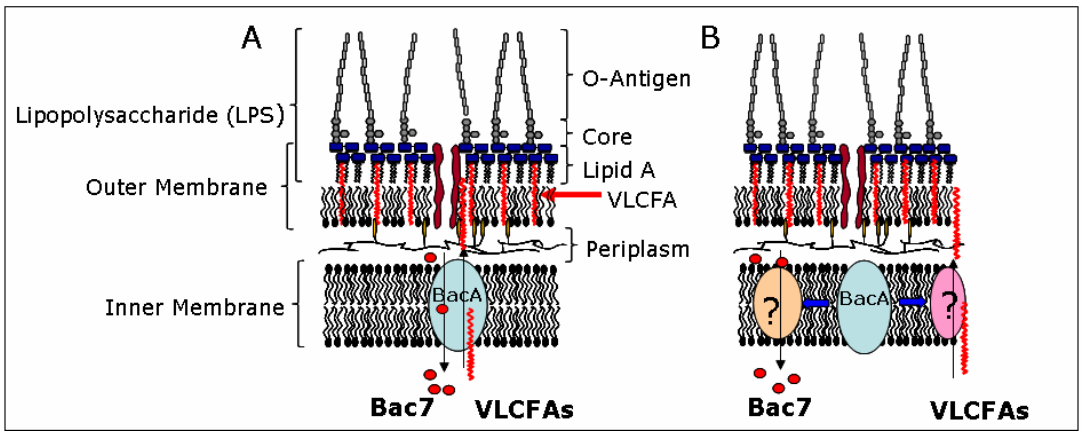


Figure 4-12. Proposed models for BacA function. (A) The BacA protein is capable of transporting two different molecules in opposite directions, the Bac7 peptide into and VLCFAs out of the cytoplasm. (B) Alternatively, the BacA protein may affect the VLCFA modification indirectly by the action of another protein and there also remains the possibility that BacA may affect Bac7 peptide uptake by some action on another inner membrane protein.

4.3.7. Future studies

As discussed hundreds of cysteine rich peptides have been shown to be produced in legume nodules (Alunni *et al.*, 2007; Mergaert *et al.*, 2003). Future work investigating these peptides should determine if their uptake is important for bacteroid differentiation. However, actual synthesis of cysteine rich peptides is very difficult (Juskowiak *et al.*, 2008) and so studies in this direction may take several years. Since the Bac7 peptide may have biological significance in *B. abortus* infection, it will be very informative to determine if the *B. abortus* BacA gene

sensitizes cells to Bac7 and is essential for uptake. Cross linking studies with BacA and selection for further *S. meliloti* Bac7 resistance mutants may help to establish if BacA plays a direct role in Bac7 transport and if any other proteins are involved in Bac7 uptake.

Interestingly, an *M. tuberculosis* mutant lacking a *bacA* like gene (*Rv1819c*) is compromised in chronic infection of mice and shows an increased resistance to the glycopeptide, bleomycin (Domenech *et al.*, 2008). Additionally, it was found previously, that expression of the *M. tuberculosis* BacA homolog in an *E. coli sbmA* mutant sensitized this mutant strain to Bac7(1-16) (Domenech *et al.*, 2008). It is unfortunate that due to lack of time it was not possible to investigate the role of the *M. tuberculosis* BacA like protein in $Bac7_{(1-16)}$-BY uptake in this work. Since it was not possible to induce sensitivity to Bac7(1-16) in the *E. coli* strain used, any future assays using this *E. coli* strain should be performed using a higher dose of the Bac7 peptide. Additionally, it may be advantageous to try a different *E. coli* parent strain and respective *sbmA* mutant for the expression of the *M. tuberculosis* BacA like protein. However, the findings published to date (Domenech *et al*., 2008) raise the possibility that the *M. tuberculosis* BacA like protein may also be involved in the uptake of a host derived peptide, which may play an important role in the outcome of *M. tuberculosis* infections which today remain a leading cause of mortality worldwide.

Chapter 5: Investigation into the Role of Glutathione in Protection of *S. meliloti* from Toxic Compounds

5.1. Introduction

In chapter 3 it was determined that the absence of the BacA protein results in a decreased accumulation of fluorescently labelled bleomycin A_5 in *S. meliloti* cells, consistent with the hypothesis that BacA plays some role in bleomycin uptake. To rule out the possibility that BacA was affecting a detoxification process, the role of the tri-peptide glutathione in protection against bleomycin A_5 by filter disc assay was investigated. It was found that glutathione did confer protection in *S. meliloti* against bleomycin. Additionally, this protection still occurred in the absence of the BacA protein, suggesting the protection by glutathione was not dependent upon the presence of BacA. In a previous study, intracellular glutathione has been shown to be necessary for a successful symbiosis of *S. meliloti* with alfalfa (Harrison *et al.*, 2005). However, little is known about the role of glutathione in free-living *S. meliloti,* in protection against toxic compounds. Hence, this chapter details a preliminary study into the role of glutathione in protection of free-living *S. meliloti* from toxic compounds and compares the findings to the well-characterised glutathione system in *E. coli* (Ferguson & Booth, 1998; Ferguson *et al.*, 1998; MacLean *et al.*, 1998)

Glutathione is the most abundant non-protein thiol found in many organisms (Fahey *et al.*, 1978; Fahey & Sundquist, 1991; Penninckx & Elskens, 1993) and is synthesized by a two-step process (Fig. 5-1).

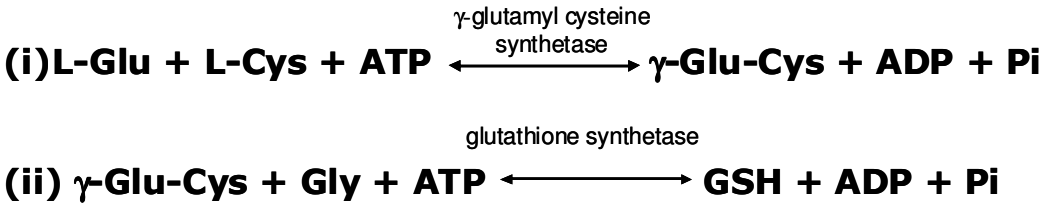


Figure 5-1. Synthesis of glutathione. In the first step; glutamate and cysteine are conjugated by γ-glutamyl cysteine synthetase (encoded by the *gshA* gene) to form γ-glutamyl cysteine (i). In a second step, glycine is added to γ-glutamyl cysteine to form glutathione in a reaction catalyzed by glutathione synthetase (encoded by the *gshB* gene) (ii) (Adapted from (Masip *et al.*, 2006).

In both eukaryotic and prokaryotic cells glutathione plays a critical role in protecting cells from oxidative damage and maintaining redox homeostasis (Forman *et al.*, 2008; Oktyabrsky & Smirnova, 2007). The exclusion of many toxic compounds from cells can be achieved through conjugation with glutathione followed by secretion of the adduct (Boyland & Chasseaud, 1969). An enzyme called glutathione *S*-transferase catalyses this conjugation reaction (Tsuchida & Sato, 1992). Glutathione is also required for the glyoxalase I and II enzymes that detoxify the toxic electrophile methylglyoxal (Inoue & Kimura, 1995). In *E. coli,* the role of glutathione in the detoxification of the toxic electrophile methylglyoxal is particularly well-characterised (Ferguson & Booth, 1998). Consistent with the findings for *S. meliloti* in chapter 4, several studies have implicated a role for glutathione in detoxification of bleomycin in eukaryotic cells. The hypersensitivity of Chinese hamster ovary cells to bleomycin was found to be due to a lack of glutathione *S*-transferase activity (Giaccia *et al.*, 1991) and cellular glutathione levels were also found to be up-regulated by bleomycin in bovine pulmonary endothelial cells (Day *et al.*, 2002).

A previous study (Harrison *et al.*, 2005), identified the genes involved in glutathione synthesis in *S. meliloti*. The open reading frame *smc00825* (*gshA*) was found to encode γ-glutamyl cysteine synthetase and the open reading frame *smc00419 (gshB*) was found to encode glutathione synthetase. The functions of these two gene products were confirmed by HPLC analysis of the total cellular content of the respective *S. meliloti* mutants. It was found the *gshA* mutant strain did not accumulate γ-glutamyl cysteine or glutathione and the *gshB* mutant accumulated γ-glutamyl cysteine. Thus, this provided evidence that no other genes code for functions able to replace the sequences altered in the mutants.

Work in this chapter begins with further investigation into the sensitivity of the *S. meliloti gshA* mutant exposed to bleomycin A_5.

5.2. Results

5.2.1. The *S. meliloti gshA* mutant displays an increased resistance to bleomycin A_5 in liquid culture

The data presented in chapter 3 had assessed only growth inhibition of the *S. meliloti* Rm1021 *gshA* mutant exposed to bleomycin on solid media (Fig. 3-12). Actual killing was next assessed by recovery of viable cells after exposure to bleomycin A_5 in LB broth (Fig. 5-2). It was anticipated that the *S. meliloti* Rm1021 *gshA* mutant would be more sensitive to killing, than the Rm1021 parent, when exposed to bleomycin A_5. Surprisingly, it was observed that in LB liquid culture, the *S. meliloti* Rm1021 *gshA* mutant displays an increased level of resistance to bleomycin A_5, relative to the Rm1021 parent strain (Fig. 5-2). As previously determined in chapter 3 (Fig. 3-4C), the *S. meliloti* Rm1021 Δ*bacA* mutant displays an increased resistance to bleomycin A_5 in liquid culture, relative to the Rm1021 parent. The Rm1021 *gshA* and Rm1021 Δ*bacA* single mutants showed a similar level of sensitivity over the first 180 minutes of the assay. However, after this point the Rm1021 *gshA* mutant showed an increased level of sensitivity relative to the Rm1021 Δ*bacA* mutant. The Rm1021 Δ*bacA/gshA* double mutant showed an increased level of resistance to bleomycin A_5, from 60 minutes onwards, relative to the respective single mutants (Fig. 5-2). This would suggest in the double mutant there is an additive effect of protection by disruption of *gshA* and *bacA*. Additionally, in this assay the Rm1021 *gshA* and Rm1021 Δ*bacA/gshA* mutant cells were recovered onto agar plates containing neomycin, since in these two mutants the *gshA* gene contains an insertional mutation with a neomycin resistance marker (Harrison *et al.*, 2005). Hence, recovery on neomycin confirmed that the *gshA* gene disruption was maintained throughout the time course of the assay. Overall, these data suggest that disruption of the *gshA* gene in *S. meliloti* results in an increased resistance of the cells to bleomycin A_5 in liquid culture.

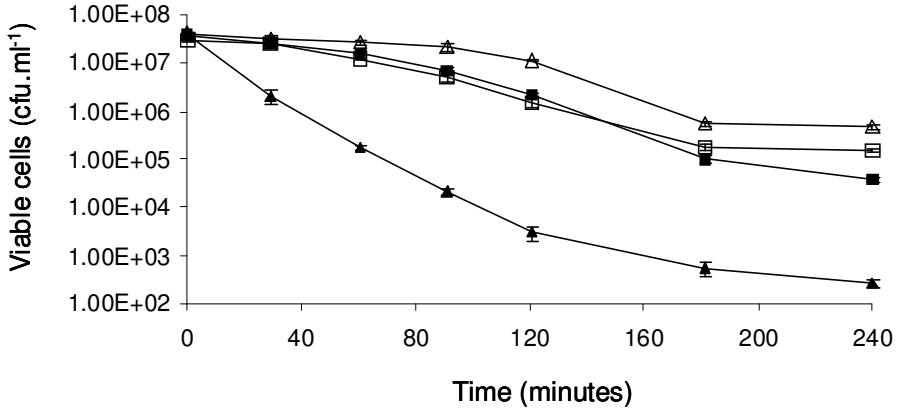


Figure 5-2. Sensitivity of the *S. meliloti* glutathione mutant strains to bleomycin A_5 in liquid culture. Cultures of Rm1021 (closed triangles), Rm1021 Δ*bacA* (open squares), Rm1021 *gshA* (filled squares) and the Rm1021 Δ*bacA/gshA* double mutant (open triangles) were exposed to bleomycin A_5 (0.72 µg.ml^{-1}) and cell viability determined at the defined times. In this case cells were recovered onto LBMC plates containing the appropriate antibiotics. A significant difference of (***P<0.001) was found when the Rm1021 parent strain and *gshA* mutant were compared and a significant difference of (**P<0.01) was found when the Rm1021 Δ*bacA* and Rm1021 Δ*bacA/gshA* double mutant were compared from 60 mins onwards. The datasets shown for the Rm1021 parent and Rm1021*gshA* mutant are representative of trends observed in two independent experiments and the data sets shown for the Rm1021 *ΔbacA* mutant and the Rm1021 Δ*bacA/gshA* double mutant show preliminary data. The error bars represent the standard deviation from the mean (n=3) for one experiment.

5.2.2. Complementation with the *S. meliloti gshA* gene increases the sensitivity of the *gshA* mutant to bleomycin

It was next important to confirm that disruption of the *gshA* gene was responsible for the liquid bleomycin resistant phenotype observed. The *S. meliloti gshA* gene is located on the chromosome and does not appear to be located in the same region as the *gshB* gene. This seems to be common in many bacteria, whereby glutathione synthesis occurs through the consecutive action of the two physically separate enzymes encoded by *gshA* and *gshB* genes (Gopal *et al*., 2005). Since the *S. meliloti gshA* gene is located nearby other genes, which are transcribed in the same direction

(Fig. 5-3), it is possible that the *gshA* gene may be part of an operon, so disruption of the *gshA* gene might have a polar effect on the downstream gene *smc00824*.

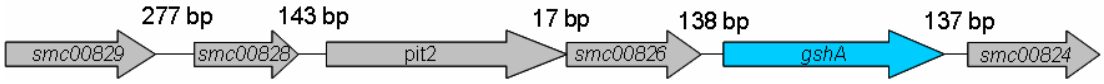


Figure 5-3. The genomic region surrounding the *S. meliloti gshA* gene. The *gshA* (*smc00825*) gene encoding γ-glutamyl cysteine synthetase could be part of a gene operon, with nearby genes encoded on the same strand. Surrounding genes are *smc00829* (encoding a probable transcriptional regulator), *smc00828* (encoding a conserved hypothetical protein), *pit2* (encoding a probable phosphate permease), *smc00826* (encoding a hypothetical protein) and *smc00824* (encoding a hypothetical protein). The numbers shown above in base pairs are the distances between each genes. (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi).

To confirm that disruption of the *S. meliloti gshA* gene was responsible for the liquid bleomycin resistence phenotype observed, the sensitivity of the Rm1021 *gshA* mutant carrying the pgshAc plasmid (pBBR1 control plasmid expressing the *S. meliloti gshA* gene constitutively) (Harrison *et al.*, 2005) to bleomycin A_5 was next assessed (Fig. 5-4). Additionally, the sensitivity of the Rm1021 *gshA* mutant carrying the pBBR1 control plasmid only was also assessed (Harrison *et al.*, 2005) (Fig. 5-4). It was observed that presence of the pgshAc plasmid carrying *gshA* results in the Rm1021 *gshA* mutant displaying an increased sensitivity to bleomycin A_5, whereas the Rm1021 *gshA* mutant strain carrying the control plasmid remained resistant to bleomycin A_5, displaying a similar level of sensitivity as the Rm1021 *gshA* mutant (Fig. 5-4). Therefore, these data confirm that disruption of the *gshA* gene in *S. meliloti* is resulting in an increased resistance to bleomycin A_5 in liquid culture, relative to the Rm1021 parent strain. Additionally, the Rm1021 *gshA* mutants carrying the pgshAc and pBBBR1 plasmids were recovered onto plates with neomycin and gentamycin, since the plasmids carry gentamycin resistance. Thus, confirming that the two mutants were maintaining both the *gshA* mutation and the plasmids.

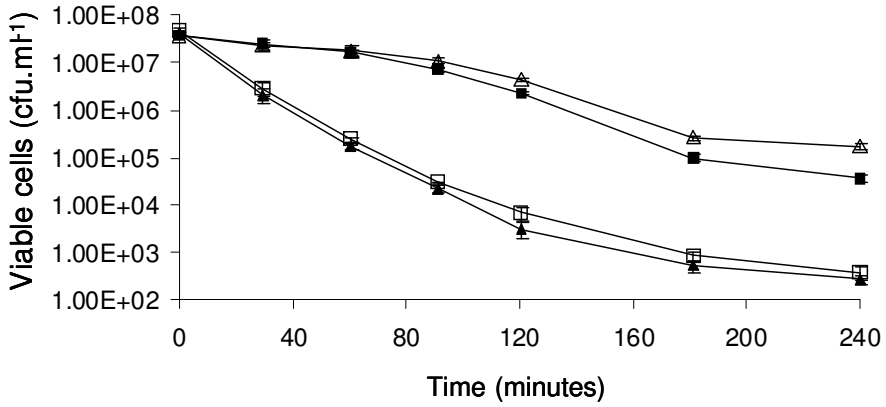


Figure 5-4. Sensitivity of the complemented Rm1021 *gshA* mutant to bleomycin A_5 in liquid culture. Cultures of the Rm1021 parent (closed triangles), the Rm1021*gshA* mutant (closed squares), the Rm1021 *gshA* mutant & pgshAc (*gshA*$^+$) (open squares) and the *gshA* mutant & the control plasmid pBBR1 (open triangles) were exposed to bleomycin A_5 (0.72 µg.ml^{-1}) and cell viability was determined at the defined times. In this case cells were recovered onto LBMC plates containing the appropriate antibiotics. A significant difference of (***P<0.001) was found when the Rm1021 *gshA* mutant & pgshAc (*gshA*$^+$) and the *gshA* mutant & the control plasmid pBBR1 were compared. The datasets for the Rm1021 parent, the Rm1021 *gshA* mutant and the Rm1021 *gshA* & pgshAc are representative of trends shown in two independent experiments. The dataset for the Rm1021 *gshA* mutant & pBBR1 shows preliminary data. The error bars represent the standard deviation from the mean (n=3) for one experiment.

A disc diffusion assay was also performed to confirm that the increased sensitivity that was observed for the Rm1021 *gshA* mutant to bleomycin A_5 on solid media in chapter 3 (Fig. 3-12) was due to disruption of the *gshA* gene. It was observed that the Rm1021 *gshA* mutant carrying the pgshAc plasmid containing the *gshA* gene had an increased level of resistance to bleomycin A_5 (Fig. 5-5), relative to the Rm1021 *gshA* mutant, not carrying the plasmid. Complementation of the *gshA* mutant resulted in a similar level of sensitivity to bleomycin A_5 as observed in the Rm1021 parent. Therefore these data confirm that disruption of the *S. meliloti gshA* gene is responsible for increased sensitivity of the Rm1021 *gshA* mutant observed on solid media, relative to the parent strain (Fig. 3-12 & Fig. 5-5).

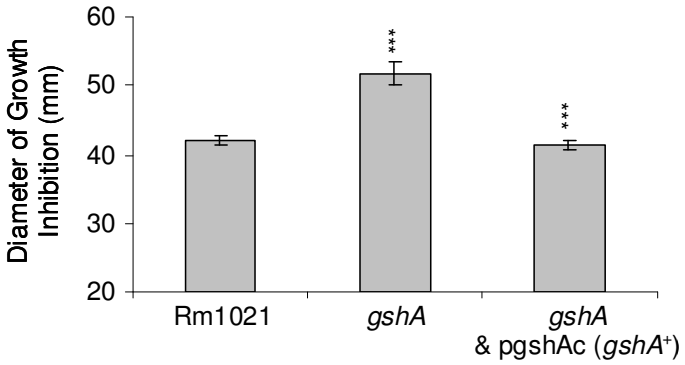


Figure 5-5. Growth inhibition of *S. meliloti gshA* complemented mutant exposed to bleomycin A_5. Cultures of the defined strains were exposed to bleomycin A_5 (2 µl of a 2mg.ml^{-1} aqueous stock solution) on LB agar. The significant values (***P<0.001) represent comparisons of the Rm1021 *gshA* mutant to the Rm1021 parent strain and the Rm1021 *gshA* mutant & pgshAc to the Rm1021 *gshA* mutant. Data shown for the Rm1021 parent and the Rm1021 *gshA* mutant is representative of the trends observed in two independent experiments. Data shown for Rm1021 *gshA* mutant & pgshAc shows preliminary data. The error bars represent the standard deviation from the mean (n=3) for one experiment.

Since the *S. meliloti gshA* liquid phenotype had been successfully complemented (Fig. 5-4), this confirmed that disruption of the *gshA* gene was resulting in the increased resistance to bleomycin A_5 observed in liquid culture, relative to the parent strain. However, it was possible to verify this further by assessing the sensitivity of a second independent *gshA* insertional mutant. This was made possible by taking advantage of an *S. meliloti* plasmid integration mutant library (http://www.cebitec. uni Bielefeld .de /transcriptomics/sm-genome/sm-mutagenesis.html), purchased from the University of Bielefeld (chapter 2, section 2.5). In this case, an *E. coli* S17-1 clone carrying the mobilizable suicide vector pK19mob2ΩHMB, containing a 330 bp internal fragment of the *S. meliloti gshA* gene was available in the library and was mobilized into Rm1021 via conjugation. The recombinant clones were then selected by purification onto the appropriate antibiotics and after purification, 3 clones were assessed for their sensitivity to bleomycin A_5 in liquid culture, alongside the Rm1021 parent and the original Rm1021 *gshA* mutant. It was observed that all the 3 putative Rm1021 *gshA* mutant clones despite having varying levels of sensitivity compared to one another, displayed an increased level of resistance to bleomycin A_5 in liquid culture (Fig. 5-6), relative to the Rm1021 parent strain. It should be noted that the putative Rm1021 *gshA* mutant clone 2, lost viability after 180 minutes, however up

until this point the putative mutant displayed an increased level of resistance, relative to the Rm1021 parent strain. The original Rm1021 *gshA* mutant (Harrison *et al.*, 2005) displayed an increased level of resistance to bleomycin A_5, as previously shown (Figs. 5-2 & 5-4). Thus, together all these data confirm that disruption of the *gshA* gene results in an increased resistance of *S. meliloti* cells to bleomycin A_5 in liquid LB medium.

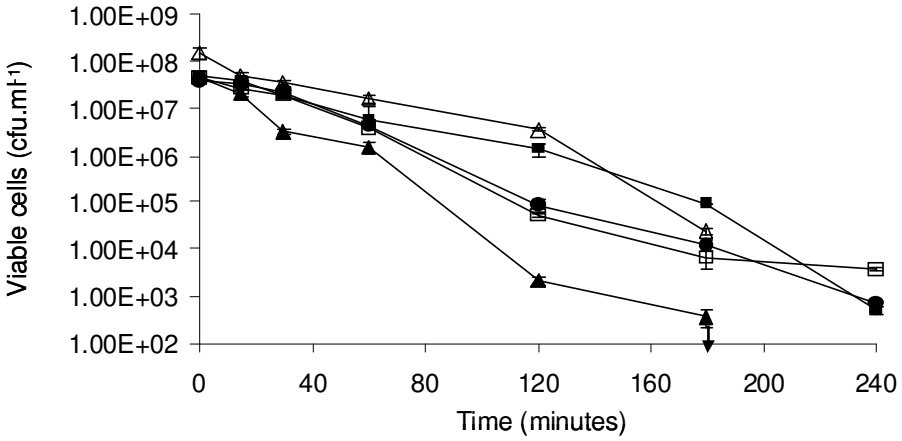


Figure 5-6. Sensitivity of the *S. meliloti* Rm1021 parent strain, the Rm1021 *gshA* mutant and 3 clones of a putative *gshA* independent mutant, to bleomycin A_5 in liquid culture. Cultures of the Rm1021 parent (closed triangles), the Rm1021 *gshA* mutant (Harrison *et al*, 2005) (closed squares) and the putative *gshA* mutants, clone 1 (closed circles), clone 2 (open triangles) and clone 3 (open squares) were all exposed to bleomycin A_5 (0.72 µg.ml^{-1}). Cell viability was determined at the defined times. In this case cells were recovered onto LBMC plates containing the appropriate antibiotics. The datasets for the Rm1021 parent and the Rm1021 *gshA* mutant (Harrison *et al.*, 2005) are representative of trends shown in two independent experiments. The datasets for the 3 putative *gshA* mutants show preliminary data. The error bars represent the standard deviation from the mean (n=3) for one experiment. The sensitivity of the Rm1021 *gshA* mutant and the 3 putative mutants were compared to that of the Rm1021 parent and in each case a significant difference of at least *P <0.05 was found.

5.2.3. The *S. meliloti gshA* mutant displays an increased resistance to methylglyoxal in liquid culture

To further investigate the liquid phenotype observed in the *S. meliloti gshA* mutant it was important to determine if this was specific to bleomycin A_5. Glutathione is known to play a major role in detoxification of the toxic electrophile methylglyoxal

(Inoue & Kimura, 1995) and like bleomycin, methylglyoxal has been shown to degrade bacterial DNA (Ferguson *et al.*, 2000). Thus, due to these similarities one might expect the *S. meliloti* Rm1021 *gshA* mutant to also have an increased resistance when exposed to methylglyoxal in liquid culture. When the cells were exposed to methylglyoxal by disc diffusion assay, the *S. meliloti* Rm1021 *gshA* mutant showed an increased level of sensitivity, relative to the Rm1021 parent, on solid media (Fig. 5-7A) as was observed for bleomycin A_5. Upon exposure of the *S. meliloti* Rm1021 *gshA* mutant to 0.7 mM methylglyoxal in liquid culture (Fig. 5-7B) the mutant was resistant to the toxic effects of the agent, whereas a decrease in cell viability was seen for the Rm1021 parent strain. The cells were next exposed to a higher dose of 1 mM methylglyoxal over a longer time period (Fig. 5-7C). In this case over the first 60 minutes of exposure the Rm1021 *gshA* mutant showed a similar level of sensitivity to methylglyoxal as the Rm1021 parent strain (Fig. 5-7C). However, after 60 minutes of exposure to methylglyoxal, the Rm1021 *gshA* strain then began to display an increased level of resistance to methylglyoxal (Fig. 5-7C).

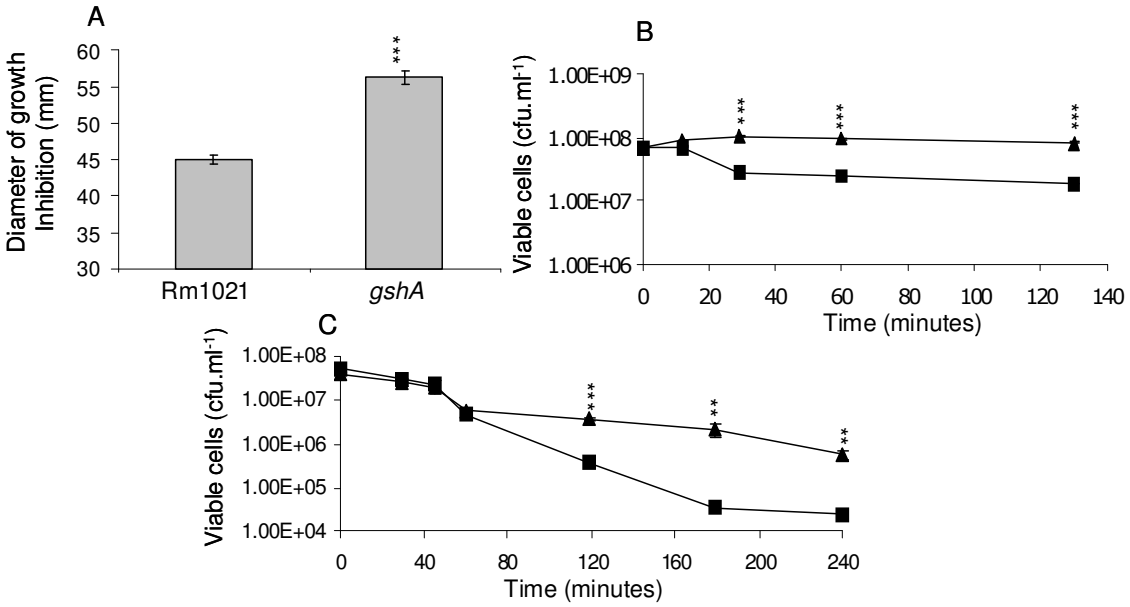


Figure 5-7. Sensitivity of the *S. meliloti* Rm1021 parent strain and the Rm1021 *gshA* mutant strain to methylglyoxal on solid media and in liquid culture. Growth inhibition of the defined strains exposed to methylglyoxal (5 µl of a 40% aqueous stock solution) on LB agar (A). Cultures of the Rm1021 parent (closed squares) and the Rm1021 *gshA* mutant (closed triangles) were exposed to 0.7 mM methylglyoxal (B) and 1mM methylglyoxal (C) and cell viability was determined at the defined times. The datasets shown in (A) are representative of trends shown in two independent experiments and (B) and (C) show preliminary data. The error bars represent the standard deviation from the mean (n=3) for one experiment. The significant values shown (**P<0.01; ***P<0.001) represent comparisons of the Rm1021 parent and the Rm1021 *gshA* mutant.

These data show in *S. meliloti* disruption of the *gshA* gene also results in an increased resistance to methylglyoxal in liquid culture, relative to the parent strain. Furthermore, it was possible to confirm that disruption of the *S. meliloti gshA* gene was responsible for the increased resistance to methylglyoxal, as sensitivity of the *gshA* mutant carrying the pgshAc plasmid (pBBR1 expressing the *S. meliloti gshA* gene constitutively) was next assessed (Fig. 5-8). Indeed it was found that presence of the pgshAc plasmid resulted in an increased sensitivity of the *gshA* mutant to methylglyoxal, relative to the Rm1021 *gshA* mutant strain not carrying the plasmid (Fig. 5-8). As previously observed the Rm1021 parent showed an increased level of sensitivity to methylglyoxal, relative to the Rm1021 *gshA* mutant.

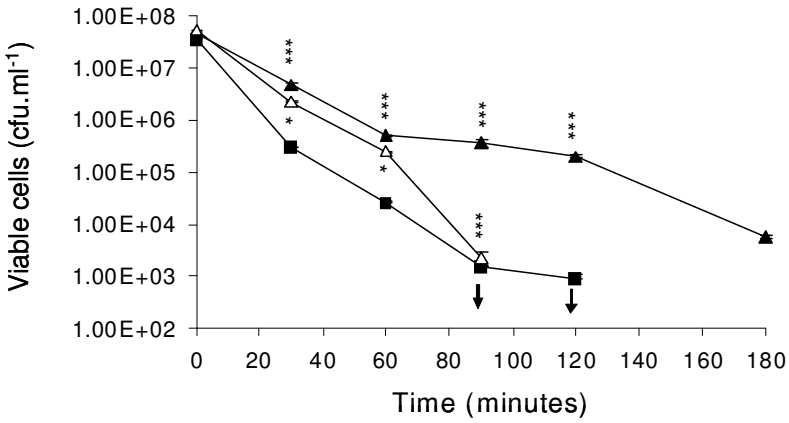


Figure 5-8. **Sensitivity the Rm1021 *gshA* complemented strain to methylglyoxal in liquid culture.** Cultures of the Rm1021 parent (closed squares), the Rm1021 *gshA* mutant (closed triangles) and the Rm1021 *gshA* & pgshAc (open triangles) were exposed to methylglyoxal (3mM aqueous stock solution) and cell viability was determined at the defined times. In this case cells were recovered onto LBMC plates containing the appropriate antibiotics. The dataset shows preliminary data. The significant values shown (*P <0.05; **P<0.01; ***P<0.001) represent comparisons of the Rm1021 *gshA* mutant with the Rm1021 parent strain and the Rm1021 *gshA* mutant & pgshAc with the *gshA* mutant without the plasmid. The error bars represent the standard deviation from the mean (n=3) for one experiment.

Taken together all the data presented so far show that disruption of the *gshA* gene in *S. meliloti* results in an increased sensitivity to the toxic compounds bleomycin A_5 and methylglyoxal on solid media. However, in LB liquid culture, disruption of the gene results in the *S. meliloti* cells displaying an increased level of resistance to both toxic compounds.

5.2.4. The *E. coli gshA* mutant does not display an increased resistance to bleomycin in liquid culture

A previous study has shown that an *E. coli gshA* mutant displays an increased sensitivity to methylglyoxal in liquid culture, relative to the parental strain (Ferguson & Booth, 1998), suggesting that the liquid phenotype observed in this work may be specific to *S. meliloti*. However, in the study the viability assay was performed in a minimal medium (Ferguson & Booth, 1998). So, it was important to next investigate the sensitivity of *E. coli* to bleomycin A_5 and methylglyoxal under the same conditions as used for *S. meliloti*, in LB medium. As observed in *S. meliloti*, the

E. coli gshA::Tn*10* mutant displayed an increased level of sensitivity to bleomycin A_5 on solid media, relative to the MJF274 parent strain (Fig. 5-9A). Two datasets (Fig. 5-9B & D) are shown for exposure of the *E. coli* strains to bleomycin A_5 in liquid culture, each done under the same conditions. In both datasets the sensitivity profile of the *E. coli gshA*::Tn*10* mutant was similar. However, in the first dataset (Fig. 5-9B) the MJF274 parent strain appeared more sensitive to bleomycin than in the second dataset (Fig. 5-9C). However, despite the experimental variation shown (Fig. 5-9B &C) overall in both datasets the *E. coli gshA*::Tn*10* mutant appeared more sensitive to bleomycin A_5 in liquid media, relative to the MJF274 parent strain. Thus, it seems unlike in the case of *S. meliloti* the *E. coli gshA* mutant displays an increased sensitivity to bleomycin A_5, both in solid and liquid media.

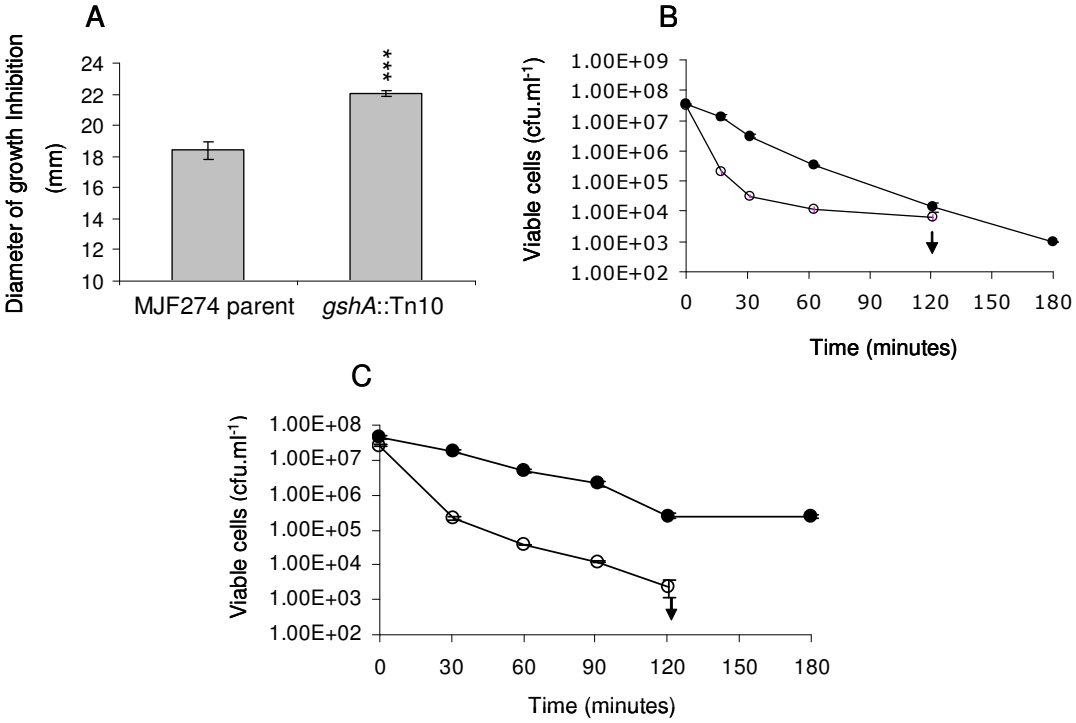


Figure 5-9. Sensitivity of the *E. coli* MJF274 parent strain the *gshA*::Tn*10* mutant to bleomycin A_5 on solid media and in liquid culture. Growth inhibition of the defined strains exposed to bleomycin A_5 (2 µl of a 2mg.ml^{-1} stock solution) on LB agar (A). Cultures of the MJF274 parent (closed circles) and the *gshA* mutant (open circles) were exposed to bleomycin A_5 (0.72 µg.ml^{-1}) and cell viability was determined at the defined times (B) and (C) The datasets shown in (A) are representative of the trends observed in two independent experiments. Datasets (B) and (C) show two biological repeats and the arrows represent complete loss of viability. The error bars represent the standard deviation from the mean (n=3) for one experiment. In (A) the significant value of ***P<0.001 represents comparisons of the MJF274 parent and the *gshA*::Tn*10* mutant in (C) the significant value of ***P<0.001 was found upon comparison of MJF274 parent and the *gshA*::Tn*10* mutant.

5.2.5 The *E. coli gshA* mutant shows the same level of sensitivity to methglyoxal in liquid culture as the parent strain

When the *E. coli* MJF274 parent strain and the *E. coli gshA*::Tn*10* mutant were exposed to methylglyoxal on solid media, the *gshA*::Tn*10* mutant showed an increased level of sensitivity (Fig. 5-10A), relative to the MJF274 parent strain, as was previously seen for *S. meliloti* (Fig. 5-7A). However, when the cells were exposed to 0.7 mM methylglyoxal in LB liquid culture the *E. coli gshA*::Tn*10* mutant

displayed a similar level of sensitivity as the MJF274 parent strain (Fig. 5-10B). The cells were next exposed to higher doses of 2 mM and 3 mM methylglyoxal (Figs. 5-10C and 5-10D, respectively) and it was observed that the *E. coli gshA*::Tn*10* mutant displayed the same level of sensitivity to methylglyoxal as was observed as for the MJF274 parent strain. Overall, these data show that loss of the *gshA* gene in *E. coli* results in an increased sensitivity to methylglyoxal when the cells are exposed on solid LB medium. Contrastingly when exposed in LB liquid the disruption of the *E. coli gshA* gene does not result in an increased sensitivity to methylglyoxal, relative to the parent strain.

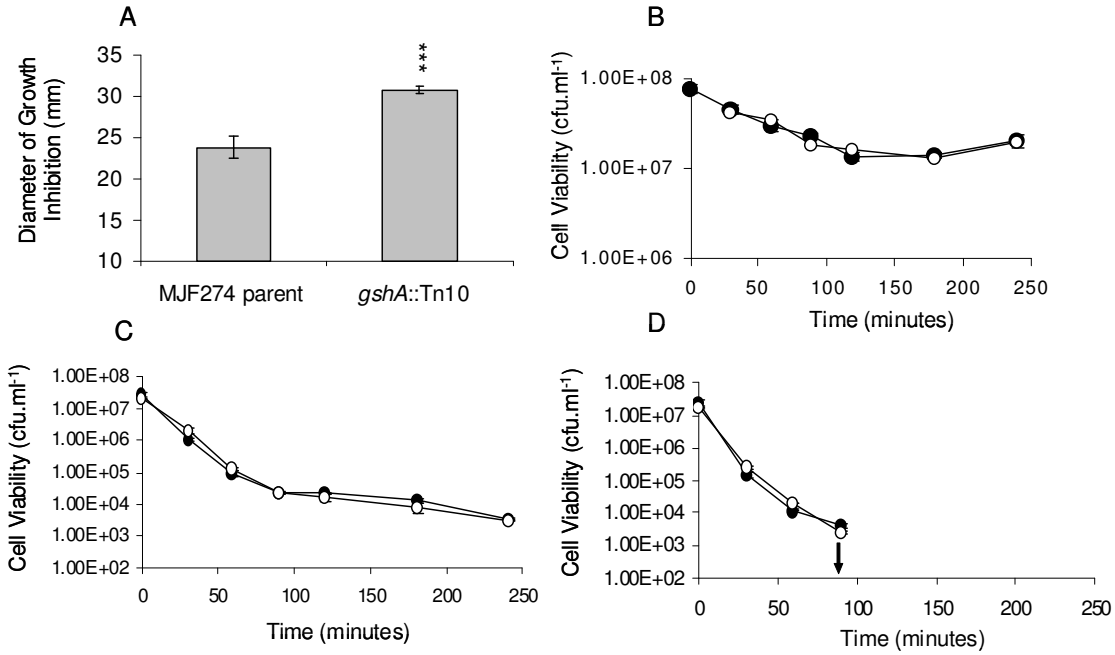


Figure 5-10. Sensitivity of the *E. coli* MJF274 parent strain and the *gshA*::Tn*10* mutant to methylglyoxal on solid media and in liquid culture. Growth inhibition of the defined strains exposed to methylglyoxal (5 µl of a 20 % aqueous stock solution) on LB agar (A). Cultures of the MJF274 parent (closed circles) and the *gshA* mutant (open circles) were exposed to 0.7 mM methylglyoxal and cell viability was determined at the defined times (B). As in (B) except cells were exposed to 2mM methylglyoxal (C). As (B) except cells were exposed to a 3 mM methylglyoxal (D). The datasets shown in (A) and (B) are representative of the trends observed in two independent experiments and datasets (C) and (D) show preliminary data. In (D) the arrow represents complete loss of viability. The error bars represent the standard deviation from the mean (n=3) for one experiment. In (A) the significant value of ***P<0.001 represents comparisons of the MJF274 parent and the *gshA*::Tn*10* mutant.

5.2.6. Preliminary data reveals the *S. meliloti gshA* mutant still displays an increased resistance to bleomycin A_5 in liquid culture under reduced oxygen conditions

The increased resistance to bleomycin A_5 and methylglyoxal observed in liquid media upon disruption of the *gshA* gene in *S. meliloti* would suggest that upon loss of GshA function, the cells are able to utilize another means to confer protection, which is not induced or effective on solid media. For *E. coli*, the data also suggests that upon disruption of GshA function another mechanism is able to protect the cells against methylglyoxal damage in liquid media.

Thus, it was next important to consider physiological differences in the environment of liquid and solid media. Undoubtedly, there will be numerous differences but one possibility is that there may be differences in aeration. When the cells are exposed to the agents on solid media they grow as a lawn in soft (0.8 % w/v) agar. In this case the cells will be more tightly packed than in liquid and so may be exposed to lower levels of oxygen. Thus, if higher levels of oxygen are available in liquid culture this may affect the way *S. meliloti* responds to bleomycin A_5 and methylglyoxal, in the absence of *gshA*. In the presence of oxygen bleomycin has been shown to generate reactive oxygen species such as superoxide and hydrogen peroxide (Sugiura & Kikuchi, 1978), which contribute to cellular damage. Additionally methylglyoxal treatment of eukaryotic cells under aerobic conditions has been shown to induce the formation of reactive oxygen species (Kalapos *et al.*, 1993). Interestingly in both *S. meliloti* and *E. coli* the presence of hydrogen peroxide has been shown to induce the expression of catalase (Luo *et al.*, 2005a), which is necessary for the decomposition of hydrogen peroxide in both *S. meliloti* (Jamet *et al.*, 2003) and *E. coli* (Claiborne & Fridovich, 1979; Claiborne *et al.*, 1979; Sak *et al.*, 1989). Taken together this led to the hypothesis that when *S. meliloti* cells are exposed to bleomycin and methylglyoxal in liquid media, the higher levels of reactive oxygen species produced, relative to those on solid medium, induce the expression of catalase in the absence of glutathione, which is able to protect against damage. Due to the preliminary nature of this study, this hypothesis was investigated using only *S. meliloti.*

To investigate this hypothesis, an assay was performed whereby the Rm1021 parent and Rm1021 *gshA* mutant were exposed to bleomycin A_5 in liquid culture with no aeration (Fig. 5-11), with the view that this would expose the cells to bleomycin A_5 under conditions of reduced oxygen, relative to previous conditions used. Under these experimental conditions, it can be seen that the Rm1021 *gshA* mutant still displays an increased level of resistance to bleomycin A_5 relative to the Rm1021 parent (Fig. 5-11). However, if the overall sensitivity of both strains is compared to a previous assay (Fig. 5-2) where the cultures were aerated, it seems under these conditions, overall less killing occurred.

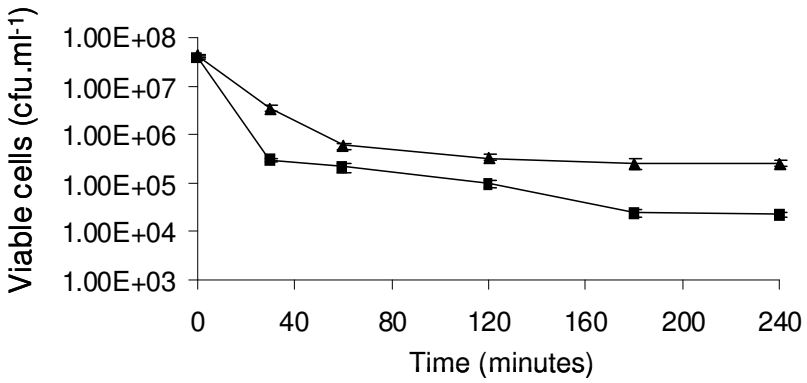


Figure 5-11. Preliminary assay assessing the sensitivity of the Rm1021 parent and the Rm1021 *gshA* mutant to bleomycin A_5 under reduced oxygen conditions. Cultures of the Rm1021 parent (closed squares), the *gshA* mutant (closed triangles) were exposed to bleomycin A_5 (0.72 µg.ml^{-1}) and viability was determined at the defined times. Cells were recovered onto LBMC plates containing the appropriate antibiotics. The dataset shows preliminary data. The error bars represent the standard deviation from the mean (n=3) for one experiment.

5.2.7. The *S. meliloti gshB* mutant displays an increased resistance to bleomycin A_5, on solid media relative to the parent strain

A previous study has shown that the *S. meliloti gshB* mutant which is defective in the glutathione synthetase enzyme has an accumulation of γ-glutamyl cysteine (Harrison *et al.*, 2005). Hence, it was next investigated if this glutathione precursor is able to confer any protection against bleomycin induced damage in *S. meliloti*. Sensitivity

to bleomycin A_5 was assessed and it was observed that the Rm1021 *gshB* mutant displayed an increased resistance to bleomycin A_5, relative to the Rm1021 parent (Fig. 5-12A). Thus, these data might suggest that under these conditions the glutathione precursor γ-glutamyl cysteine can protect against bleomycin A_5. When the *bacA* mutation was transduced into the Rm1021 *gshB* mutant by M12 phage , it could be observed that loss of the BacA protein in a *gshB* mutant background still conferred further protection against bleomycin A_5 (Fig. 5-12A). It was next investigated if γ-glutamyl cysteine could also protect *S. meliloti* against the toxic effects of methylglyoxal. It was observed that relative to the parent strain, the Rm1021 *gshB* mutant did display an increased sensitivity to methylglyoxal (Fig. 5-12B). However, since the Rm1021 *gshB* mutant was not as sensitive to methylglyoxal as the Rm1021 *gshA* mutant on solid media (Fig. 5-12B) this would suggest γ-glutamyl cysteine does confer some protection against methylglyoxal.

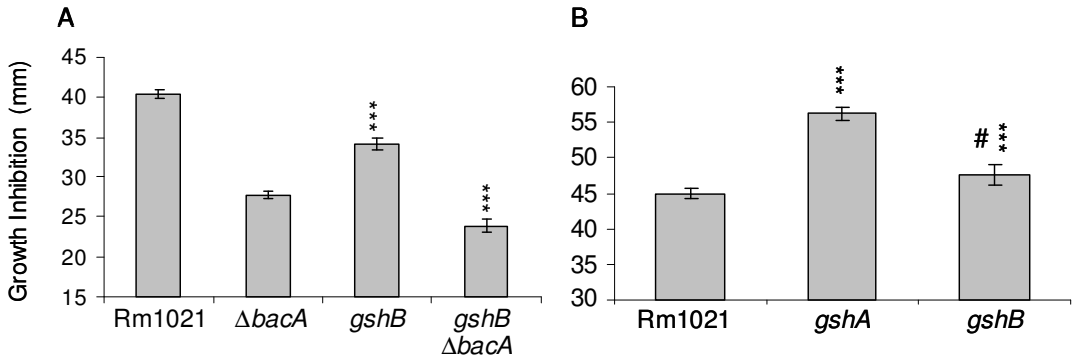
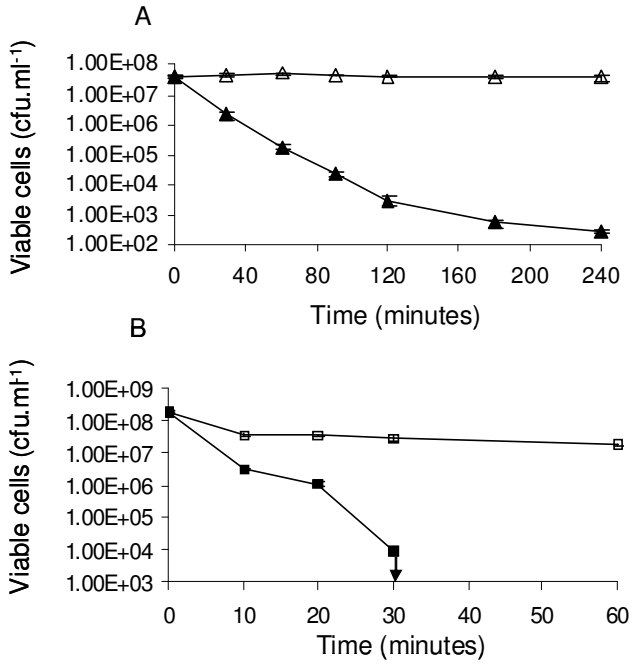


Figure 5-12. Growth inhibition of *S. meliloti* glutathione mutants exposed to bleomycin A_5 and methylglyoxal. Cultures of the defined strains were exposed to bleomycin A_5 (2 µl of a 2mg.ml^{-1} aqueous stock solution) (A) and methylglyoxal (5 µl of a 40% aqueous stock solution) (B) on LB using a filter disc assay. In (A) the significant values (***P<0.001) represent comparisons of the *gshB* mutant to the Rm1021 parent strain and the *gshB/ΔbacA* double mutant to that of the *gshB* single mutant. In (B) the significant values (***P<0.001) represent comparisons of the *gshA* mutant to the Rm1021 parent strain and comparison of the *gshB* mutant to the Rm1021 parent strain. The significant value (# P<0.001) represents a comparison of the *gshB* and *gshA* mutants. Dataset (A) shows preliminary data and dataset (B) is representative of the trends observed in two independent experiments. In each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

5.2.8. The *S. meliloti gshB* mutant displays an increased resistance to bleomycin A_5 in liquid culture

The liquid sensitivity of the Rm1021 *gshB* mutant was next assessed by recovery of viable cells after exposure to bleomycin A_5 in LB broth (Fig. 5-13). Upon exposure of the cells to 0.72 $\mu g.ml^{-1}$ of bleomycin A_5 (Fig. 5-13A), the Rm1021 *gshB* mutant appeared resistant to the killing effects of the drug, contrastingly for the Rm1021 parent as previously observed, the cells were killed over the time course of the experiment (Fig. 5-13A). Next, a higher dose of 3 $\mu g.ml^{-1}$ of bleomycin was used and in this case the Rm1021 *gshB* mutant still displayed an increased level of resistance to bleomycin A_5 relative to the Rm1021 parent, which was rapidly killed during the first 30 minutes of the assay (Fig. 5-13B). Overall, these data show that the Rm1021 *gshB* mutant like the Rm1021 *gshA* mutant displayed an increased level of resistance to bleomycin A_5, relative to the parent strain.



5-13. Sensitivity of the *S. meliloti* Rm1021 parent strain and the *gshB* mutant, to bleomycin A_5 in liquid culture. Cultures of the Rm1021 parent (closed triangles) and the *gshB* mutant (open triangles) were exposed to bleomycin A_5 (0.72 $\mu g.ml^{-1}$) (A). Cultures of the Rm1021 parent (closed squares) and the *gshB* mutant (open squares) were exposed to bleomycin A_5 (3 $\mu g.ml^{-1}$). (B) Cell viability was determined at the defined times. In this case cells were recovered onto LBMC plates containing the appropriate antibiotics. The dataset is representative of the trends observed in two independent experiments. The error bars represent the standard deviation from the mean (n=3) for one experiment.

Additionally, comparison of figures 5-2 and 5-6 with 5-13B, would suggest that the *S. meliloti gshB* mutant appears more resistant to bleomycin A_5 in liquid culture than the Rm1021 *gshA* mutant.

5.3. Discussion

The work presented in this chapter describes a preliminary investigation into the role of glutathione in the protection of free-living *S. meliloti* against bleomycin and methylglyoxal toxicity and compares it to the well characterised system of *E. coli* (Ferguson & Booth, 1998; Ferguson *et al.*, 1998; MacLean *et al.*, 1998). The key finding in this study was that although glutathione appears to be important in *S. meliloti* for detoxification of bleomycin and methylglyoxal on solid media, cells lacking glutathione actually appeared more resistant to these two toxic compounds, when exposed in liquid culture.

5.3.1. Intracellular glutathione protects both *S. meliloti* and *E. coli* against methylglyoxal on solid media

In both *S. meliloti* and *E. coli*, loss of the *gshA* gene, encoding γ-glutamylcysteine synthetase, results in an increased level of sensitivity to methylglyoxal on solid media, thus suggesting in both bacterial species that the presence of intracellular glutathione is important for protection against methylglyoxal under these conditions (Fig. 5-14). Detoxification of methylglyoxal by the glutathione dependent glyoxalase I and II enzymes is very well characterised in *E. coli* (Inoue & Kimura, 1995). Although to date this system has not been characterised in *S. meliloti*, the genome contains two genes, *Smc00290* and *Smc00708*, annotated putatively as encoding glyoxalase I and glyoxalase II respectively (http://iant.toulouse.inra.fr/ bacteria/ annotation /cgi/rhime.cgi), which could potentially be involved in glutathione dependent methylglyoxal detoxification (Fig. 5-14).

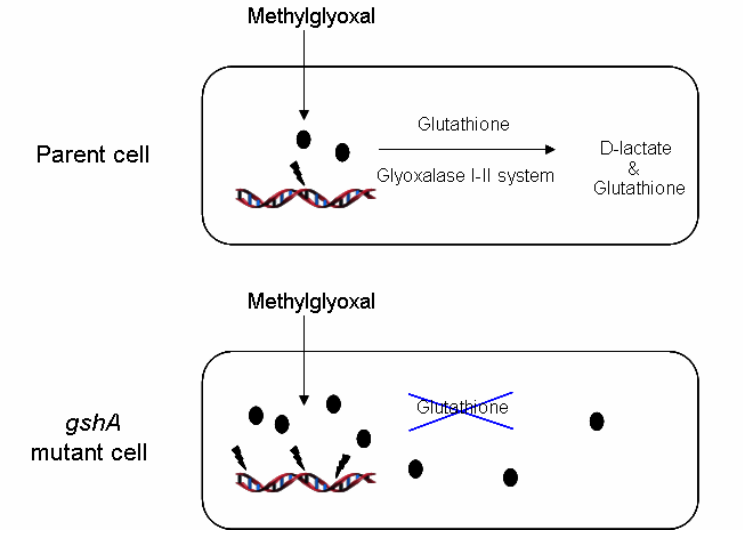


Figure 5-14. Proposed model for protection of *S. meliloti* from methylglyoxal on solid media. In *E. coli* the detoxification of methylglyoxal to D-lactate and free glutathione by the glutathione dependent glyoxalase I and II enzymes is well characterised (Inoue & Kimura, 1995). Additionally it has been shown in *E. coli* that exposure to methylglyoxal results in DNA degradation (Ferguson *et al.*, 2000). Hence, it is proposed in the *S. meliloti* parent the glutathione dependent glyoxalase I-II detoxification system may play an important role in protection against methylglyoxal on solid media. However, in the *S. meliloti gshA* mutant due to the absence of intracellular glutathione, methylglyoxal will not be effectively detoxified. Therefore, the accumulation in methylglyoxal will result in an increased level of sensitivity, perhaps due to DNA damage.

5.3.2. Intracellular glutathione protects both *S. meliloti* and *E. coli* against bleomycin damage on solid media

Loss of the *gshA* gene in *S. meliloti* and *E. coli* also results in an increased sensitivity to bleomycin A_5 on solid media. It was anticipated that glutathione may play a role in bleomycin detoxification in *S. meliloti* and *E. coli* since several studies have suggested a role for glutathione in detoxification of bleomycin in eukaryotic cells. One study found that in pulmonary endothelial cells bleomycin treatment was shown to result in up-regulation of the cellular levels of glutathione and expression of γ-glutamylcysteine synthetase (Day *et al.*, 2002). Additionally, the hypersensitivity of Chinese hamster ovary cells to bleomycin was found to be due to lack of glutathione

S-transferase activity (Giaccia *et al.*, 1991). Glutathione *S*-transferases catalyse the nucleophilic conjugation of both xenobiotic and endogenous electrophiles with glutathione, thereby decreasing their reactivity (Armstrong, 1997). Both *S. meliloti* and *E. coli* have several glutathione *S*-tranferases annotated in their genomes (http://iant.toulouse.inra. fr/bacteria/annotation /cgi/rhime.cgi and http://www.genome. wisc.edu /sequencing/k12.htm, respectively). Thus, it may be possible in *S. meliloti* and *E. coli* that detoxification of bleomycin may be catalyzed by glutathione-*S*-transferases, perhaps by catalyzing the conjugation of bleomycin to glutathione (Fig. 5-15). Alternatively or additionally glutathione *S*-transferases may catalyse the addition of glutathione to a metabolic by-product of bleomycin damage (Fig. 5-15), for example base propenals are derived after cleavage of DNA by bleomcyin (Grollman *et al.*, 1985; Steighner & Povirk, 1990) and preliminary analysis has revealed they would be likely substrates for glutathione-*S*-transferases (Giaccia *et al.*, 1991).

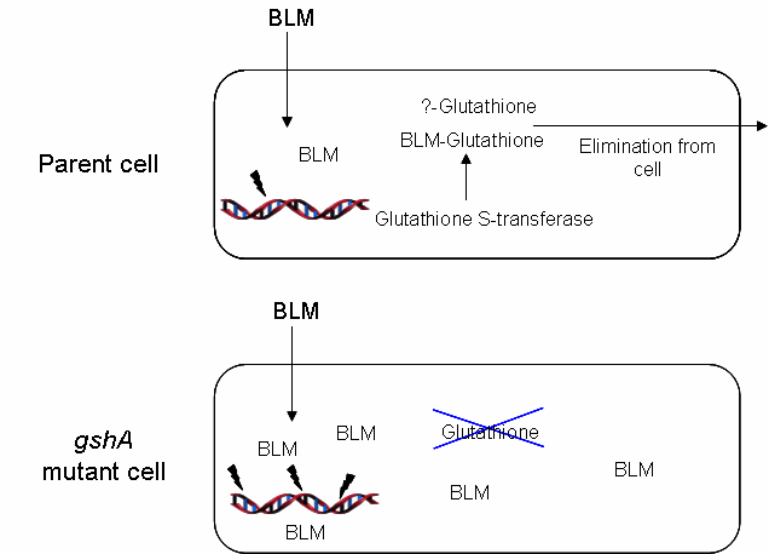


Figure 5-15. Proposed model for the protection of *S. meliloti* and *E. coli* against bleomycin damage on solid media. Upon exposure of the *S. meliloti* and *E. coli* parent cells to bleomycin it is thought that glutathione-*S*-transferases may catalase the addition of glutathione to bleomycin, thereby decreasing its toxicity and possibly resulting in its elimination from the cell. Alternatively or additionally glutathione *S*-transferases may catalyse the addition of glutathione to a metabolic by-product of bleomycin damage. However in the absence of glutathione, bleomycin and/or its metabolic products will not be effectively detoxified in the *gshA* mutant cells, which may lead to an increased level of bleomycin induced DNA damage, relative to the parent strain. Key: BLM=bleomycin.

5.3.3. Could the increased resistance of the *S. meliloti gshA* mutant to bleomycin in liquid media be due to higher levels of oxygen, relative to those on solid media?

The finding that *S. meliloti* lacking a functional *gshA* gene displayed an increased resistance to bleomycin in liquid culture was a surprising result. It was proposed during this study that this phenotype may arise from physiological differences between liquid and solid LB media. LB media contains yeast extract and thus some glutathione (Helbig *et al.*, 2008), thus one could spectulate perhaps in liquid culture that this glutathione is more accessible to the bacteria than on solid media, hence the *S. meliloti gshA* mutant is protected against bleomycin. However, a study assessed the cytoplasmic contents of an *E.coli gshA* mutant grown in LB and found that glutathione levels were less than 5% of the levels found in the parent strain (Helbig *et al.*, 2008), making this unlikely. Perhaps in liquid media higher levels of oxygen may be present than on solid media. These higher oxygen levels may affect the toxicity of bleomycin.

In *vitro* studies with bleomycin have postulated that following its interaction with Fe (II) in the presence of oxygen an activated bleomycin complex is formed (Sugiura *et al.*, 1982). The activated complex is then thought to cause double DNA strand breaks, during which superoxide, hydroxyl radicals and hydrogen peroxide are generated (Oberley & Buettner, 1979; Sugiura *et al.*, 1982). It has been shown that the *S. meliloti gshA* mutant has a seven fold higher catalase enzyme activity at the end of the exponential growth phase, relative to the Rm1021 parent strain (Harrison *et al.*, 2005). Since late exponential phase cells were used for the liquid assays in this work, it is possible the increased levels of catalase activity in the Rm1021 *gshA* mutant may breakdown the hydrogen peroxide generated by the bleomycin damage, lessening its toxic effect. Overall this may provide more protection against bleomycin damage than the intracellular glutathione present in the parent strain (Fig. 5-16). Additionally, it has been shown that the presence of superoxide and hydrogen peroxide may reactivate bleomycin to cause more damage (Oberley & Buettner, 1979; Sugiura *et al.*, 1982). Hence, an up-regulation of catalase, resulting in breakdown of hydrogen peroxide, under these conditions could be very beneficial to *S. meliloti.*

Three catalase eyzymes in *S. meliloti* known to be involved in the breakdown of hydrogen peroxide are KatA, KatB and KatC (Herouart *et al.*, 1996; Jamet *et al.*, 2003; Sigaud *et al.*, 1999). Using catalase activity gels it was determined that late exponential phase Rm1021 *gshA* mutant cells express enhanced levels of KatA and KatB, relative to the Rm1021 parent strain. Additionally, some KatC activity was detected, that was not present in the parent strain (Harrison *et al.*, 2005). Furthermore, Kat A activity in *S. meliloti* has been shown to be induced by the presence of hydrogen peroxide (Herouart *et al.*, 1996). Therefore, this increased level of KatA activity may further contribute to the increased resistance of the *S. meliloti gshA* mutant to bleomycin.

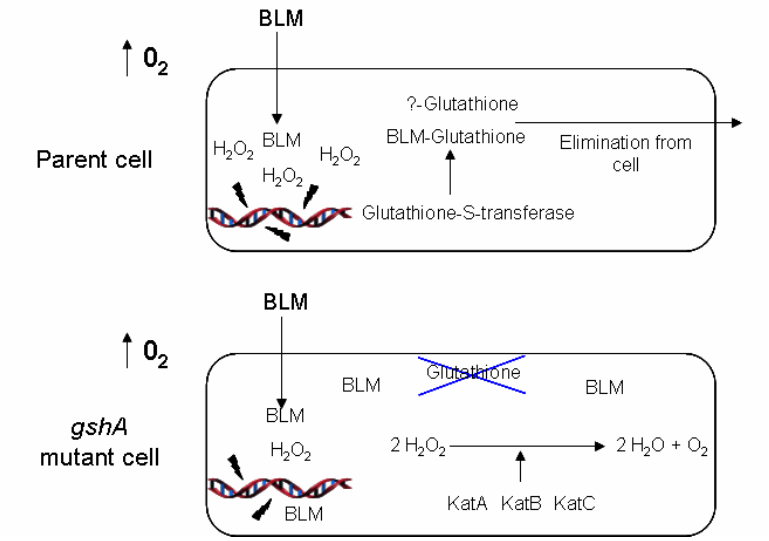


Figure 5-16. Proposed model to account for the increased resistance of the *S. meliloti gshA* mutant to bleomycin in liquid media. When *S. meliloti* cells are exposed to bleomycin in liquid media, the higher levels of oxygen contribute to the production of reactive oxygen species such as hydrogen peroxide which damage the cell and can also re-activate bleomycin (Oberley & Buettner, 1979; Sugiura *et al.*, 1982). In the *S. meliloti* parent cell glutathione will protect against bleomycin damage. However, in the *gshA* mutant cells the enhanced expression of the catalase enzymes will result in the breakdown of hydrogen peroxide, so conferring increased protection to bleomycin damage, relative to that provided by glutathione in the parent strain. Key: BLM=bleomycin.

5.3.4. Up regulation of catalase activity in the *S. meliloti gshA* mutant may not be beneficial when exposed to bleomycin on solid media

Contrastingly, when *S. meliloti gshA* cells were exposed to bleomycin on solid medium where lower levels of oxygen may be present, it is possible that lower levels of reactive oxygen species are formed. Thus, although late exponential phase cells were used, the enhanced level of catalase expression, relative to the parent, under these conditions may be less beneficial in protection against bleomycin. Therefore the Rm1021 *gshA* mutant displays an increased sensitivity to bleomycin, relative to the parent.

Another important consideration is that although it is hypothesized based on previous studies (Harrison *et al.*, 2005) that the Rm1021 *gshA* mutant cells harvested for use in the assays would already have an up-regulation of catalase activity, it is likely the Rm1021 *gshA* cells in the liquid assay will be under oxidative stress due to the higher oxygen levels in the liquid and would thus continue to express higher levels of catalase, independently from that induced by bleomycin, compared to the cells placed on solid agar where the oxygen levels are hypothesized to be lower.

5.3.5. Preliminary data reveal that the *S. meliloti gshA* mutant still displays an increased resistance to bleomycin A_5 in liquid culture with no aeration

To investigate if the increased resistance of the *S. meliloti gshA* mutant to bleomycin in liquid culture was as a result of higher oxygen levels, relative to those on solid media, a bleomycin liquid assay was performed with the Rm1021 *gshA* mutant and the Rm1021 parent but under conditions of reduced oxygen. Instead of exposing the cells to bleomycin with aeration, in this case the cells were exposed to bleomycin in an eppendorf tube, with no airspace and incubated in a water bath, rather than a shaking incubator. However, under these conditions the Rm1021 *gshA* mutant still displayed an increased resistance to bleomycin, relative to the parent strain. Thus, this would suggest that if the protective effect observed is due to enhanced catalase activity, then this is still occurring in the Rm1021 *gshA* mutant cells under these

conditions and/or is still having a beneficial effect i.e. reactive oxygen species are still being formed.

In retrospect, this was a fairly crude assay with no means of measuring actual oxygen levels. If time had allowed a genetic approach may have been a more direct way to test this hypothesis. As discussed, it has been shown the *katA* and *katB* genes are up-regulated in the *gshA* mutant, additionally the *katA* gene is induced by hydrogen peroxide (Herouart *et al.*, 1996). Thus, the construction of Rm1021 *gshA/katA* and *gshA/katB* double mutants and a *gshA/katA/katB* treble mutant and subsequent investigation into their sensitivity to bleomycin and methylglyoxal, relative to the Rm1021 *gshA* mutant would be very informative. Additionally, catalase activity gels could be performed on *S. meliloti* cells harvested from liquid and solid media in the presence and absence of bleomycin to determine if there is an increased level of catalase activity in liquid media, relative to solid media upon exposure to the toxic compounds.

5.3.6. Catalases have also been shown to protect against methylglyoxal damage

It was also observed that the *S. meliloti gshA* mutant displayed an increased resistance to methylglyoxal in liquid media, relative to the parent strain. Interestingly, a study with the plant pathogen *Xanthomonas campestris* pv. *phaseoli* found that bacteria harbouring an expression vector carrying a catalase gene were over 100 fold more resistant to methylglyoxal than bacteria without the plasmid; although in this case it is less clear how catalase induces protection against methylglyoxal toxicity (Vattanaviboon *et al.*, 2001). Additionally, it also was found that addition of 10 mM sodium pyruvate, which chemically inactivates peroxide (Nath *et al*., 1995), to the growth medium, increased *X. campestris* pv. *phaseoli* resistance levels more than 100-fold to methylglyoxal killing (Vattanaviboon *et al.*, 2001). Thus, this study would be consistent with catalase also playing a role in protection against methylglyoxal induced damage in *S. meliloti* in liquid media.

The presence of methylglyoxal can be determined in a spectrophotometric assay using the chemical compound 2,4-dinitrophenylhydrazine (Gilbert & Brandt, 1975). This has been successfully used in a previous study to measure the

disappearance of methylglyoxal from a culture medium with *E. coli* cells in the presence and absence of intracellular glutathione (Ferguson & Booth, 1998). In this work it was attempted to perform this assay to determine if methylglyoxal was been detoxified in the Rm1021 *gshA* mutant and to compare this to the detoxification rate of the Rm1021 parent strain. However, problems were encountered with the use of LB as a medium, since the LB appeared to react with 2,4-dinitrophenylhydrazine. So in future studies, a different growth medium would have to be used.

5.3.7. The *E. coli gshA* mutant also displays an altered phenotype in liquid culture when exposed to methylglyoxal, relative to that observed on solid media

In *E. coli* disruption of the *gshA* gene did not result in an increased sensitivity to methylglyoxal in liquid culture, relative to the parent strain. Therefore, this would suggest in the absence of glutathione, like *S. meliloti*, the *E. coli* cells have another mechanism to protect against methylglyoxal damage. Indeed, a previous study has shown that an *E. coli gshA* mutant was also found to have an up-regulation of catalase expression, both in the presence and absence of hydrogen peroxide (Oktyabrsky *et al.*, 2001). Therefore, one can hypothesize that catalase induced protection may too be occurring in the *E. coli gshA* mutant when exposed to methylglyoxal in liquid culture. A previous study, which assessed the sensitivity of an *E. coli gshA*::Tn*10* mutant to methylglyoxal in liquid media, found that the *gshA* mutant cells were more sensitive to methylglyoxal, relative to the parent strain. However, different conditions were used in this assay, one of which was that a minimal medium was used containing 0.2% (w/v) glucose as a carbon source (Ferguson & Booth, 1998). Interestingly it has been shown that in *E. coli,* glucose actually represses catalase expression (Hassan & Fridovich, 1978). However, the assays performed in this chapter were done in LB, which does not contain glucose (Sezonov *et al.*, 2007). Thus, these findings are consistent with the hypothesis that the protective effect against methylglyoxal observed in liquid is due to an up-regulation of catalase genes. However, in *E. coli* the same was not true of bleomycin treatment as the *gshA* mutant was more sensitive to bleomycin, relative to the parent

strain in liquid culture. Perhaps this can be accounted for by differences in the catalase systems in *E. coli* and *S. meliloti*, as in contrast to *S. meliloti,* which has three characterised catalase enzymes, only two catalases have been characterised in *E. coli*, the *katG* gene which is induced in response to hydrogen peroxide (Storz *et al.*, 1990) and also *katE*, which is activated in the stationary phase of growth (Sak *et al.*, 1989).

5.3.8. Disruption of the *S. meliloti gshB* gene results in an increased resistance to bleomycin both on solid and in liquid culture

When the *S. meliloti gshB* mutant (lacking the enzyme glutathione synthetase) was exposed to bleomycin on solid media, an increased level of resistance, relative to the Rm1021 parent was observed. This mutant has previously been shown to accumulate γ-glutamyl cysteine (Harrison *et al.*, 2005). However, to the best of my knowledge there is no known literature suggesting a role for γ-glutamyl cysteine in detoxification of toxic compounds. It may be possible that the increased levels of γ-glutamyl cysteine in the *S. meliloti gshB* mutant result in another change i.e. up-regulation of another gene which may encode a protein conferring resistance to bleomycin (Fig. 5-17). However, this phenotype would need to be complemented to confirm that it is definitely due to disruption of the *gshB* gene.

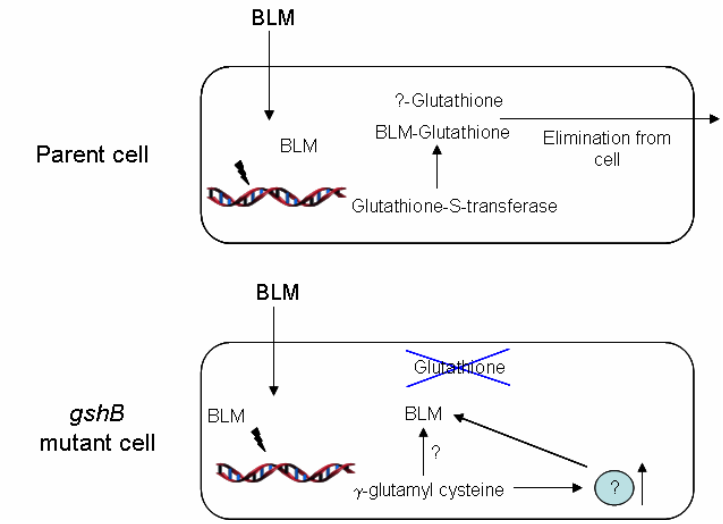


Figure 5-17. Proposed model to account for the increased resistance of the *S. meliloti gshB* mutant to bleomycin on solid media. In the absence of glutathione in the *S. meliloti gshB* mutant the accumulation of γ-glutamyl cysteine protects the cells against bleomycin damage either directly by some unknown mechanism or indirectly i.e. by up-regulation of another gene which may encode a protein conferring resistance to bleomycin. This mechanism of protection in the *S. meliloti gshB* mutant appears more effective than glutathione induced protection in the parent cell. Key: BLM=bleomycin.

The *S. meliloti gshB* mutant was also found to be resistant to bleomycin in liquid culture, relative to the parent strain. In fact, when the Rm1021 *gshB* mutant cells were exposed to same concentration of bleomycin under the same conditions as the Rm1021 *gshA* mutant they appeared much more resistant. Late exponential phase cells of the Rm1021 *gshB* mutant have also been shown to have a three to four fold up-regulation of catalase activity (Harrison *et al.*, 2005). Additionally, catalase activity gels revealed increased activity of all three catalases (Harrison *et al.*, 2005), relative to the parent strain. Thus, it is possible that the increased catalase activity combined with the as yet unknown protective effect of γ-glutamyl cysteine results in the increased resistence of *S. meliloti gshB* mutant to bleomycin (Fig. 5-18).

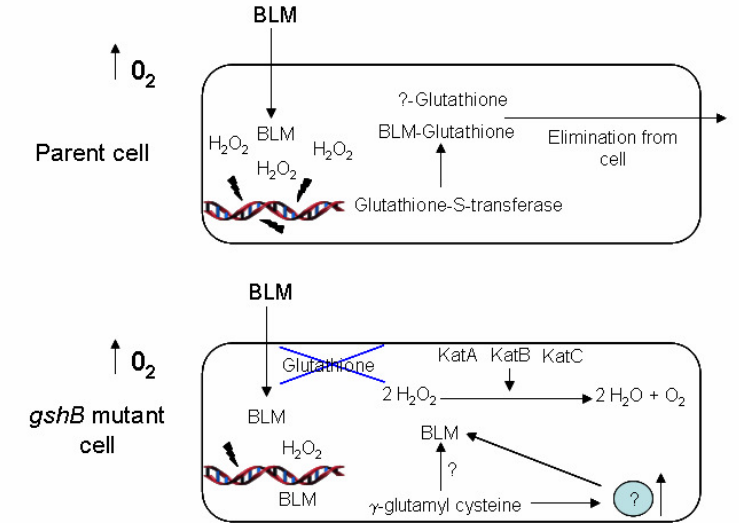


Figure 5-18. Proposed model to account for the increased resistance of the *S. meliloti gshB* mutant to bleomycin in liquid culture. In the *S. meliloti* parent cell glutathione will protect cells against bleomycin damage. However in the *gshB* mutant the combined protective effect of γ-glutamyl cysteine (as described in Fig. 5-17) and also the enhanced expression of the catalase enzymes, resulting in the breakdown of hydrogen peroxide, will confer enhanced protection against bleomycin. Key: BLM=bleomycin.

Interestingly there is a second *gshB* like gene (*smb21586*) named *gshB2*, annotated as a putative glutathione synthetase in the *S. meliloti* genome (iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi), which is 34% identical and 50% similar (http://blast.ncbi.nlm.nih.gov/Blast.cgi) at the protein sequence level, to the GshB protein identified in the previous study (Harrison *et al.*, 2005). Since the *gshB* mutant has been shown to lack intracellular glutathione under normal growth conditions (Harrison *et al*, 2005), this suggests no other gene product is able to compensate for lack of *gshB*. However, under some stress conditions i.e. exposure to toxic compounds the *gshB2* gene may play a role in glutathione synthesis. To investigate this hypothesis, a *gshB/gshB2* double mutant was created by transduction with M12 phage. Unfortunately due to time constraints it was not possible to assess the sensitivity of this double mutant to bleomycin. However, this can be undertaken in future studies within the laboratory.

5.3.9. Future work

Overall this preliminary study into the role of glutathione in protection of free-living *S. meliloti* from bleomycin and methylglyoxal revealed some unexpected and interesting phenotypes. To continue this study, further physiological assays could be performed to attempt to gain clues as to what is occurring in the absence of the *S. meliloti gshA* and *gshB* genes. However, the quickest way to address this question would be to perform microarray analysis on the two *S. meliloti* glutathione mutants under varying conditions i.e. taken from growth on solid and in liquid media, in the presence and absence of both bleomycin and methylglyoxal. This should be happening in the laboratory in the near future and may provide very valuable insights into the mechanisms of protection occurring in the absence of glutathione in the *S. meliloti gshA* and *gshB* mutants, under certain conditions.

Chapter 6: Investigating the Role and Biosynthesis of the Lipopolysaccharide in Free-Living and Symbiotic *S. meliloti*

6.1. Introduction

In both *S. meliloti* and *B. abortus* loss of BacA function results in a ~50% reduction in the amount of LPS lipid A molecules that are modified with very-long-chain fatty acids (VLCFAs) (Ferguson *et al.*, 2004). Interestingly the BacA protein is distantly related to the adrenoleukodystrophy family of eukaryotic proteins, thought to be involved in the transport of VLCFAs (Ferguson *et al.*, 2004). Combined these findings led to the proposal that BacA could be involved in the transport of VLCFAs out of the cytoplasm where they could then be used to modify the lipid A in the outer membrane.

Since the mechanism by which BacA leads to the lipid A VLCFA modification is still unresolved *S. meliloti* mutants were constructed in the *acpXL* and *lpxXL* genes (Ferguson *et al.*, 2005), which are directly involved in the biosynthesis of VLCFA-modified LPS. The *lpxXL* gene encodes an VLCFA acyl transferase (Basu *et al.*, 2002) and the *acpXL* gene encodes an VLCFA acyl carrier protein (Brozek *et al.*, 1996) (Fig. 6-1). Although the LPS of the *acpXL* and *lpxXL* free-living mutants completely lack the VLCFA modification in complex media they are able to form a successful symbiosis with alfalfa (Ferguson *et al.*, 2005). However, since the *acpXL* and *lpxXL* mutants are substantially less competitive in co-inoculation experiments with the Rm1021 parent strain (Ferguson *et al.*, 2005), this would suggest the AcpXL and LpxXL proteins play important roles in at least one stage of the alfalfa symbiosis.

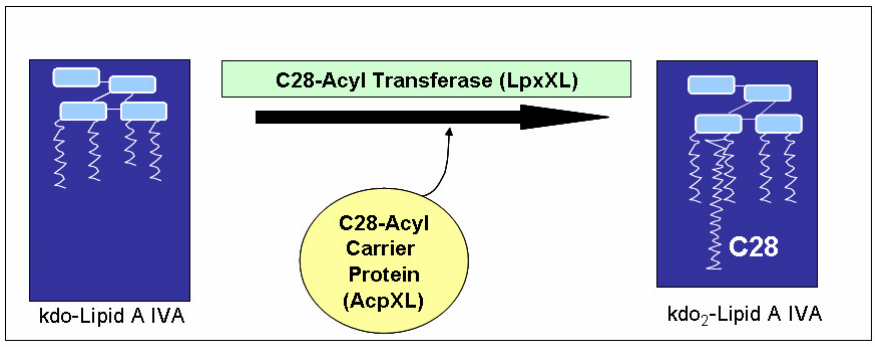


Figure 6-1. The Biosynthesis of the VLCFA-modified Lipid A. The VLCFA is synthesised on the acyl carrier protein AcpXL and attached to a lipid A precursor by the acyl transferase LpxXL. Lack of any of these two proteins results in the complete loss of all VLCFAs (Ferguson *et al.*, 2005; Sharypova *et al.*, 2003).

A study performed on the related bacterium *Rhizobium leguminosarum* found that although the *acpXL* mutant completely lacks the VLCFA modification in the free-living state, the lipid A of this mutant becomes partially modified with VLCFA during passage though peas (Vedam *et al.*, 2006). Experimental evidence in *S. meliloti* has also shown that the LPS hydrophobity increases in *S. meliloti* during the alfalfa symbiosis (Ferguson *et al.*, 2005), which may be indicative of VLCFA addition. Therefore, these findings raise the possibility that the *S. meliloti acpXL* and *lpxXL* mutants may undergo further changes in the plant. Additionally, unlike *E. coli* which has only one acyl carrier protein, the genome of *S. meliloti* encodes multiple acyl carrier and transferase genes whose products could potentially be involved in host induced LPS changes (Geiger & Lopez-Lara, 2002). Therefore it may be possible that one or more of these additional proteins could partially compensate for the loss of AcpXL and LpxXL in planta.

If the proposed model that BacA plays some role in the transport of activated VLCFAs out of the cytoplasm onto the lipid A in the outer membrane (Ferguson *et al.*, 2004) is correct then the LPS would need to be transported across the inner membrane before the lipid A could be modified with a VLCFA. In *E. coli*, the transport of newly synthesized rough LPS (containing lipid A and the core oligosaccharide) from the inner to the outer membrane is dependent upon the inner membrane ABC transporter MsbA protein (Doerrler *et al.*, 2001; Zhou *et al.*, 1998). The *S. meliloti* Rm1021 genome (Galibert *et al.*, 2001) encodes multiple proteins, which share protein similarity and identity over their entire length with the *E. coli* MsbA proteins (Beck *et al.*, 2008). Thus, it may be possible that *S. meliloti* MsbA-like proteins could also be playing a role in the transport of polysaccharide or lipid-containing polysaccharide such as LPS and these processes could play an important role in the host interaction.

Thus, the aims of the work presented in this chapter were as follows: firstly, to determine the importance of the VLCFA modifications in the *S. meliloti* alfalfa symbiosis by investigating the free-living and *in planta* phenotypes of the *S. meliloti acpXL* and *lpxXL* mutants and a putative acyl carrier protein mutant. Secondly, to investigate the roles of potential MsbA-like proteins in free-living and symbiotic

S. meliloti which may eventually lead to further clues as to the proposed role of BacA in the transport of VLCFAs.

6.2. Results

6.2.1. Investigation into the importance of VLCFA modifications in free-living and symbiotic *S. meliloti*

6.2.1.1. Free-living *acpXL* and *lpxXL* mutants display an altered LPS profile by SDS PAGE

It has been previously shown that the *S. meliloti acpXL* and *lpxXL* mutants display an increased level of sensitivity to cell envelope disrupting agents such as DOC and SDS, relative to the Rm1021 parent strain (Ferguson *et al.*, 2005), thus suggesting that the AcpXL/LpxXL-dependent VLCFA modifications of *S. meliloti* LPS are important for free-living stress resistance (Ferguson *et al.*, 2005). As a first step to investigate any other LPS changes in the free-living *S. meliloti acpXL* and *lpxXL* mutants, LPS samples were extracted from the mutants using an SDS lysis method. The samples were then analyzed by SDS-PAGE, followed by staining with sodium-m-periodate, which stains oxidized sugar residues (Fig. 6-2). As previously shown using this method of analysis (Ferguson *et al.*, 2002) there are no detectable differences in the LPS profile of the Rm1021 Δ*bacA* mutant, relative to the Rm1021 parent strain (Fig. 6-2, lanes 1 and 2, respectively). Contrastingly, in the Rm1021 *acpXL* and *lpxXL* mutants, it could be observed that the higher molecular weight band (I) was absent (Fig. 6-2, lanes 3 and 4, respectively), relative to the Rm1021 parent. The LPS profile observed for the *S. meliloti acpXL* mutant in this work is consistent with previous published findings (Sharypova *et al.*, 2003). However, this work shows for the first time that the *S. meliloti lpxXL* mutant too displays an altered LPS profile, relative to the Rm1021 parent strain. This higher molecular weight band (I) may correspond to smooth LPS (lipid A, core polysaccharide and the O-antigen), whereas the lower molecular weight band (II) may represent rough LPS, lacking the O-antigen. Thus, these data showing that the LPS profiles of the Rm1021 *acpXL* and *lpxXL* mutants are missing the higher molecular weight band

could be interpreted as an inability of these mutants to express O antigen. However, the glycosyl composition of the LPS of these mutants has been analysed and was found to be very similar to that of the Rm1021 parent strain (R.W. Carlson and G.P Ferguson, unpublished data) suggesting there are no differences in the O-antigen of these mutants, relative to the Rm1021 parent strain.

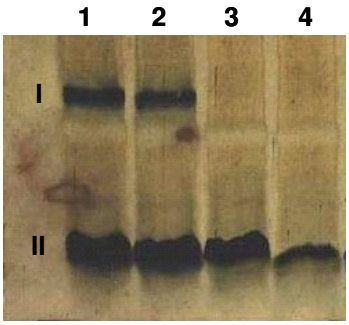


Figure 6-2. SDS-PAGE gel of LPS extracted from the Rm1021 parent strain, Δ*bacA, acpXL* and *lpxXL* mutants. Profiles shown are the Rm1021 parent stain (1), the Δ*bacA* mutant (2), the *acpXl*::pk18mobGII mutant (3) and the *lpxXL*::pJH104 mutant (4). The gel was then stained using the periodate-silver staining method. Band I represents a high-molecular weight form of LPS and band II represents the lower molecular weight, faster migrating form.

6.2.1.2. Within the host the *S. meliloti acpXL* mutant is delayed in infection droplet release and the nodules prematurely senesce

As previously discussed, work performed with the related bacterium *R. leguminosarum* found an *acpXL* mutant also lacks the VLCFA modification in the free-living state but like the *S. meliloti acpXL* mutant can form a successful symbiosis (Vedam *et al.*, 2003). However, TEM of pea infected nodules revealed that *R. leguminosarum* lacking the AcpXL protein showed defects in the host, relative to the parent, suggesting an important role for AcpXL in bacteroid development (Vedam *et al.*, 2004). Thus, it may be possible that the *S. meliloti* AcpXL protein may also be involved in bacteroid development. To investigate this, the Rm1021 *acpXL* mutant and the Rm1021 parent strain were inoculated onto alfalfa plants. Nodules were removed from plants at 1 and 4 weeks post infection and fixed and analysed by TEM (Fig. 6-3). At 1 week post infection very few

Rm1021 *acpXL* mutant bacteroid containing cells were observed in the nodule sections (Fig. 6-3A & B), relative to the Rm1021 parent strain (Fig. 6-3C). Instead the majority of Rm1021 *acpXL* mutant bacteroids were still in the infection droplets (Fig. 6-3A & B), of which there were multiple.

At four weeks post infection defects observed in the Rm1021 *acpXL* induced nodules (Fig. 6-3D & E), relative to the Rm1021 parent (Fig. 6-3F) were even more pronounced. At this time point the plant cells appeared quite disorganised (Fig. 6-3D & E), relative to the plant cells induced by the Rm1021 parent (Fig. 6-3F) strain. The presence of multiple Rm1021 *acpXL* mutant bacteroids per symbiosome could also be observed (Fig. 6-3D & E), here the bacteroids could be seen to be retracting from the peribacteroid membrane. Contrastingly, in the parent strain (Fig. 6-3F), only one bacteroid per symbiosome was ever observed. Additionally, lytic vesicles could be observed (Fig. 6-3D) containing multiple odd shaped bacteroids. Taken together these data would suggest that the *S. meliloti* AcpXL protein plays an important role in the host interaction at both the early and late stages of the symbiosis.

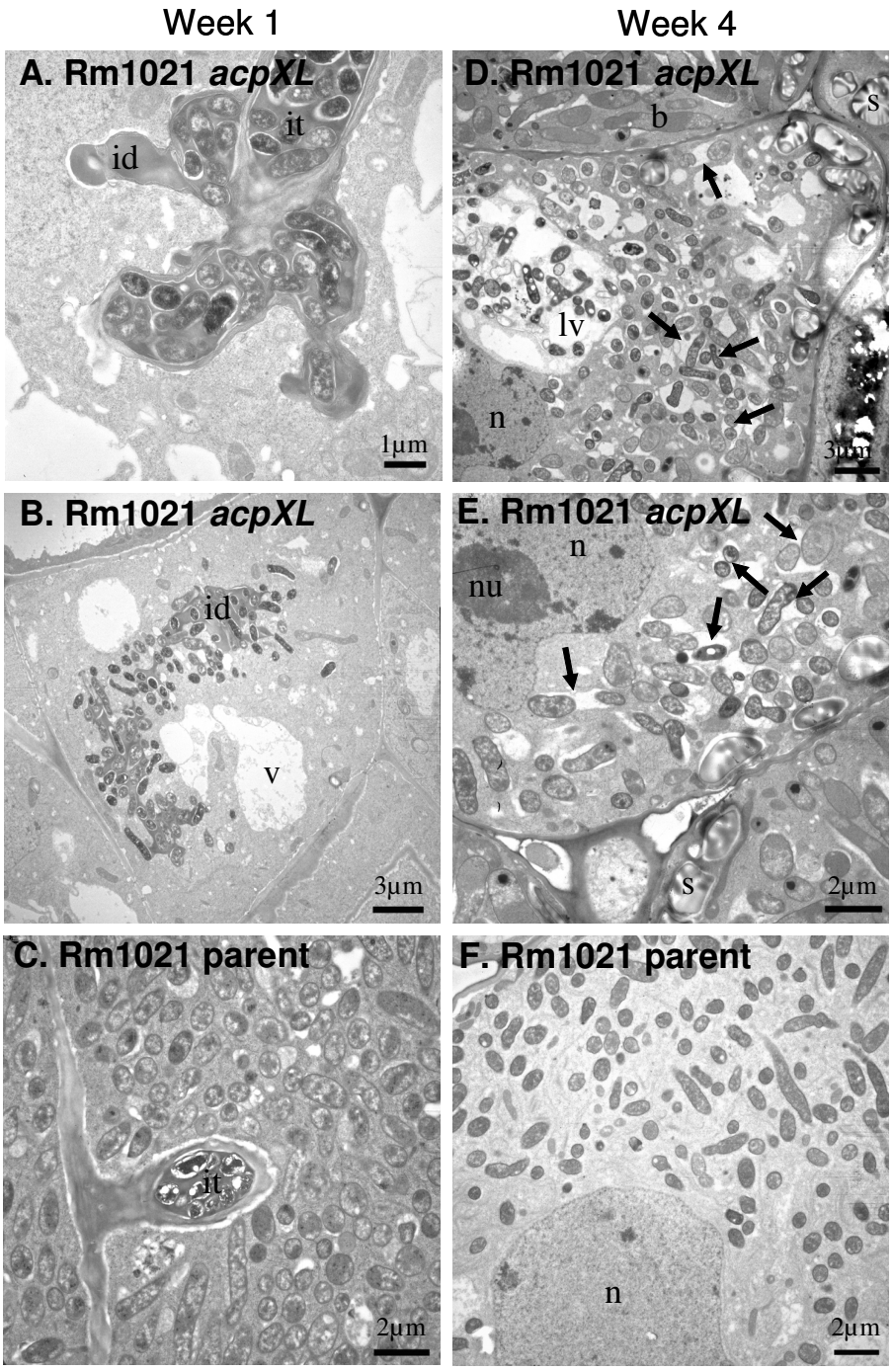


Figure 6-3. TEM Micrographs of the Rm1021 parent and Rm1021 *acpXL* mutant. The alfalfa nodules induced by the defined strains one (A-C) and four (D-F) weeks post infection were analyzed by TEM. The arrows in D & E show the presence of multiple bacteroids within a single membrane compartment. Abbreviations: bacteroid (b), infection droplet (id), infection thread (it), nucleus (n), nucleolus (nu), lytic vesicle (lv), starch grain (s), and vacuole (v). TEM analysis was performed by Euan James, at the University of Dundee. This project was performed in collaboration with Andreas Haag at the University of Aberdeen.

6.2.1.3. The *S. meliloti* Rm1021 *lpxXL* mutant bacteroids also show abnormalities throughout the entire infection process

Previous work had shown that the free-living Rm1021 *lpxXL* mutant was found to be more sensitive than the Rm1021 *acpXL* mutant to cell envelope disrupting agents SDS and DOC (Ferguson *et al.*, 2005), since despite lacking the VLCFA modification, the Rm1021 *acpXL* mutant is still able to transfer a shorter chain C16:0 or C18:0 unhydroxylated fatty acid not normally found in the *S. meliloti* LPS, onto a portion of its lipid A molecules (Ferguson *et al.*, 2002). Therefore, the Rm1021 *acpXL* mutant is still able to produce a significant portion of its lipid A molecules in the pentaacylated state (Ferguson *et al.*, 2005), relative to the Rm1021 *lpxXL* mutant which does not. Thus, it may be possible that due to the altered lipid A in the Rm1021 *lpxXL* mutant, differences may be observed relative to the Rm1021 *acpXL* mutant in the host symbiosis. To investigate this possibility, the Rm1021 *lpxXL* mutant was inoculated onto alfalfa plants. Nodules were removed from plants at 1 to 4 weeks post infection and fixed and analysed by TEM (Fig. 6-4). At 1 week post infection, there appeared to be more Rm1021 *lpxXL* mutant bacteroids present within the plant cells (Fig. 6-4A), relative to what was observed for the Rm1021 *acpXL* mutant (Fig. 6-3A & B). However, throughout the 4 week period very odd shaped bacteroids were observed, often appearing very enlarged (Fig. 6-4B & C) and showing branching and bulges (Fig. 6-4A, B, C &D). Contrastingly, these very odd shaped bacteroids were never present in the Rm1021 parent strain (Fig. 6-3C&F and 6-4E&F) or the Rm1021 *acpXL* mutant (Fig. 6-3A, B, D & E). These data therefore suggest that the *S. meliloti* LpxXL protein also has an important role in bacteroid development. However, since the Rm1021 *lpxXL* mutant had a phenotype distinct from the Rm1021 *acpXL* mutant, this could suggest the AcpXL and LpxXL proteins play important but different roles in bacteroid development.

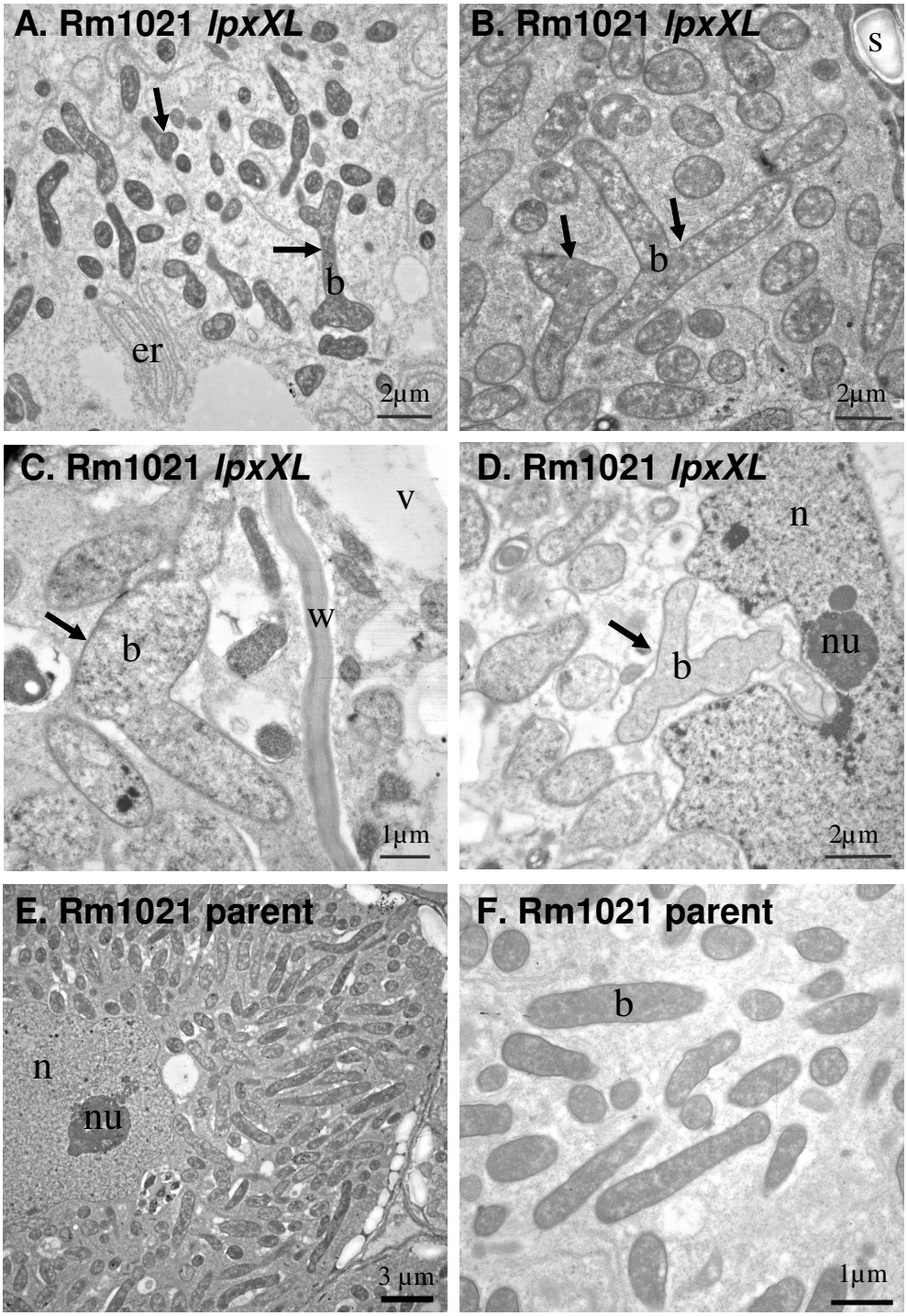


Figure 6-4. TEM-Micrographs of the Rm1021 *lpxXL* mutant and the Rm1021 parent. The alfalfa nodules induced by the defined strains either one (A & E), two (B), three (C) or four (D & F) weeks post infection were analysed by TEM. The arrows shown in A-D indicate the abnormally shaped bacteroids. Abbreviations: bacteroid (b), endoplasmic reticulum (er), mitochondria (m),nucleus (n), nucleolus (nu), starch grain (s), cell wall (w) and vacuole (v). TEM analysis was performed by Euan James, at the University of Dundee. This project was performed in collaboration with Andreas Haag at the University of Aberdeen.

6.2.1.4. The *S. meliloti acpXL/lpxXL* double mutant bacteroids display similar phenotypes to both the *acpXL* and *lpxXL* single mutants

It had previously been shown that the Rm1021 *lpxXL* mutant and the Rm1021 *acpXL/lpxXL* double mutant have identical lipid A profiles in their free-living forms (Ferguson *et al.*, 2005). However, the free-living Rm1021 *acpXL/lpxXL* double mutant showed a slight increased sensitivity to certain stresses i.e. detergents DOC and SDS, compared to the Rm1021 *lpxXL* single mutant. Thus, this raised the possibility that the double mutant may display more pronounced defects in the plant. To investigate this, TEM analysis was performed on 1 and 4 week nodules induced by the *S. meliloti acpXL/lpxXL* double mutant (Fig. 6-5). At 1 week post infection it could be observed that in nodules induced by the Rm1021 *acpXL/lpxXL* double mutant there were less bacteria present in the plant cells (Fig. 6-5A & B) relative to the Rm1021 *lpxXL* single mutant (Fig. 6-4A) and the Rm1021 parent strain (Fig. 6-3C). However, this phenotype was not as pronounced as had been observed in the Rm1021 *acpXL* mutant (Fig. 6-3 A & B). Additionally at week 1 post infection there was also the presence of odd-shaped bacteroids (Fig. 6-5B), as was observed for the Rm1021 *lpxXL* single mutant. These odd shaped bacteroids were still present at 4 weeks post infection (Fig. 6-5C &D) and additionally, lytic vesicles and multiple bacteroids per symbiosome were also evident (Fig. 6-5C &D), as observed in the Rm1021 *acpXL* mutant. Thus, these data show that interestingly the Rm1021 *acpXL/lpxXL* mutant displays phenotypes of both the Rm1021 *acpXL* and Rm1021 *lpxXL* single mutants.

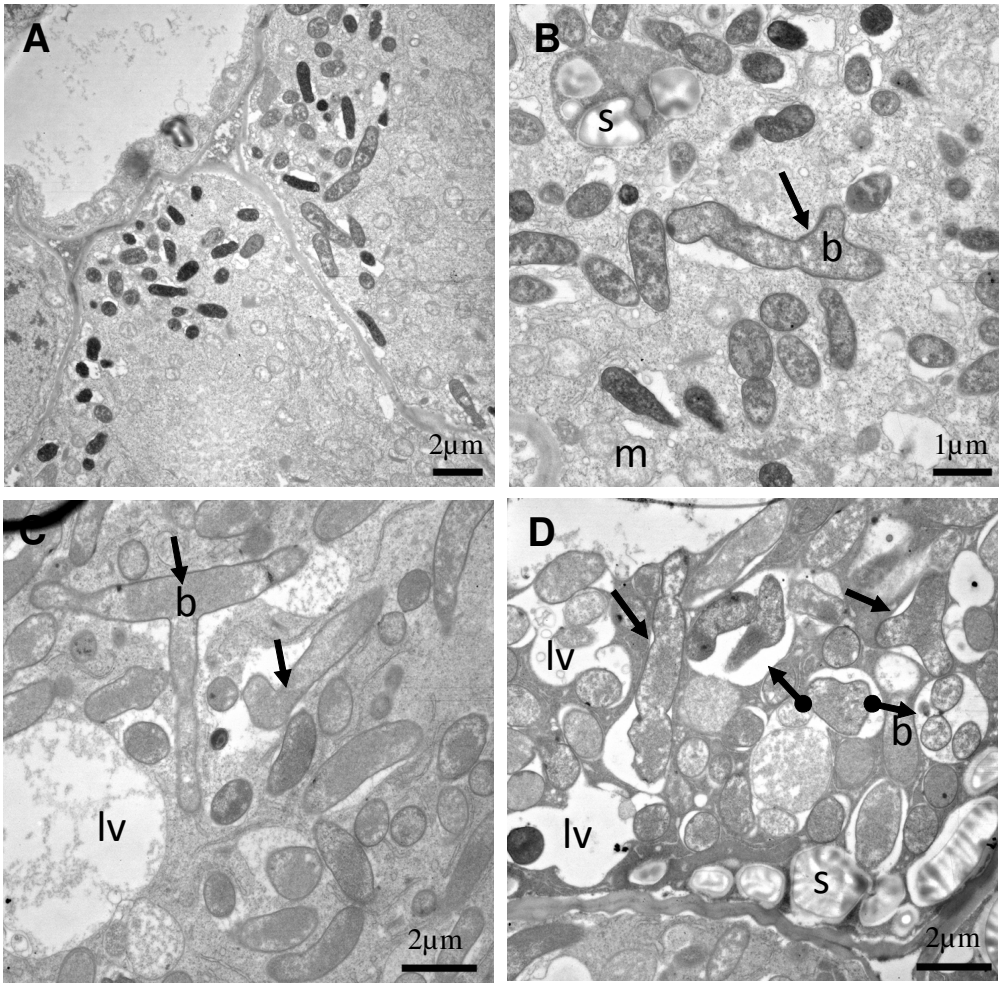


Figure 6-5. TEM-Micrographs of the Rm1021 *acpXL/lpxXL* double mutant. The alfalfa nodules induced the *S. meliloti* Rm1021 *acpXL/ lpxXL* double mutant either at one (A & B) or four (C & D) weeks post infection were analysed by TEM. The abnormally shaped bacteroids are indicated by arrows (B-D) and the arrows with dots (D) show the presence of multiple bacteroids within a single membrane compartment. Abbreviations: bacteroid (b) endoplasmic reticulum (er), mitochondria (m), lytic vesicle (lv), nucleus (n), nucleolus (nu), starch grain (s). TEM analysis was performed by Euan James, at the University of Dundee. This project was performed in collaboration with Andreas Haag at the University of Aberdeen.

6.2.1.5. Preliminary *in planta* analysis suggests the putative acyl carrier protein Smb20651 does not compensate for the loss of the AcpXL protein

Previous work with the *R. leguminosarum acpXL* mutant showed that despite lacking the VLCFA modification in the free-living state, the lipid A of this mutant was partially modified with VLCFA when extracted back from the host (Vedam *et al.*, 2006). Analysis of the bacteroid lipid A revealed that 56% of the lipid A was found to contain the VLCFA modification (Vedam *et al.*, 2006). Thus, it was possible that the *S. meliloti acpXL* mutant may also undergo further lipid A changes in the plant. Although a role has been proposed for the BacA protein in these host induced changes, additional proteins could also be involved in the biosynthesis of host-induced LPS VLCFA modifications (Geiger & Lopez-Lara, 2002). It is possible that one or more proteins could partially compensate for the loss of AcpXL *in planta*. Intriguingly in *S. meliloti* the *smb20651* gene encodes a potential acyl carrier protein which is located in an operon with putative long-chain fatty acid CoA ligase (Geiger & Lopez-Lara, 2002) (Fig. 6-6). Thus, the protein encoded by *smb20651* could be a potential candidate for host induced lipid A changes in the *S. meliloti acpXL* mutant (Geiger & Lopez-Lara, 2002). To investigate this possibility, the free-living and *in planta* phenotype of the *S. meliloti* Rm1021 *smb20651* mutant (Ramos-Vega *et al.*, 2009) in the presence and absence of the AcpXL protein was assessed.

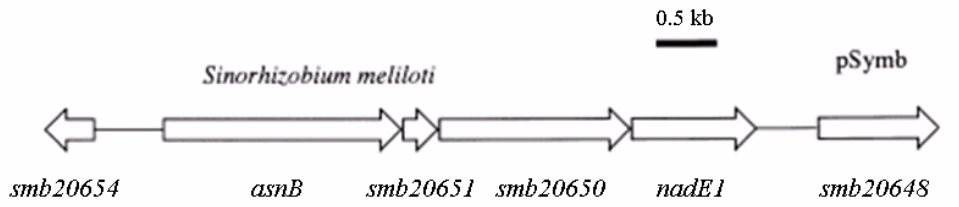


Figure 6-6. Genetic organization around the genes coding for the proposed acyl carrier protein Smb20651. *smb20654*: encodes hypothetical protein, *asnB*: encodes putative asparagine synthetase, *smb20650*: putative long-chain fatty acid CoA ligase, *nadE1*: encodes putative NH_3-dependent NAD^+ synthetase, and *smb20648*: encodes putative oxido/reductase. (Geiger & Lopez-Lara, 2002).

Initially, an LPS sample was extracted from the free-living Rm1021 Δ*smb20651* mutant using the SDS lysis method and analyzed by SDS-PAGE to determine if loss of this putative acyl carrier protein Smb20651 resulted in an altered LPS profile. However, it was observed that there were no detectable differences in the banding pattern of the Rm1021Δ*smb20651* mutant relative to the Rm1021 parent strain (Fig. 6-7 lanes 2 and 1, respectively), with the Rm1021 Δ*smb20651* mutant profile displaying both the upper and lower migrating bands (Fig. 6-7 lane 2). The Δ*smb20651* background was next transduced into the Rm1021 *acpXL* mutant, using M12 phage. The resulting Rm1021 *acpXL/*Δ*Smb20651* double mutant displayed an LPS profile identical to that of the *acpXL* single mutant (Fig. 6-2). Therefore, if loss of the Smb20651 protein in *S. meliloti* affects the LPS, it is not detectable by this means of analysis.

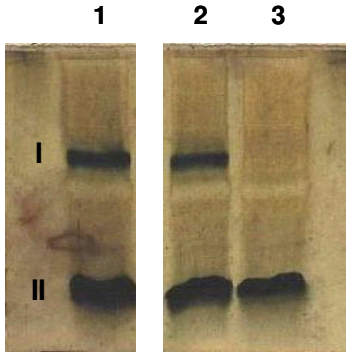


Figure 6-7. SDS-PAGE gels of LPS extracted from the Rm1021 parent strain, the putative acyl carrier protein mutant Δ*smb20651* and the Δ*smb20651*/*acpXL* double mutant. Profiles shown are the Rm1021 parent stain (1), the Rm1021 Δ*smb20651* mutant (2), and the Rm1021 Δ*smb20651/acpXL* double mutant (3). The gel was then stained using the periodate-silver staining method. Band I represents a high-molecular weight form of LPS and band II represents the lower molecular weight, faster migrating form.

To investigate the possibility that the Smb20651 protein may be involved in host-induced LPS changes in the absence of the *acpXL* gene, alfalfa seedlings were inoculated with the Rm1021 Δ*smb20651* single mutant and Rm1021 *acpXL/* Δ*smb20651* double mutant and plant growth and nodule characteristics were recorded after a four week period. Alfalfa seedlings were also inoculated with the Rm1021 parent strain and the Rm1021 *acpXL* mutant. Despite the disruption of the

smb20651 gene, in both the Rm1021 parent and Rm1021 *acpXL* mutant background, both mutant strains were able to form a symbiosis with alfalfa plants (Table 6-1). Thus, these data would suggest that in *S. meliloti* the putative acyl carrier protein Smb20651 does not appear to compensate for the loss of the AcpXL protein in the alfalfa symbiosis.

Table 6-1. Effect of the loss of putative acyl carrier protein Smb20651 on the alfalfa symbiosis

Bacterial Strain	Symbiosis	Plant height[a] (cm)	Plant colour	Mean No. of pink nodules per plant	Mean No. of white nodules per plant
Rm1021 parent	Yes	9.2 ± 1.6	Dark green	9.3 ± 2.1	0
acpXL	Yes	7.9 ± 1.8	Dark green	5.6 ± 2.2	0.8 ± 1.1
Δ*smb20651*	Yes	9.0 ± 1.8	Dark green	8.9 ± 4.2	3.3 ± 2.2
acpXL/Δ*smb20651*	Yes	7.2 ± 1.4	Dark & lighter* green	7.3 ± 3.2	0.9 ± 1.2

± shows the standard derivation from the mean
[a] 10 plants were analysed per *S. meliloti* strain
* lighter leaves present on 2 out of 10 plants analysed, relative to dark green leaves of plants infected with the parent strain which are indicative of a successful symbiosis

6.2.1.6. The Smb20651 protein confers a significant competitive advantage to *S. meliloti* in the alfalfa symbiosis

Since previous work had shown that the Rm1021 *acpXL* mutant was able to form a symbiosis but was substantially less competitive than the Rm1021 parent strain (Ferguson *et al.*, 2005), it was next investigated if Smb20651 function conferred a competitive advantage during the symbiosis. A series of competition assays were set up with alfalfa plants. In each case two different *S. meliloti* strains were inoculated simultaneously in a 1:1 ratio onto the alfalfa seedlings. After 4 weeks, the bacteria were recovered from pink nodules and the numbers of each *S. meliloti* strain present in each nodule were identified by selection of the appropriate antibiotic resistance markers (table 6-2).

Analysis of nodules inoculated with the Rm1021 parent strain and the Rm1021 Δ*smb20651* mutant found that for each nodule analysed, the Rm1021 parent strain represented over 99% of bacteria recovered, and the same was true of the Rm1021 parent strain versus the Rm1021 *acpXL*/Δ*smb20651* double mutant competition. The Rm1021 *acpXL*/Δ*smb20651* double mutant was also found to be significantly less competitive than either respective single mutant alone (table 6-2). Although the presence of the Smb20651 protein does not appear to be essential for *S. meliloti* to form a successful symbiosis these data suggest it confers a very significant competitive advantage. Upon recovery of nodules inoculated with the Rm1021 *acpXL* and Rm1021 Δ*smb20651* single mutants, it was observed that in 4 out of 5 nodules analysed the Rm1021 Δ*smb20651* mutant represented over 99% of the recovered bacteria. Therefore it appears that under the conditions tested loss of the *acpXL* gene was more detrimental in the alfalfa symbiosis than loss of the *smb20651* gene.

Table 6-2. Effect of loss of the putative acyl carrier protein Smb20651 on the alfalfa symbiosis

Competition	Percentage of each strain recovered from nodule[a]	
Rm1021 versus *smb20651*	Rm1021 (99.9 ± 0.14)	*smb20651* (0.1 ± 0.14)
Rm1021 versus *smb20651/acpXL*	Rm1021 (99.9±0.13)	*smb20651/acpXL* (0.1 ±0.13)
acpXL versus *smb20651/acpXL*	*acpXL* (98.7±1.3) *	*smb20651/acpXL* (1.3 ±1.3)*
smb20651 versus *smb20651/acpXL*	*smb20651* (99.8 ± 0.17)	*smb20651/acpXL* (0.2± 0.17)
acpXL versus *smb20651*	*smb20651* (99.7± 0.53)†	*acpXL* (0.3 ± 0.53)

± shows the standard derivation from the mean

[a] 10 plant nodules were analysed per competition assay, with the exception of *acpXL* versus *Smb20651* where only 5 nodules were analysed.

* With the exception that one nodule analysed, 84% of the *acpXL* mutant and 16% of the *Smb20651/acpXL* mutant were recovered.

† With the exception that one nodule analysed, 75.1% of the *acpXL* mutant and 24.9% of the *Smb20651* mutant were recovered.

6.2.1.7. Preliminary data suggests that passage through the host does not restore the NaCl tolerance of the Rm1021 *lpxXL* mutant

As previously discussed, analysis of the lipid A from *R. leguminosarum acpXL* mutant bacteroids revealed unlike the free-living *acpXL* mutant, the bacteroid lipid A was found to contain some VLCFA (Vedam *et al.*, 2006). However, when *R. leguminosarum acpXL* ex-nodule isolates were grown both under standard laboratory conditions and in the presence of 0.5% (w/v) NaCl, no VLCFA was found on the lipid A of these mutants. So these data suggest that whatever mechanism is responsible for the addition of the VLCFA in the absence of the AcpXL protein, it appears to be only induced in the host environment. However, the finding that the *R. leguminosarum acpXL* ex-nodule isolates were able to grow in the presence of 0.5% NaCl unlike the free-living *acpXL* mutant, suggested that passage through the plant had restored the salt tolerance of the *acpXL* mutant. Therefore, it was next investigated if passage though the plant could restore the salt sensitivity of the *S. meliloti lpxXL* mutant.

Growth of free-living and ex-nodule *S. meliloti* was assessed on different salt concentrations (Fig. 6-8). However, it was observed that relative to the free-living *lpxXL* mutant the ex-planta *lpxXL* mutant strain did not display an increased salt resistance. Like the free-living mutant, the ex-planta *lpxXL* mutant was also unable to grow in the presence of 0.25 % NaCl (Fig. 6-8D). Thus, these preliminary data suggested that at least under the conditions tested passage through the plant did not appear to restore the NaCl resistance of the *S. meliloti lpxXL* mutant.

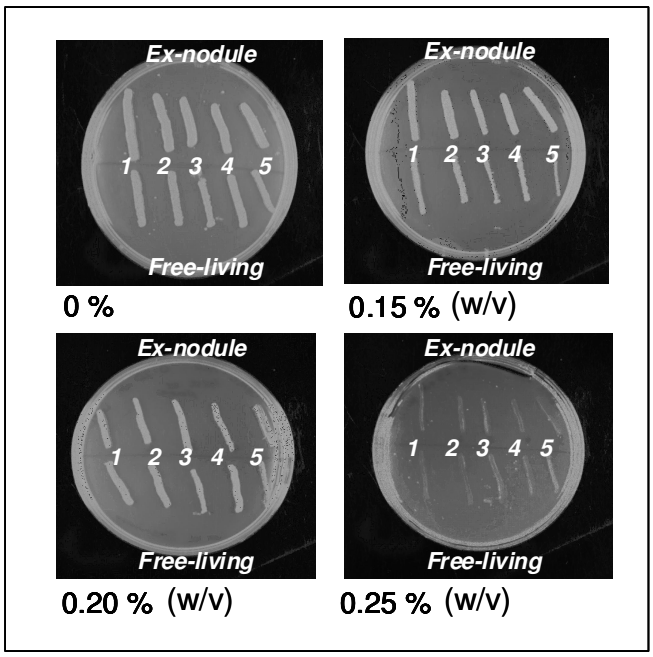


Figure 6-8. NaCl tolerances of free-living and host-extracted *S. meliloti lpxXL* cells. The bacteria were extracted from alfalfa nodules four weeks post inoculation and were recovered and pre-grown on LBMC plates. Single ex-nodule and free-living colonies were then screened on LBMC plates containing different salt concentrations as indicated by the percentages.

6.2.2. Investigation into the role of the MsbA like proteins in *S. meliloti*

6.2.2.1. *S. meliloti msbA1* and *msbA2* mutants do not have an increased sensitivity to cell envelope disrupting agents

Thus far in this chapter it has been determined that the AcpXL and LpxXL proteins play important roles in bacteroid development. Additionally, a preliminary study suggested that the putative acyl carrier protein Smb20651 does not appear to compensate in the host for loss of the AcpXL protein in *S. meliloti*. However, there still remained the possibility that further host-induced lipid A changes were occurring in the *S. meliloti acpXL* and *lpxXL* mutants and the BacA protein may play some role in these changes. If the proposed model that BacA is involved in the transport of activated VLCFAs out of the cytoplasm onto the lipid A (Ferguson *et al.*, 2004) is correct then the LPS would need to be transported across the inner membrane before the lipid A could be modified with a VLCFA. In *E. coli*, the

transport of newly synthesized rough LPS (containing lipid A and the core oligosaccharide) and phosolipids from the inner to the outer membrane of is dependent upon the inner membrane ABC transporter MsbA protein (Doerrler *et al.*, 2001; Zhou *et al.*, 1998). Interestingly, the *S. meliloti* Rm1021 genome contains four genes thought to encode MsbA like proteins, *msbA1* (*smb20813*), *msbA2* (*smb21191*), *exsA* (*smb20941*) and *y02836* (*smc02836*) (Galibert *et al.*, 2001). These *S. meliloti* MsbA like proteins share between 26-34% identity and 47-58% similarity over their entire length with the *E. coli* MsbA protein (Beck *et al.*, 2008). Therefore, it may be possible that *S. meliloti* MsbA-like proteins could also be playing a role in the transport of polysaccharide or lipid-containing polysaccharide such as LPS and these processes could play an important role in the host interaction. Thus, investigation into the role of the MsbA like proteins in lipid trafficking in *S. meliloti* may eventually lead to more clues about the proposed role of the BacA protein in the VLCFA modification and where in the cell envelope this modification occurs.

To understand more about the roles of potential MsbA-like proteins in *S. meliloti, msbA1* and *msbA2* insertional mutants were constructed by Sebastian Beck, a postdoctoral researcher in the laboratory. To do this internal fragments of the respective genes were cloned into the suicide vector pJH104 (Davies & Walker, 2007). Upon successful construction of the mutants, both were transduced into a clean Rm1021 parent background.

Previous research has shown that *S. meliloti* LPS mutants display an altered sensitivity to cell envelope-disrupting agents such as SDS, DOC and Crystal Violet (Davies & Walker, 2007; Ferguson *et al.*, 2002; Ferguson *et al.*, 2005; Ferguson *et al.*, 2006). As a first means to determine if the *S. meliloti* Rm1021 *msbA1*::pHJ104 and *msbA2*::pHJ104 mutants had LPS alterations, sensitivity of the mutants to cell envelope disrupting agents was assessed (Fig. 6-9). It was observed that unlike the Rm1021 Δ*bacA* mutant, neither the Rm1021 *msbA1*::pHJ104 or Rm1021 *msbA2*::pHJ104 mutants displayed an increased sensitivity to SDS (Fig. 6-9A) or Crystal Violet (Fig. 6-9B), when assessed by filter disc assay. Additionally, when the cells were exposed to a DOC gradient, unlike the Rm1021 Δ*bacA* mutant which displays a zone of growth inhibition, relative to the Rm1021 parent, no growth inhibition was observed for *msbA1*::pHJ104 or *msbA2*::pHJ104 mutants (Fig. 6-9C).

These findings would therefore suggest that the Rm1021 *msbA1*::pHJ104 and Rm1021 *msbA2*::pHJ104 mutants do not appear to have LPS alterations, relative to the Rm1021 parent which were detectable under these stress assay conditions.

The Δ*bacA* mutation was next transduced into the Rm1021 *msbA1* and *msbA2* mutant backgrounds using M12 phage, to determine if loss of BacA increased the sensitivity of the *msbA* mutants to cell envelope disrupting agents. It could be observed that loss of *bacA* in the *msbA1* and *msbA2* mutant backgrounds did result in an increased sensitivity to SDS (Fig. 6-9A). Interestingly, it was also observed that the Rm1021 *msbA1*::pHJ104 /Δ*bacA* and *msbA2*::pHJ104 /Δ*bacA* double mutants displayed a reduced level of sensitivity to SDS and Crystal Violet relative to the Rm1021 Δ*bacA* single mutant (Fig. 6-9A & B, respectively). This phenotype seemed to be specific for SDS and Crystal Violet since the *msbA1*::pHJ104 /Δ*bacA* and *msbA2*::pHJ104 /Δ*bacA* double mutants displayed the same level of sensitivity to DOC as observed for the Rm1021 Δ*bacA* single mutant (Fig. 6-9C). However, these data would suggest that disruption of the *msbA1* and *msbA2* genes partially suppresses the sensitivity phenotype of the Rm1021 Δ*bacA* mutant to SDS and Crystal Violet.

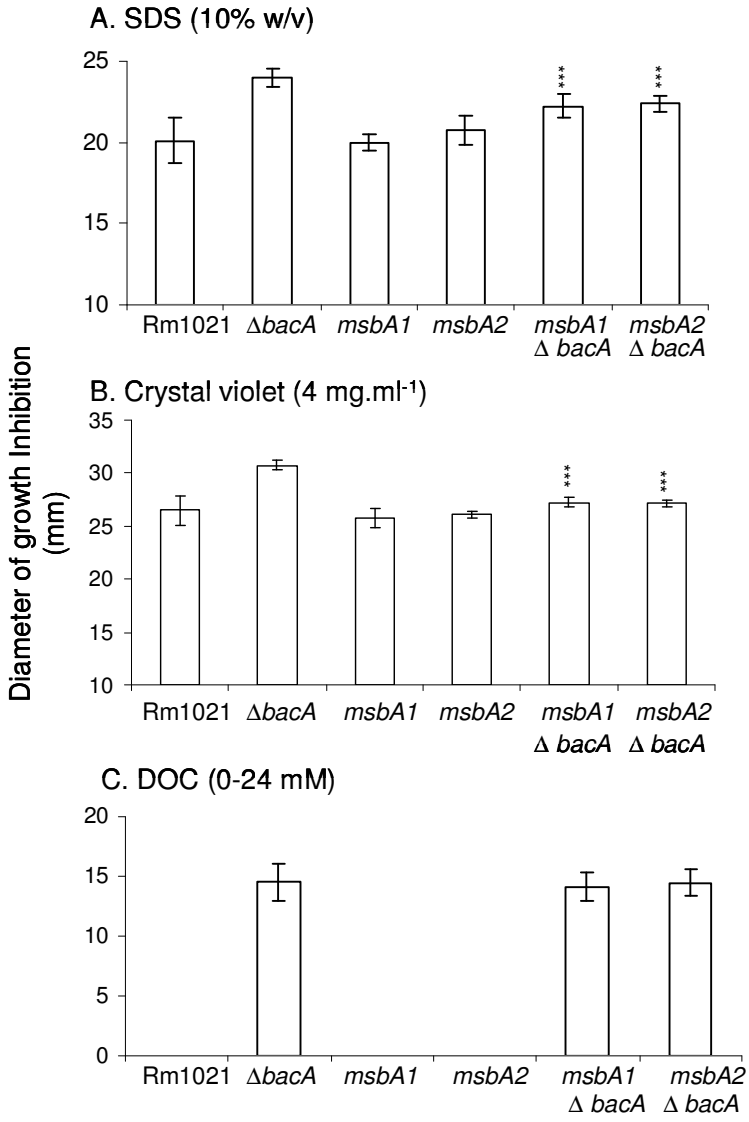


Figure 6-9. Sensitivity of the Rm1021 Δ*bacA, msbA1* and *msbA2* mutants to SDS, Crystal Violet and DOC. The defined strains were exposed to SDS (5 µl of a 10% w/v stock solution) using a filter disc assay on LB agar (A). As in (A), except cells were exposed to crystal violet (5 µl of a 4 mg.ml^{-1}stock solution) (B). The defined strains were exposed to DOC (0-24 mM) using a gradient assay on LB agar (C). In (A) and (B) the significant values (***P<0.001) represent comparisons of the *msbA1*/Δ*bacA* and *msbA2*/Δ*bacA* to the Δ*bacA* mutant. All datasets show are representative of the trends observed in two independent experiments. The error bars represent the standard deviation from the mean (n=3) in one experiment.

6.2.2.2. The *S. meliloti msbA2* mutant is defective in the legume symbiosis

To investigate if the MsbA1 or MsbA2 proteins were playing a role in the *S. meliloti* legume symbiosis, alfalfa seedlings were inoculated with cultures of the Rm1021 parent strain, the *msbA1*::pJH104 and the *msbA2*::pJH104 mutant. Plant growth and nodule development was assessed 4 weeks post inoculation. This work was performed by Sebastian Beck. No defects in the alfalfa symbiosis were observed for the Rm1021 *msbA1*::pJH104 mutant, relative to the Rm1021 parent strain (S.Beck and G.P.Ferguson, unpublished data). Contrastingly, plants inoculated with the Rm1021 *msbA2*::pJH104 mutant were stunted and the leaves were paler green/yellowish, relative to the dark green leaves of plants induced by the parent strain (Beck *et al.*, 2008). The characteristics displayed by the plants inoculated with the Rm1021 *msbA2*::pJH104 are indicative of an unsuccessful symbiosis (Fig. 6-10). Additionally, compared to the pink, elongated, nitrogen fixing root nodules induced by the Rm1021 parent strain, the root nodules induced by the Rm1021 *msbA2*::pJH104 mutant were shorter and white and stumpy like in appearance with brown tinges (Fig. 6-10) (Beck *et al.*, 2008), again indicative of an unsuccessful symbiosis.

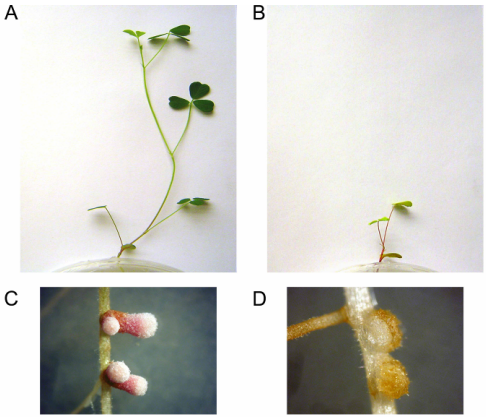


Figure 6-10. Alfalfa seedlings after inoculation with the *S. meliloti* Rm1021 parent and the *S. meliloti* Rm1021 *msbA2*::pJH104. Alfalfa seedlings were inoculated with either the Rm1021 parent (A & C) or the Rm1021 *msbA2*::pJH104 mutant (B & D). Plant growth (A & B) and nodule development (C & D) were photographed 4 weeks post infection. Pictures were taken 28 days post inoculation. Work performed by Sebastian Beck. Figure adapted from Beck *et al.*, 2008.

Since an interesting in planta phenotype had been observed for the Rm1021 *msbA2*:: pJH104 mutant, it was decided to now focus on characterisation of this mutant. Additionally, the *msbA2* gene is located on the pSymB megaplasmid of *S. meliloti* Rm1021 (Beck *et al.*, 2008), which contains many genes whose products are involved in the synthesis of cell surface carbohydrates (Finan *et al.*, 2001), and is immediately downstream of genes whose products are predicted to be involved in the biosynthesis of a lipid linked polysaccharide (Beck *et al.*, 2008) (Fig. 6-11).

Figure 6-11. Genomic organisation of the *S. meliloti msbA2* gene. The *msbA2* gene (*smb21191*) is likely to be the last gene in a multi-gene operon with *smb21188* (encoding a putative acyltransferase) and *smb21189/smb21190* (encoding putative glycosyltransferases). The predicted operon is surrounded by the upstream genes *gabT* (*smb21186*, encoding a putative 4-aminobutyrate aminotransferase) and *smb21187* (encoding a putative transcriptional regulator) and the downstream gene *cbbA2* (*smb21192*, encoding a putative fructose-bisphosphate aldolase) (Beck *et al.*, 2008).

Genes located downstream of the *S. meliloti msbA2* gene are on the opposite strand in the Rm1021 genome (Fig. 6-11) (Galibert et al., 2001). Therefore, it seemed unlikely that disruption of the *msbA2* gene would exert a polar effect on any of these genes. Moreover, a second study, using transposon mutagenesis had independently shown that the Rm1021 *msbA2* gene was essential for a successful legume symbiosis (Griffitts & Long, 2008). Unfortunately, work performed by Sebastian Beck in the laboratory, attempting to clone a wild-type copy of the *S. meliloti msbA2* gene into several broad-host-range vectors in *E. coli* was unsuccessful (Beck *et al.*, 2008). It was only possible to clone a mutated form of the *msbA2* gene (pmsbA2G97A) into pJN105 (Newman & Fuqua, 1999), under control of an arabinose-inducible promoter in the presence of 0.1% (w/v) glucose (Beck *et al.*, 2008). The cloned *msbA2* gene was found to contain a point mutation (G97A), which produced a mutated form of the MsbA2 protein, with a subsitiution of asparagine, instead of a serine residue, at amino acid position 33 (Beck *et al.*, 2008).

In work performed in collaboration with Gail Ferguson, alfalfa seedlings were inoculated with the *S. meliloti msbA2* mutant carrying pmsbA2G97A in the presence of 0.1% (w/v) arabinose. As a control alfalfa seedlings were inoculated with the Rm1021 *msbA2* mutant carrying the pJN105 vector only. At four weeks post infection plant growth was assessed. It was observed that in 7 out of 15 plants inoculated with the *S. meliloti* Rm1021 *msbA2* mutant containing pmsbA2G97A in the presence of 0.1% (w/v) arabinose, the leaf colour remained light green/yellow with only brown 7.0 (± 0.7) and white nodules 2 (± 3) evident. However, 8 out of 15 plants revealed dark green leaves, indicative of a healthy symbiosis and had an average of 2.6 (± 1.0) pink nitrogen fixing nodules per plant root (Fig. 6-12A & C, respectively). Contrastingly, on plants inoculated with the *S. meliloti* Rm1021 *msbA2* mutant carrying only the pJN105 control vector (n=9) the leaves remained light green/yellow with an average of 5.8 (± 3.5) brown and 1.66 (± 0.52) white nodules per plant. No pink nodules were present (Fig. 6-12B & D).

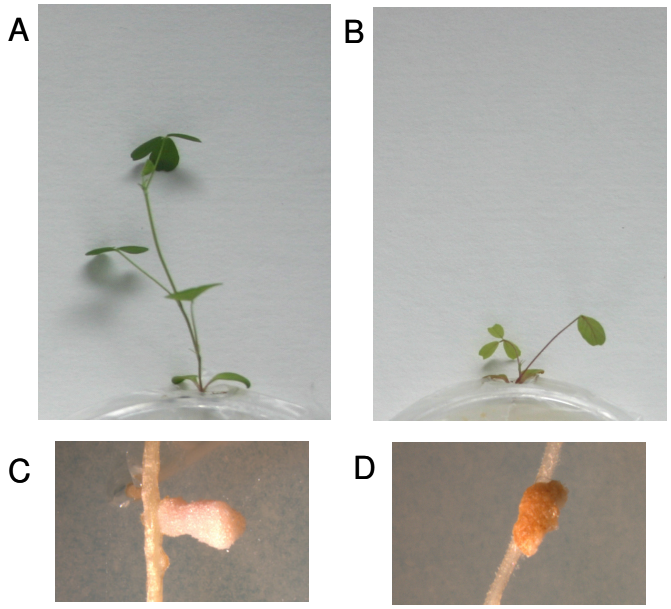


Figure 6-12. Alfalfa seedlings after inoculation with the Rm1021 *msbA2*::pJH104 mutant carrying pmsbA2G97A and the pJN105 control vector. Alfalfa seedlings were inoculated with either the *msbA2*::pJH104 mutant carrying pmsbA2G97A (A and C) or the Rm1021 *msbA2*::pJH104 mutant carrying the pJN105 control vector (B and D). Plant growth (A and B) and nodule development (C and D) were photographed 4 weeks post infection. All plants were grown in the presence of 0.1 % (w/v) arabinose.

However, in the independent study previously discussed, which had also identified the *S. meliloti* Rm1021 *msbA2* gene as essential for the alfalfa symbiosis (Griffitts & Long, 2008), complementation of the *msbA2* mutant, using the wild type *msbA2* gene was successfully achieved. In this study, a 1.8 kb fragment of the *msbA2* open reading frame, including the putative ribosomal binding site was cloned into the broad host range vector pRF771 (Wells & Long, 2002). The resulting plasmid pJG176 (Griffitts & Long, 2008), contains the *msbA2* gene under the control of the constitutive P*trp* promoter. *E. coli* DH5α cells carrying the pRF771 and pJG176 vectors were received as a kind gift from Joel Griffitts, Brigham Young University, USA. These two plasmids were mobilized into the Rm1021 *msbA2*::pJH104 mutant by triparental mating and transconjugants were isolated using antibiotic selection. The purified transconjugants were then inoculated into alfalfa seedlings alongside the Rm1021 parent and *msbA2*::pJH104 mutant and plant growth and nodule development was assessed after a four week period (Table 6-4 and Fig. 6-13). Plants inoculated with the Rm1021 *msbA2*::pJH104 mutant carrying pJG176 were tall and dark green in colour (Fig. 6-13A and table 6-4) with pink nitrogen fixing nodules, the same characteristics as observed for plants inoculated with the Rm1021 parent strain (Table 6-3). Contrastingly, plants inoculated with the *msbA2*::pJH104 carrying the pRF771 control plasmid only, remained short with light green and yellow leaves and brown and white nodules evident (Fig. 6-13B and table 6-4). It should be noted that on 1 out of the 10 plants inoculated with *msbA2*::pJH104 & pRF771 a pink nodule was observed. This phenotype had also been noted in the independent study (Griffitts & Long, 2008), where it was observed that in 1 out of 10 plants inoculated, the *S. meliloti msbA2* mutant would induce a delayed pink nitrogen fixing nodule. However, these data show that a cloned wild type copy of the *S. meliloti* Rm1021 *msbA2* gene successfully complements the symbiotic defect displayed by the Rm1021 *msbA2*:: pJH104 mutant in this work. Thus, these data further confirm that the *S. meliloti msbA2* gene is essential for a successful symbiosis.

Table 6-3. Effect of pRF771 and pJG176 (*msbA2*$^{+}$) plasmids on the symbiotic efficiency of the *S. meliloti msbA*2::pJH104 mutant.

Bacterial Strain	Symbiosis	Plant height[a] (cm)	Plant colour	Mean no. of pink nodules per plant	Mean no. of white nodules per plant	Mean no. of brown nodules per plant
Rm1021 parent	Yes	12.4 ± 2.1	Dark green	9.3 ± 4.2	0	0
msbA2::pJH104	No	3.5 ± 1.1	Light green/yellow	0	1 ± 1.1	6.6 ± 4.0
msbA2::pJH104 & pRF771	No	3.6 ± 1.0	Light green/yellow	0[#]	1.1 ± 1.1	6.4 ± 2.1
msbA2::pJH104 & pJG176	Yes	11.8 ± 1.9	Dark green	9.5 ± 3.9	0	0

± shows the standard derivation from the mean
[a] 10 plants were analysed per *S. meliloti* strain
[#] with the exception that 1 plant contained 1 pink nodule.

Figure 6-13. Alfalfa seedlings after inoculation with Rm1021 *msbA2*::pJH104 & pJG176 carrying *msbA2* and the pRF771 control vector. Alfalfa seedlings were inoculated with either with Rm1021 *msbA2*::pJH104 & pJG176 carrying *msbA2* (A) or *msbA2*::pJH104 carrying the pRF771 control vector (B). Plant growth was photographed 4 weeks post infection.

6.2.2.3. The *S. meliloti msbA2* mutant induces a plant defence response

It had been determined that nodules induced by the Rm1021 *msbA2*::pJH104 mutant were brown and stumpy in appearance (Beck *et al.*, 2008). Brown nodules have previously been shown to be indicative of a host defence response (Veereshlingam *et al.*, 2004). Light microscopy analysis of Rm1021 *msbA2*::pJH104 mutant nodules had revealed a thickened endodermal layer, relative to nodules induced by the

Rm1021 parent (Beck *et al.*, 2008), which is also indicative of a plant defence response. Additionally TEM analysis of nodule sections induced by the Rm1021 *msbA2*::pJH104 mutant revealed that the mutant was defective in host cell entry, with abortion of the infection occurring whilst the bacteria were still within the infection thread (Beck *et al.*, 2008). Interestingly, a previous study had shown that *S. meliloti* exopolysaccharide mutants which were defective in infection thread development, induced a plant defence response in the host plant nodules (Niehaus *et al.*, 1998). In the study the plant defence response was evident by accumulation of phenolic compounds in the nodule wall and the presence of autofluorescent material (Niehaus *et al.*, 1998).

Thus, to investigate if a plant defence response could be detected in nodules induced in the *S. meliloti msbA2* mutant, alfalfa seedlings were inoculated with the *S. meliloti msbA2* mutant and the Rm1021 parent to serve as a control. After 3 weeks post infection nodules were removed from the plants and finely sliced and fixed. After fixing the nodules, slices were analysed by fluorescence microscopy (Fig 6-14A & C) or under went histochemical staining (Fig. 6-14B &D) for detection of plant polyphenolics. Indeed it could be observed that nodule slices induced by the *S. meliloti msbA2* mutant showed a considerable increased level of auto fluorescence (Fig. 6-14A), relative to the Rm1021 parent strain (Fig. 6-14C). Additionally, following the histochemical staining with potassium permanganate/methylene blue, the nodule slices of the *S. meliloti msbA2* mutant were stained blue (Fig. 6-14B), relative to the unstained nodule slices induced by the Rm1021 parent strain (Fig. 6-14D). Hence these data, combined with TEM analysis showing a thickened endodermal layer in nodules induced by the *S. meliloti msbA2* mutant (Beck *et al.*, 2008), provide evidence that the *S. meliloti msbA2* mutant induces a plant defence response in alfalfa.

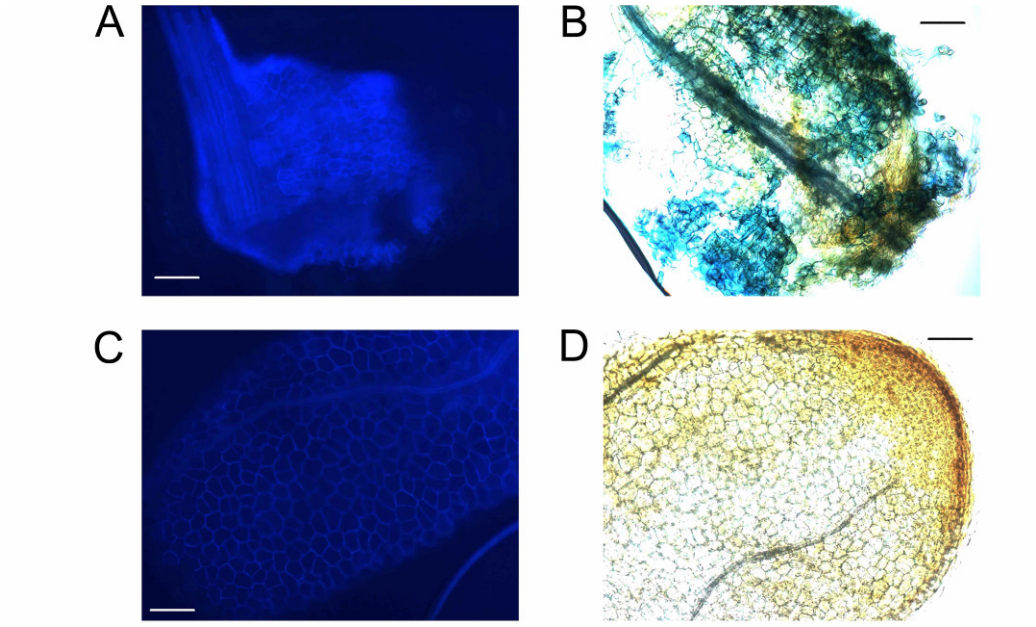


Figure 6-14. Microscopic pictures of thin sections of alfalfa nodules showing an increased plant defence reaction. Nodules were removed 3 weeks post-infection from alfalfa seedlings inoculated with either the *S. meliloti* Rm1021 *msbA*2 mutant (A & B) or the *S. meliloti* Rm1021 parent (C & D). The nodules were thinly sliced and polyphenolic compounds were detected by either auto fluorescence (A & C) or histrochemical staining (B & D). All bars 10 µm. Taken from Beck *et al*, 2008.

6.3. Discussion

The work in this chapter has shown that the presence of the lipid A VLCFA modification is important for the *S. meliloti*-alfalfa symbiosis and that the AcpXL and LpxXL proteins play important but distinct roles in *S. meliloti* bacteroid development. It was also determined that the *S. meliloti* MsbA2 protein is essential for the legume symbiosis. Additionally, in the absence of the MsbA2 protein *S. meliloti* induces a defence response in alfalfa more characteristic of a pathogen, causing browning of plant tissue and an increased production of polyphenolic defence compounds.

6.3.1. Free-living *acpXL* and *lpxXL* mutants display an altered LPS profile, relative to the Rm1021 parent

The free-living *S. meliloti acpXL* and *lpxXL* mutants showed an altered LPS profile by SDS-PAGE, displaying only a lower molecular weight band, relative to the Rm1021 parent, where a higher molecular weight band was also observed. These findings confirm previously published work (Sharypova *et al.*, 2003), where the same LPS profile was observed for an independent *S. meliloti* Rm1021 *acpXL* mutant (L994). Additionally, in the published study two independent methods of LPS isolation were used and complementation analysis confirmed that disruption of the *S. meliloti acpXL* gene was responsible for this altered LPS profile (Sharypova *et al.*, 2003). In this work it was also shown that the free-living *S. meliloti lpxXL* mutant also has an altered LPS profile, relative to the Rm1021 parent strain.

An immediate interpretation of the difference observed could be that the LPS of the *S. meliloti acpXL* and *lpxXL* mutants is missing the O-antigen, since previous work has proposed that the higher molecular weight band corresponds to the lipid A, core and O–antigen and the lower molecular weight band corresponds to rough LPS consisting of only lipid A and core only (Niehaus *et al.*, 1998). However, it was determined that the *S. meliloti acpXL* and *lpxXL* mutants do not have an altered glycosyl composition, relative to the Rm1021 parent strain (G.P. Ferguson and R.W. Carlson, unpublished data), suggesting there are no changes in the O-antigen. Moreover, the published study also found that the *S. meliloti acpXL* mutant (L994) does not have an altered glycosyl composition relative to the parent strain (Sharypova *et al.*, 2003).

Interestingly, the authors of the published study have hypothesized an alternative explanation for the missing higher molecular weight band in the *S. meliloti acpXL* mutant (Sharypova *et al.*, 2003). The authors rationalise that, the higher molecular weight form of the LPS could in fact be an aggregated form of the lower molecular weight LPS, stabilized by the hydrophobic interactions between the VLCFA portion of the lipid A. Subsequently the *S. meliloti acpXL* and *lpxXL* mutants completely lacking the VLCFA modification would express only the faster migrating form of the LPS. This is an interesting hypothesis, which remains a

possibility since the structure of the *S. meliloti* O-antigen is still unknown and to date it has not been demonstrated that the higher molecular weight LPS band contains a unique structural domain not present in the lower molecular weight band (Reuhs *et al.*, 1999).

6.3.2. The *S. meliloti* AcpXL and LpxXL proteins play important but distinct roles in bacteroid development

It was determined that the *S. meliloti* AcpXL and LpxXL proteins play important roles in *S. meliloti* bacteroid development. At 1 week post infection the majority of *S. meliloti acpXL* mutant bacteroids were still found within infection droplets, relative to the Rm1021 parent bacteroids, which had been released into the plant cells. Thus, this would suggest that loss of the AcpXL protein in *S. meliloti* results in delayed infection thread release. These findings are consistent with the delayed nodulation phenotype previously observed for the *S. meliloti acpXL* (L994) mutant (Sharypova *et al.*, 2003). At 4 weeks post infection the defects observed in the *S. meliloti acpXL* mutant were even more pronounced, with disorganised plant cells evident, which have previously been shown to be indicative of nodule senescence (Paau *et al.*, 1980). Moreover the presence of lytic vesicles and multiple bacteroids per symbiosome, in which the bacteroids were no longer tightly associated with the symbiosome compartment, would suggest the *S. meliloti acpXL* mutant bacteroids undergo pre-mature senescence. In nodules induced by the Rm1021 parent strain, these phenotypes were never observed.

Contrastingly, in nodules induced by the *S. meliloti lpxXL* mutant the bacteroids appeared swollen, with major morphological abnormalities throughout the symbiosis, relative to the Rm1021 parent strain. These data would therefore suggest the *S. meliloti* AcpXL and LpxXL proteins both play important but distinct roles in bacteroid development. Interestingly, in a previous TEM study of pea nodules induced by the *R. leguminosarum acpXL* mutant also revealed enlarged, abnormally branched and irregular shaped bacteroids (Vedam *et al.*, 2004). Additionally, in the early stages of infection there were low numbers of bacteria present in the plant cells, (Vedam *et al.*, 2004) and multiple bacteroids per symbiosome observed, relative to the *R. leguminosarum* parent strain. Thus, although the *S. meliloti acpXL*

and *lpxXL* mutants display distinct defects in the symbiosis, both share similarities with defects observed in the *R. leguminosarum acpXL* mutant bacteroids (Vedam *et al.*, 2004).

TEM of nodules induced by the *S. meliloti acpXL/lpxXL* double mutant revealed phenotypes similar to both the *S. meliloti acpXL* and *lpxXL* single mutants. Like the *S. meliloti acpXL* mutant, the *S. meliloti acpXL/lpxXL* double mutant was delayed in infection thread release. Additionally, odd shaped *S. meliloti acpXL/lpxXL* mutant bacteroids were observed, similar to those seen in nodules induced by the *S. meliloti lpxXL* mutant bacteroids. Therefore, since the free-living *S. meliloti acpXL/lpxXL* double mutant has an identical lipid A profile to that of the *S. meliloti lpxXL* single mutant (Ferguson *et al.*, 2005), yet shows bacteroid phenotypes of both the *S. meliloti lpxXL* and *acpXL* single mutants, the free-living lipid A profiles cannot fully account for the symbiotic phenotype of the double mutant. It was previously shown that the *S. meliloti acpXL/lpxXL* double mutant displays an increased sensitivity towards detergents and NaCl in its free-living state, relative to the *S. meliloti lpxXL* single mutant (Ferguson *et al.*, 2005). These findings provide evidence that disruption of *acpXL* also leads to lipid A independent changes in free-living *S. meliloti*. Additionally, in the host loss of the AcpXL protein in *S. meliloti* may result in further changes that are independent of the LpxXL protein which may account for the phenotypic differences between the *acpXL* and *lpxXL* mutant bacteroids.

6.3.3. Preliminary data suggests the putative acyl carrier protein Smb20561 does not appear to compensate for loss of the AcpXL protein

Since it had been shown that despite lacking the VLCFA modification in the free-living state, the lipid A of the *R. leguminosarum acpXL* mutant was partially modified with VLCFA when extracted back from the host (Vedam *et al.*, 2006), it remained a possibility that the *S. meliloti acpXL* mutant may too undergo further host induced lipid A changes. One potential candidate for these host induced lipid A changes was the *smb20651* gene encoding a potential acyl carrier protein which is located in an operon with a putative long-chain fatty acid CoA ligase (Geiger &

Lopez-Lara, 2002).

No differences were detected in the free-living LPS profile of the *S. meliloti smb20651* mutant, relative to the Rm1021 parent. Additionally, *S. meliloti* cells lacking the Smb20651 protein in both the Rm1021 and *acpXL* mutant background were still able to form a successful symbiosis. Therefore, these data suggest that the Smb20651 protein does not play an essential role in the symbiosis. However, the presence of the Smb20651 protein did appear to confer a significant competitive advantage to *S. meliloti.*

Subsequent to completion of this work, a study has been published characterising the *S. meliloti* Smb20651 protein (Ramos-Vega *et al.*, 2009). Findings of the study were able to confirm that the Smb20651 protein is expressed in free-living *S. meliloti* and is likely to be involved in the formation and transfer of a fatty acid. However, both the function and the target of this molecule are currently unknown (Ramos-Vega *et al.*, 2009). The published study also investigated the symbiotic phenotype of *S. meliloti* Δ*smb20651* mutant and confirmed the findings in this work that the Smb20651 protein is not essential for a successful symbiosis. Additionally, the study also investigated free-living phenotypes of the *S. meliloti* Δ*smb20651* mutant and found that loss of the Smb20651 protein does not result in increased sensitivity to cell envelope disrupting agents, relative to the parent (Ramos-Vega *et al.*, 2009). This is consistent with findings in this work showing that loss of the Smb20651 protein in free-living *S. meliloti* does not result in an altered LPS profile. However, a previous study using microarray analysis found that the *S. meliloti* Smb20651 protein is repressed during the symbiosis (Becker *et al.*, 2004), which is surprising, since preliminary data in this chapter suggests presence of the protein confers competitive advantage to *S. meliloti* in the symbiosis. However, there is a second candidate region of the *S. meliloti* genome that could potentially be involved in potential host induced VLCFA modifications. This region is a cluster of four genes (*smc04277, smc04275*, *smc04273* and *smc04270*) located between *acpXL* and *lpxXL*, whose products are proposed to be involved in the incorporation of the VLCFA onto the lipid A (Sharypova *et al.*, 2003). *S. meliloti* mutants have been constructed in these genes and their free-living and symbiotic phenotypes are currently being investigated in the laboratory.

6.3.4. The presence of the VLCFA is important for the legume symbiosis

The precise reason why loss of the *S. meliloti* VLCFA modification affects bacteroid development requires further investigation. However, it has previously been observed that the free-living *S. meliloti acpXL* and *lpxXL* mutants display increased sensitivity to a number of stresses, relative to the parent strain (Ferguson *et al.*, 2005). Since VLCFAs have the potential to span the whole outer membrane (Ferguson *et al.*, 2004), *S. meliloti* cells lacking VLCFAs may have an increased sensitivity to stresses encountered in the nodule environment such as low pH and reactive oxygen species (Hérouart *et al.*, 2002). Interestingly, another *S. meliloti* LPS mutant which displayed irregular shaped, swollen bacteroids during the *M. truncatula* host interaction (Niehaus *et al.*, 1998) was found to induce a plant defence response. Therefore another possibility may be that the altered lipid A in the *S. meliloti lpxXL* mutant could also induce a plant defence response, which may affect bacteroid development.

Work is currently underway in the laboratory to determine more precisely if there are host-induced lipid A changes in the *S. meliloti acpXL* and *lpxXL* mutants. Recent GC-MS analysis of the *S. meliloti lpxXL* mutant bacteroid LPS was unable to detect the presence of any VLCFA (S.Wehimeier and G.P.Ferguson, unpublished data). Thus, these findings suggest that the LpxXL protein plays an essential role in the modification of *S. meliloti* bacteroid LPS with VLCFA. Additionally, the finding that *S. meliloti* mutant bacteroids completely lack the VLCFA, yet can still undergo a successful symbiosis, shows that the VLCFA modification is not essential for the host interaction. However, the very odd shaped bacteroids that were evident in nodules induced by the *S. meliloti lpxXL* mutant suggests the VLCFA do play a role in bacteroid development. These data would suggest that the BacA-mediated lipid A VLCFA modification is unlikely to be solely responsible for the essential role of BacA in the *S. meliloti*-alflalfa symbiosis. In chapter 4, it was found that BacA plays an essential role in the uptake of a truncated eukaryotic peptide, Bac7(1-16) in *S. meliloti*. Since it has been shown that there are hundreds of root nodule-specific cysteine-rich peptides produced by the plant host *M. truncatula* (Mergaert et al., 2003; Mergaert et al., 2006), the BacA mediated uptake of one of these peptides

combined with the BacA-mediated lipid A VLCFA modification may account for the essential role of BacA in the legume symbiosis.

6.3.5. The *S. meliloti msbA1* and *msbA2* mutants do not show increased sensitivity to cell envelope disrupting agents relative to the Rm1021 parent strain

As an initial means to determine if the *S. meliloti* MsbA1 or MsbA2 proteins are involved in the transport of phosphate containing lipids such as LPS across the inner membrane, sensitivity of the *S. meliloti msbA1* and *msbA2* mutants to cell envelope disrupting agents was assessed. However no altered sensitivities were observed in either mutant to DOC, SDS or Crystal Violet, relative to the Rm1021 parent strain. Consistent with these data, it was determined that the *S. meliloti msbA1* and *msbA2* mutants were not affected in the transport of phosphate containing lipids across the inner membrane relative to the Rm1021 parent strain (W.Doerrler and G.P.Ferguson, unpublished data and Beck *et al*., 2008, respectively). These data therefore suggest that neither the *S. meliloti* MsbA1 nor MsbA2 proteins are essential for transport of LPS in *S. meliloti*. However, since the genome of *S. meliloti* encodes other MsbA like proteins (Galibert *et al.*, 2001) it may be possible that one of these proteins could be masking a role for MsbA1 or MsbA2. Previous work has characterised the roles of the MsbA-like proteins ExsA and NdvA in *S. meliloti* (Becker *et al.*, 1995; Dickstein *et al.*, 1988; Stanfield *et al.*, 1988). However, the function of other *S. meliloti* MsbA like proteins still remains to be investigated. The MsbA1 and Y02836 proteins, show the highest degree of similarity to the *E. coli* MsbA protein (Beck *et al.*, 2008). Interestingly, the MsbA1 and Y02836 proteins are 91% similar and 84 % identical to each other (Beck *et al.*, 2008), therefore it remains a possibility that the Y02836 protein is able to compensate for loss of the MsbA1 protein. Work is currently under way in the laboratory on the construction of an *S. meliloti* mutant lacking the Y02836 protein. Moreover, the creation of *S. meliloti* mutants with disruptions in multiple MsbA like genes will allow the individual importance of these gene products to be determined.

6.3.6. The *S. meliloti msbA2* mutant is defective in the legume symbiosis and induces a defence like response in the plant

In the absence of the MsbA2 protein *S. meliloti* was no longer able to form a successful symbiosis with the host. It was determined that the *S. meliloti msbA2* interaction was aborted at the level of the infection threads (Beck *et al.*, 2008). It was evident that loss of the MsbA2 protein resulted in *S. meliloti* inducing a defence response in the plant more characteristic of a pathogen, causing browning of plant tissue and a heightened production of polyphenolic defence compounds. Moreover, light microscopy and TEM analysis had revealed that in nodules induced by the *S. meliloti msbA2* mutant there was a substantial thickening of the plant endodermis, preventing bacterial entry into the plant cell (Beck *et al.*, 2008), also indicative of a plant defence response. Previous studies showing similar plant defence responses, along with aborted infection threads has been observed for *S. meliloti* and *R. leguminosarum* mutants, which are known to have polysaccharide defects (Niehaus *et al.*, 1993; Niehaus *et al.*, 1998; Perotto *et al.*, 1994). Interestingly DOC-PAGE analysis of the free-living *S. meliloti msbA2* mutant revealed the presence of a phenol extractable polysaccharide, not detected in the extracts from the Rm1021 cells (Beck *et al.*, 2008). Thus, it may be possible that this polysaccharide alteration accounts at least partly for the symbiotic defects observed. However, further biochemical studies will be necessary to determine the precise polysaccharide affected in the *S. meliloti msbA2* mutant, since the *msbA2* gene appears to be associated with two glycosyltransferases and an acyltransferase gene (Fig. 6-11), it is possible that these genes form an operon and that the MsbA2 protein may be involved in the transport of novel polysaccharide. Thus, the alteration observed on the DOC-PAGE gel could be due to accumulation of this polysaccharide species. Indeed this would be consistent with findings in the second published study characterising the *S. meliloti msbA2* mutant, since in this study it was determined that deletion of the *msbA2* linked transferase genes (Fig. 6-11) suppressed the symbiotic defect in the *msbA2* mutant (Griffitts & Long, 2008). Moreover, deletion of the entire *msbA2* gene region did not result in a symbiotic defect in *S. meliloti.* Thus, these findings support the hypothesis that the host defect observed in the *S. meliloti* could be due to the cytoplasmic

accumulation of a novel polysaccharide, encoded by upstream genes, which somehow interferes with the legume interaction.

However, it remains to be determined how the altered polysaccharide observed in the *S. meliloti msbA2* mutant may account for the symbiotic defect. Previous work has suggested that *S. meliloti* polysaccharides are essential to suppress the host defence response and enable proper infection thread development (Niehaus *et al.*, 1993; Niehaus *et al.*, 1998; Perotto *et al.*, 1994). Thus, it is interesting to speculate that the altered polysaccharides in the *S. meliloti msbA2* mutant may be less effective at suppressing the plant defence response and that the plant would perceive this mutant as a pathogen, rather than a symbiont.

6.3.7. Future studies

Although work in this chapter would suggest that the VLCFA modification is not essential for the *S. meliloti* legume symbiosis, it has been demonstrated that *S. meliloti* lacking these VLCFA modifications display significant defects in the host. In addition to *Brucella* species, lipid A VLCFA modifications are also present on several facultative intracellular pathogens that form chronic infections, e.g. *Bartonella henselae*, and *Legionella pneumophila* (Bhat *et al.*, 1991). Thus, learning more about the biosynthesis of VLCFA and their role in the chronic infection process will be very beneficial. The complete genome sequences of *B. melitensis* and *B. suis* (DelVecchio *et al.*, 2002; Paulsen *et al.*, 2002) have revealed that both bacteria possess sequences highly similar to the *S. meliloti acpXL* and *lpxXL* genes (Ferguson *et al.*, 2005). Indeed, the construction of *Brucella* species *acpXL* and *lpxXL* mutants could hold potential as vaccine candidates if they exhibit the correct balance between attenuation and immunogenicity.

Interestingly polysaccharides are also thought to play a role in the interaction of *Brucella* species with their host, for instance, cyclic glucans have been proposed to be essential for the intracellular trafficking of *Brucella* species within their host (Arellano-Reynoso *et al.*, 2005). Thus, future investigations into the role of MsbA like proteins in *Brucella* species will be very informative. Future studies investigating the potential role of the uncharacterised MsbA like proteins in lipid trafficking in *S. meliloti* may ultimately enable more to be learnt regarding the role of

the BacA protein in the lipid A VLCFA modification and where precisely in the *S. meliloti* cell envelope this modification occurs.

Chapter 7: Investigation into Other Lipid A Independent Changes Occurring in the *S. meliloti bacA* Mutant

7.1. Introduction

The *S. meliloti* and *B. abortus bacA* mutants display a wide range of phenotypes in their free-living forms (Ferguson *et al.*, 2002; Roop *et al.*, 2002) (Fig. 7-1), relative to their respective parent strains. It has been shown that the increased resistance of the *S. meliloti* Δ*bacA* mutant to the glycopeptide bleomycin is independent of the altered lipid A (Ferguson *et al.*, 2006). However, the remaining free-living phenotypes have not been further investigated. It has been hypothesized that the increased sensitivity of the *S. meliloti* Δ*bacA* mutant to cell envelope disrupting agents is as a result of the VLCFA alteration. However, sensitivity of the *S. meliloti* Δ*bacA* mutant to various agents such as deoxycholate (DOC), sodium dodecyl sulphate (SDS) and crystal violet has never been demonstrated to be solely due to the VLCFA modification. Hence, it may be possible that disruption of *bacA* in *S. meliloti* results in other cellular alterations, independent of the VLCFA alteration, which may contribute to these free-living phenotypes. Additionally, it was determined in chapter 3 that the *S. meliloti* Δ*bacA* mutant displays an increased sensitivity to the DNA damaging agent mitomycin C, relative to the Rm1021 parent strain.

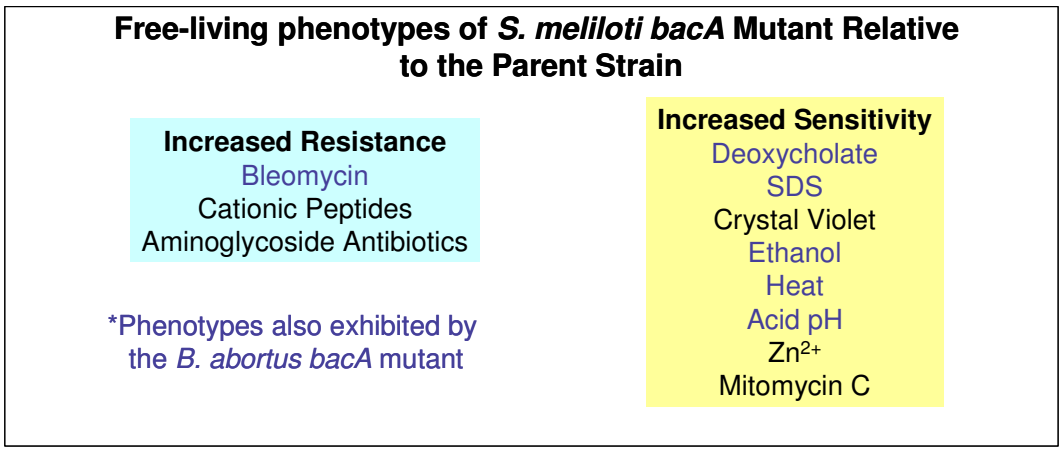


Figure 7-1. The free-living phenotypes of the *S. meliloti* and B. *abortus bacA* mutants, relative to the Rm1021 parent strain (Ferguson *et al.*, 2002; Roop *et al.*, 2002).

Interestingly, it has been determined that the BacA homologue SbmA in *Salmonella enterica* serovar Typhimurium is encoded in an operon with *yaiW* (Fig. 7-2A), thought to encode a lipoprotein (K. Tan and G.P. Ferguson, unpublished data).

Hence it remains a possibility that in addition to affecting the lipid A BacA may also affect the lipid modification of another cell envelope component(s). Although a *yaiW* homologue has not been found in *S. meliloti* and *bacA* is not part of an operon (Fig. 7-2B), the genome of *S. meliloti* encodes multiple putative outer membrane lipoproteins (Galibert *et al.*, 2001), which could potentially be affected by the action of the BacA protein.

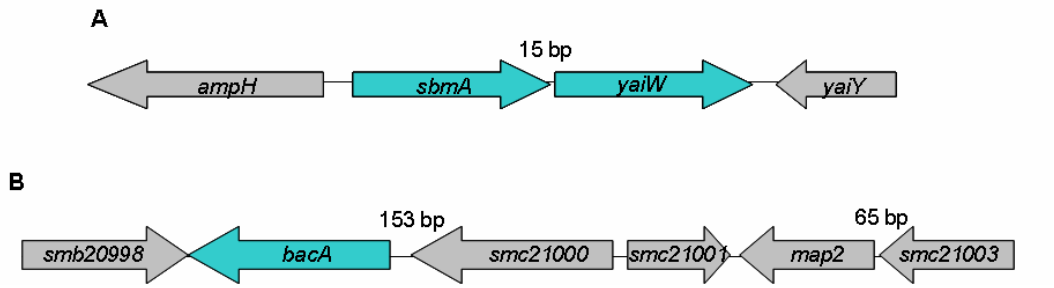


Figure 7-2. Diagrammatic representation of the genomic region surrounding the *S.* Typhimurium *sbmA* gene and the *S. meliloti bacA* gene. (A) *sbmA* is in a two gene operon with the lipoprotein *yaiW* (K.Tan and G.P.Ferguson, unpublished data). The surrounding genes include *ampH* (encoding a penicillin binding protein) and *yaiY* (encoding a putative inner membrane protein) (http://genome.wustl.edu/projects/bacterial /styphimurium/). (B) The *S. meliloti bacA* gene does not appear to be in an operon. Genes surrounding *bacA* on the same strand are *smc21000* (encoding a putative transport protein), *map2* (encoding a putative methionine aminopeptidase) and *smc21003* (encoding a probable oxidoreductase). Genes on the opposite strand include *smb20998* (encoding a hypothetical exported protein) and *smc21001* (encoding a hypothetical protein). (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi).

Hence, it remains a possibility that disruption of *bacA* could be resulting in alterations in *S. meliloti,* independent of the VLCFA lipid A modification, which may also contribute to the host persistence defect. To investigate if any lipid A independent alterations were occurring in *S. meliloti* upon disruption of *bacA,* the first aim of this chapter was to determine if the altered sensitivity of the Rm1021 Δ*bacA* mutant to a selection of the agents shown in Fig 7-1 is dependent or independent of the altered VLCFA. Additionally, to investigate other potential alterations in the free-living *S. meliloti* Δ*bacA* mutant on a genome wide scale, the second aim of this chapter was to identify any genes or proteins that were differentially expressed, relative to the Rm1021 parent strain. This was undertaken

by performing DNA microarrays and two dimensional (2D) gel analyses on the Rm1021 Δ*bacA* mutant, relative to the Rm1021 parent. It may be possible that data obtained from the microarray and 2D gel analysis may help to account for some of the free-living phenotypes of the *S. meliloti bacA* mutant.

7.2. Results

7.2.1. The increased sensitivity to DOC and SDS observed in the *S. meliloti bacA* mutant appears to be due to the VLCFA modification

The free-living Rm1021 Δ*bacA* mutant has been shown to exhibit an increased sensitivity to cell envelope disrupting agents, relative to the parent strain (Ferguson *et al.*, 2002). Thus, to determine if the increased sensitivity to SDS and DOC was exclusively due to the reduction in the VLCFA content, sensitivity of the *S. meliloti acpXL* mutant to these two agents was assessed. The AcpXL protein encodes an acyl carrier protein, essential for the biosynthesis of the lipid A VLCFA in free-living *S. meliloti* (Brozek *et al.,* 1996). Consequently, the *S. meliloti acpXL* mutant completely lacks the VLCFA modification (Ferguson *et al.*, 2005). It could be observed that like the Rm1021 Δ*bacA* mutant, the Rm1021 *acpXL* mutant also displayed an increased sensitivity to SDS and DOC (Fig. 7-3A &C, respectively), relative to the Rm1021 parent strain. Therefore these data would suggest the reduction in the lipid A VLCFA content in the Rm1021 Δ*bacA* mutant most probably accounts for the increased resistance to SDS and DOC. Additionally, it was observed that the Rm1021 *acpXL* mutant was found to be significantly more sensitive to DOC than the Rm1021 Δ*bacA* mutant (Fig. 7-3C), suggesting that complete loss of the VLCFA modification leads to a greater sensitivity to DOC.

To provide further evidence that the SDS and DOC sensitivity phenotype of the Rm1021 Δ*bacA* mutant is due to the VLCFA alteration, the sensitivity of the Rm1021 *acpXL* mutant and the Rm1021 Δ*bacA/acpXL* double mutants were compared (Fig. 7-3B & D). Previous work has shown that the Rm1021 Δ*bacA/acpXL* double mutant has an identical lipid A profile to the Rm1021 *acpXL* single mutant (Ferguson *et al.*, 2005). Thus, since the VLCFA modification appears to be responsible for these phenotypes one would expect the two mutants to display

the same level of sensitivity to SDS and DOC. It was observed that deletion of *bacA* in the Rm1021 *acpXL* background completely lacking the VLCFA modification resulted in a slight increase in sensitivity to DOC and SDS (Fig. 7-3B & D). Thus, the VLCFA alteration in the Rm1021 Δ*bacA* mutant is likely to account the majority of the SDS and DOC sensitivity phenotype. However, the slight increase observed in the Rm1021 Δ*bacA/acpXL* mutant, relative to the Rm1021 *acpXL* mutant single mutant may suggest disruption of *bacA* is resulting in an additional alteration to the cell which may play a very minor role in the sensitivity to SDS and DOC.

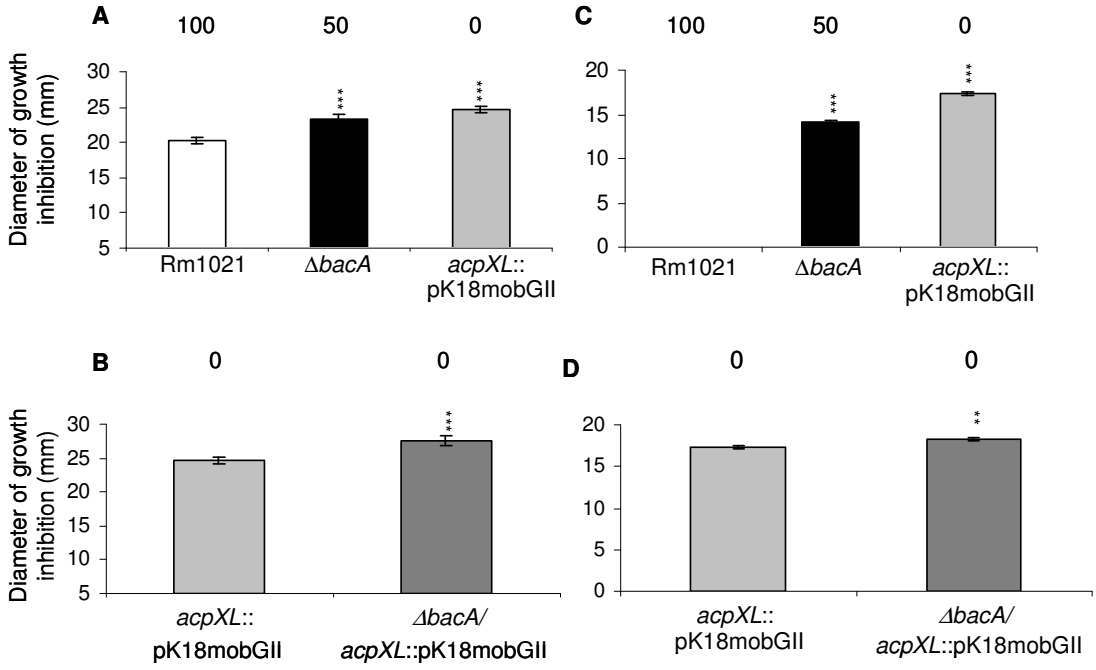


Figure 7-3. Sensitivity of the *S. meliloti acpXL* mutant to the cell envelope disrupting agents SDS and DOC, in the presence and absence of the *bacA* gene. The defined *S. meliloti* strains were exposed to 5 µl of 10% (w/v) SDS by filter disc assay (A & B) or 0-24 mM of DOC, (C & D) using a gradient assay. In A & C the significant values shown (***P< 0.001), represent comparisons of the Δ*bacA* mutant and *acpXL*::pK18mobGII mutant to the Rm1021 parent strain. In B & D the significant values (***P<0.001) and (**P<0.01) shown represent comparisons of the Δ*bacA/acpXL*::pK18mobGII mutant to that of the *acpXL*::pK18mobGII mutant. For each dataset the numbers above the bars indicate the percentage of the lipid A modified with VLCFAs in each strain (Ferguson *et al*., 2005). Each dataset shown is representative of the trends observed in two independent experiments and in each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

7.2.2. The increased sensitivity to Crystal Violet seen in the absence of the BacA protein is independent of the VLCFA modification

The toxicity of the hydrophobic dye Crystal Violet is thought to be due to its ability to enter bacterial cells, where it binds ribosomes which then function less efficiently in protein synthesis. The dye has therefore been used as an indicator of alterations in the cell envelope (Gustafsson *et al.*, 1973). Previous work has shown that the Rm1021 Δ*bacA* mutant has an increased sensitivity to Crystal Violet, relative to the Rm1021 parent strain (Ferguson *et al.*, 2002). Thus, in this case too, comparison of the Crystal Violet sensitivity of the Rm1021 *acpXL* mutant with that of the Rm1021 Δ*bacA* mutant provided a means to investigate if the increased sensitivity to crystal violet is as a result of the reduction of the VLCFA lipid A content. However, in contrast to the Rm1021 Δ*bacA* mutant, which displays an increased sensitivity to crystal violet, no significant increase in sensitivity to Crystal Violet was observed in the Rm1021 *acpXL* mutant (Fig. 7-4A), relative to the Rm1021 parent strain. These data would therefore suggest that the reduction in the VLCFA content does not lead to an increased sensitivity to Crystal Violet. To further confirm that the increased sensitivity of the Rm1021 Δ*bacA* mutant to Crystal Violet occurs independently of the VLCFA modification, the Crystal Violet sensitivity of the Rm1021 Δ*bacA/acpXL* double mutant was compared to that of the Rm1021 *acpXL* single mutant (Fig.7-4B). Despite the Rm1021 *acpXL* and Rm1021 Δ*bacA /acpXL* mutant having identical lipid A profiles, disruption of the *bacA* gene increases the sensitivity of the Rm1021 *acpXL* mutant to Crystal Violet. Thus these data suggest that the Crystal Violet phenotype of the *S. meliloti bacA* mutant is independent of the VLCFA modification, suggesting some other component of the cell envelope may be altered, resulting in this increased sensitivity.

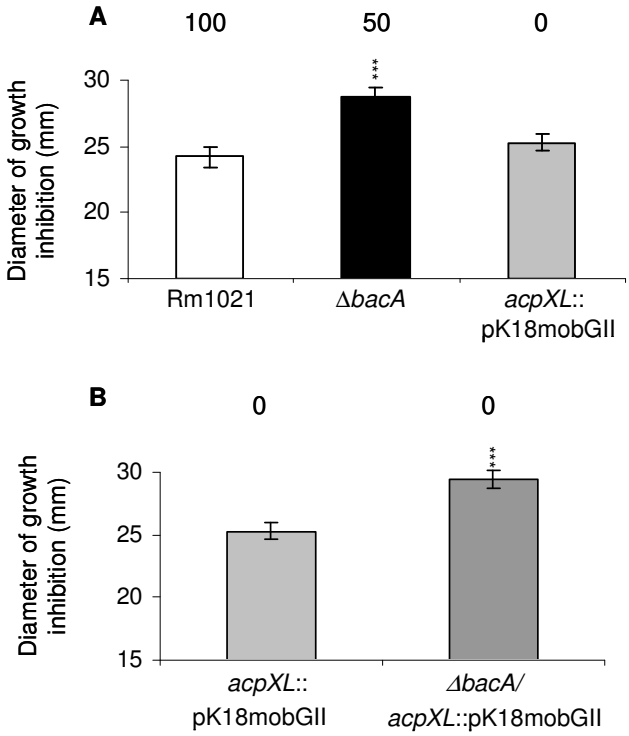


Figure 7-4. Sensitivity of the *S. meliloti acpXL* mutant to the hydrophobic dye Crystal Violet, in the presence and absence of the *bacA* gene. The defined *S. meliloti* strains were exposed to Crystal Violet (5 µl of a 4 mg.ml^{-1} stock) on LB by filter disc assay (A & B). In (A) the significant value (P***<0.001) shown, represents a comparison of the Δ*bacA* mutant to the Rm1021 parent strain. In (B) the significant value (P***<0.001) shown, represents a comparison of the Δ*bacA/acpXL*::pK18mobGII mutant to that of the *acpXL*::pK18mobGII mutant. For each dataset the numbers above the bars indicate the percentage of the lipid A modified with VLCFAs in each strain (Ferguson *et al*., 2005). Each dataset shown is representative of the trends observed in two independent experiments and in each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

7.2.3. The increased sensitivity of the *S. meliloti bacA* mutant to the DNA damaging agent mitomycin C appears to be due to the altered VLCFA

Mitomycin C is an alkylating antibiotic which shows strong activity against bacteria and tumour cells (Hata *et al.*, 1956; Sugiura, 1959; Wakaki, 1958) and has been shown to induce DNA damage in *E. coli* (Otsuji & Murayama, 1972). In chapter 3, whilst investigating the increased resistance of the *S. meliloti bacA* mutant to bleomycin induced DNA damage it was determined that the *S. meliloti* Δ*bacA* mutant

displayed an increased sensitivity to mitomycin C, relative to the parent strain. Thus, it was next investigated if this was as a result of the reduction in the VLCFA lipid A content. It was observed that like the Rm1021 Δ*bacA* mutant the Rm1021 *acpXL* mutant also displays an increased sensitivity to mitomycin C (Fig. 7-5A), suggesting that the increased sensitivity observed is as a result of the altered lipid A. The sensitivity of the Rm1021 *acpXL* mutant was next compared to that of the Rm1021 Δ*bacA/ acpXL* double mutant (Fig. 7-5B), which both have identical lipid A profiles. In this case loss of *bacA* in the Rm1021 *acpXL* mutant background appeared to result in a slightly increased level of sensitivity, suggesting that disruption of *bacA* may result in an additional cellular alteration, which also contributes to the increased sensitivity.

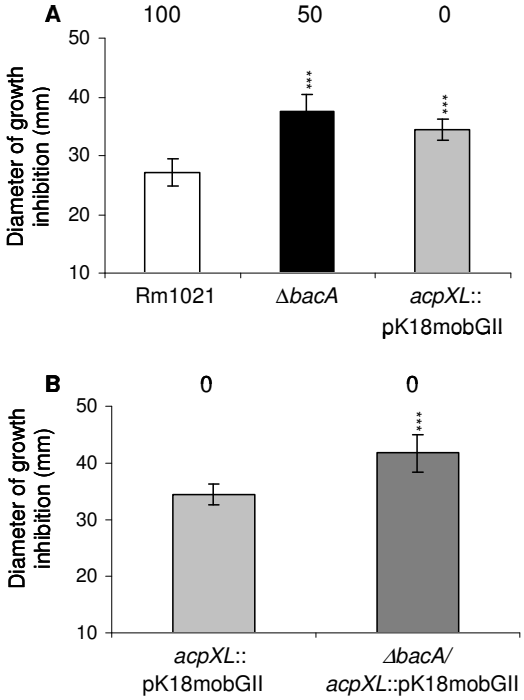


Figure 7-5. Sensitivity of the *S. meliloti acpXL* mutant to the DNA damaging agent mitomycin C, in the presence and absence of the *bacA* gene. The defined *S. meliloti* strains were exposed to mitomycin C (5 µl of a 0.3 mg.ml^{-1} aqueous stock solution) by filter disc assay (A & B). In (A) the significant value (***P <0.001) shown, represents a comparison of the Δ*bacA* mutant to the Rm1021 parent strain. In (B) the significant value (***<P 0.001) shown, represents a comparison of the Δ*bacA/acpXL*::pK18mobGII mutant to that of the *acpXL*::pK18mobGII mutant. For each dataset the numbers above the bars indicate the percentage of the lipid A modified with VLCFAs in each strain (Ferguson *et al.*, 2005). Each dataset shown is representative of the trends observed in two independent experiments and in each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

7.2.4. The increased resistance of the *S. meliloti bacA* mutant to gentamycin is only partially due to the VLCFA alteration

Previous work has shown that disruption of *bacA* in *S. meliloti* resulted in an increased resistance to the aminoglycoside antibiotics gentamycin, netilmicin and tobramycin. However, this study was performed using the *S. meliloti* Rm8002 strain background, in which the first *bacA* mutant was isolated (Glazebrook *et al.*, 1993; Ichige & Walker, 1997; LeVier & Walker, 2001; Long *et al.*, 1988). Thus, it was initially important to determine if the same phenotype was observed upon disruption of *bacA* in the Rm1021 strain background used throughout this work. To determine this, the sensitivity to the aminoglycoside antibiotic gentamycin was investigated. It was observed that the Rm1021 Δ*bacA* mutant did display an increased resistance to gentamycin, relative to the parent strain (Fig. 7-6A), consistent with the findings in the Rm8002 background (Ichige & Walker, 1997). In several bacteria, mutations that affect the integrity of the cell envelope have been shown to result in an increased resistance to aminoglycoside antibiotics (Taber *et al.*, 1987). Therefore, it was possible the increased resistance observed in the Rm1021 Δ*bacA* mutant was a result of the altered VLCFA. To investigate this, the sensitivity of the Rm1021 *acpXL* mutant was next assessed. It was determined that the Rm1021 *acpXL* mutant did display a slightly increased resistance to gentamycin (Fig. 7-6A), however the resistance phenotype was not as great as that displayed by the Rm1021 Δ*bacA* mutant. Since the Rm1021 *acpXL* mutant has a complete loss of the VLCFA modification (Ferguson *et al.*, 2005), relative to the 50% reduction seen in the Rm1021 Δ*bacA* mutant (Ferguson *et al.*, 2004), this would suggest that loss of *bacA* may be resulting in another cellular alteration that results in an increased resistance to aminoglycoside antibiotics. To confirm this further the sensitivity of the Rm1021 *acpXL* and Rm1021 Δ*bacA/acpXL* to gentamycin was next determined. It could be shown that despite having identical lipid A profiles disruption of *bacA* in the Rm1021 *acpXL* mutant background, did result in an increased level of resistance to gentamycin (Fig. 7-6B). Therefore these data would confirm that the VLCFA alteration only partially accounts for the increased resistance to gentamycin making it possible that loss of the BacA protein is having another affect on the cell that contributes to this phenotype.

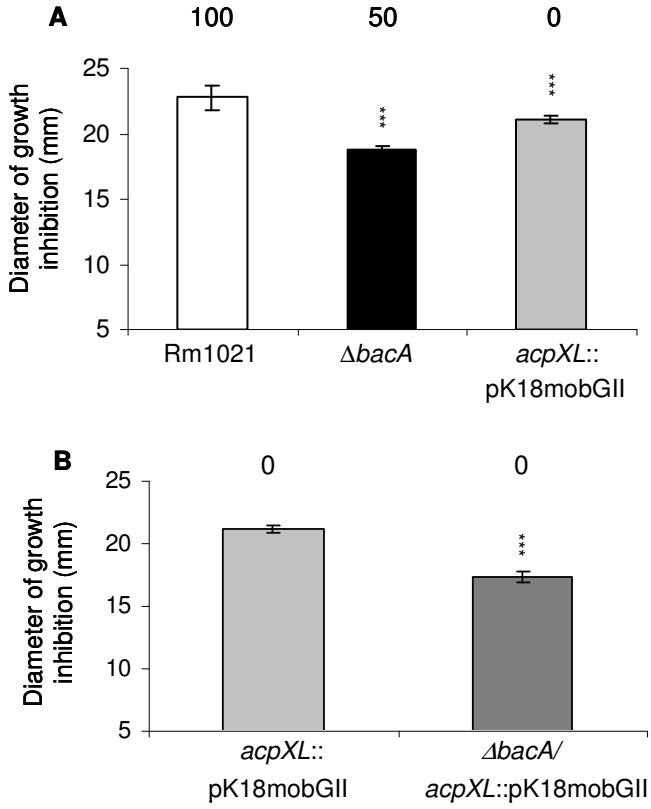


Figure 7-6. Sensitivity of the *S. meliloti acpXL* mutant to the aminoglycoside antibiotic gentamycin, in the presence and absence of the *bacA* gene. The defined *S. meliloti* strains were exposed to gentamycin (5 µl of a 5 mg.ml^{-1} aqueous stock solution) by filter disc assay (A & B). In (A) the significant value (P***<0.001) shown, represents a comparison of the Δ*bacA* mutant to the Rm1021 parent strain. In (B) the significant value (P***<0.001) shown, represents a comparison of the Δ*bacA/acpXL*::pK18mobGII mutant to that of the *acpXL*::pK18mobGII mutant. For each dataset the numbers above the bars indicate the percentage of the lipid A modified with VLCFAs in each strain (Ferguson *et al.*, 2005). The dataset shows preliminary data and in each case the error bars represent the standard deviation from the mean (n=3) for one experiment.

7.2.5. Only a few out of some ~6000 genes in the *S. meliloti* genome showed altered expression in the Δ*bacA* mutant, relative to the Rm1021 parent strain

To determine if there was altered expression of any *S. meliloti* genes upon disruption of the *bacA* gene whole genome microarray analysis was performed. This was done in collaboration with Dr Anke Becker at the University of Bielefeld, where all work was performed. In brief, *S. meliloti* Rm1021 parent and Δ*bacA* cells were grown to

mid-exponential phase (OD_{600} ~0.8) in LB media, harvested, RNA isolated, fluorescently labelled cDNA prepared and then microarray hybrization and data analysis was performed. Out of some ~6000 genes in the *S. meliloti* genome disruption of *bacA* resulted in the altered expression of just 28 of these genes, with 21 genes showing increased expression and 7 genes showing decreased expression (tables 7-1 and 7-2, respectively).

Interestingly, several of the genes with altered expression had related functions. The up-regulated genes (table 7-1) included 4 genes encoding proteins involved in respiration/metabolic processes, 3 genes annotated as encoding transporter proteins, 1 gene encoding a pilus assembly protein, 1 gene annotated as encoding a regulatory protein and a gene encoding a symbiotically induced protein. Additionally, 10 genes encoding hypothetical proteins were up-regulated. BLAST searches were performed (http://www.ncbi.nlm.nih.gov/sutils/genom_table .cgi) using the protein sequences of the 10 hypothetical proteins, against all known microbial protein databases. It was determined that 4 of the hypothetical proteins have a high percentage of similarity and identity to bacterial proteins with known functions (table 7-3).

The down-regulated genes (table 7-2) consisted of 3 genes involved in respiration/metabolic processes, 3 genes which are part of a fructose uptake operon and 1 hypothetical protein.

7.2.5.1. Genes involved in metabolism and respiration are both up and down regulated in the *S. meliloti* Δ*bacA* mutant, relative to the Rm1021 parent strain

Upon disruption of *bacA* there was up-regulation of genes encoding a probable oxidoreductase (*smb20103*), two dehydrogenases (*smb1483* and *sma0335*) and an adenlyate cyclase (*sma0464/cyaI2*), along with the down regulation of genes encoding a quinol oxidase (*smc02255/qxtA*) and two dehydrogenases (*smc02689* and *sma1296/adhA1*). The altered expression of these genes would suggest there are some changes, albeit small occurring in respiratory pathways and metabolism, upon disruption of the *bacA* protein.

7.2.5.2. Genes encoding ABC transport proteins are both up and down regulated in the *S. meliloti* Δ*bacA* mutant, relative to the Rm1021 parent strain

Three genes, each proposed to be part of ABC transport systems were observed to be up-regulated in *S. meliloti* upon disruption of *bacA*. These genes encode a putative sugar transporter (*smb20672*), a periplasmic solute binding protein forming part of a putative myo-inositol ABC transporter (*smb20712/ibpA*) and a periplasmic nitrate binding protein (*sma0585/nrtA*).

Additionally, there was down-regulation of 3 genes (*smc02173/frcS*, *smc02167/frcK* and *smc02171/frcB*) thought to be part of putative fructose ABC-type transport system (Fig. 7-7). This transport system has been characterized in *S. meliloti* (Lambert *et al.*, 2001) and consists of six genes, identified as forming two putative transcriptional units, composed respectively of two (*frcRS*) and four (*frcBCAK*) genes transcribed divergently. The genes have been shown to be induced by mannitol and fructose and repressed by succinate (Lambert *et al.*, 2001). The *frcB* and *frcK* genes are proposed to be a periplasmic component and transport system kinase respectively (table 7-2 and fig 7-7). However, the function of the *frcS* encoded protein remains unknown. Its amino acid sequence shows 37% identity to the *E. coli* protein FucU, involved in metabolism of fructose and 23% identity to the *E. coli* RbsD protein, part of the D-ribose high-affinity transport system (Bell *et al.*, 1986; Lambert *et al.*, 2001; Lu & Lin, 1989).

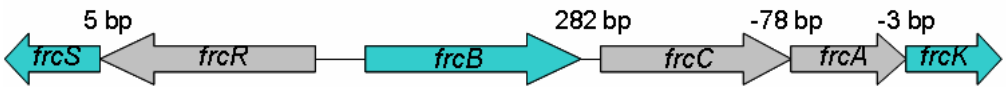


Figure 7-7. Diagrammatic representation of the *S. meliloti* fructose binding ABC transporter operon. The genes highlighted in blue are those shown to be down regulated in the Rm1021 Δ*bacA* mutant, relative to the Rm1021 parent. The genes are *frcS* (encoding a conserved hypothetical protein), *frcR* (encoding a transcriptional regulator), *frcB* (encoding periplasmic component), *frcC* (encoding periplasmic component), *frcA* (encoding ATPase component) and *frcK* (encoding a transport system kinase).

Table 7-1. *S. meliloti* genes showing increased expression in the Rm1021 Δ*bacA* mutant, compared to the Rm1021 parent

Gene[a]	Fold change in expression (+)	M value[b]	Function/Annotation[c]
smb20103	3.4	1.70	**Probable FAD-dependent oxidoreductase**
sma0585[#] **(*nrtA*)**	2.82	1.41	**Nitrate transporter, periplasmic nitrate binding protein**
smb20672[#]	2.56	1.28	**Putative sugar ABC transporter**
sma1568 **(*cpaF2*)**	2.44	1.22	**CpaF2 pilus assembly protein**
sma0310	2.36	1.18	***lysR* transcriptional regulator**
smb20451[#]	2.34	1.17	Conserved hypothetical protein
smc01774	2.28	1.14	Hypothetical protein signal peptide
sma1093	2.26	1.13	Hypothetical protein
sma0464 **(*cyaI2*)**	**2.24**	**1.12**	**Adenylate/guanylate cyclase**
smb20712 **(*ibpA*)**	**2.18**	**1.09**	**Putative myo-inositol ABC transporter, periplasmic solute-binding protein**
sma1077[#] **(*nex18*)**	**2.16**	**1.08**	**Nex18 symbiotically induced conserved protein**
smb21123	**2.14**	**1.07**	**Hypothetical membrane-anchored protein**
smb20133[#]	**2.08**	**1.04**	**Conserved hypothetical protein**
smb1483	**2.08**	**1.04**	**Dehydrogenase**
sma2061[#]	**2.08**	**1.04**	**Conserved hypothetical protein**
smc01788	2.06	1.03	Hypothetical protein
sma0335	**2.04**	**1.02**	**Short chain alcohol dehydrogenase-related dehydrogenase**
smb20521	**2.04**	**1.02**	**Conserved hypothetical protein**
smb21483	1.94	0.97	Hypothetical protein
smb20359	1.97	0.97	Hypothetical protein
smc00252	1.92	0.96	Conserved hypothetical protein, signal peptide

[a] sma, smb, and smc indicate location of genes on *S. meliloti* megaplasmids pSymA, pSymB and the chromosome, respectively

[b] Genes displaying M-values (log2 ratio) ≥0.9 were regarded as induced

[c] Gene function/annotations are taken from http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/

[#] Gene thought to be part of operon.

Bold text highlights genes encoding proteins with known function/annotation or genes encoding hypothetical proteins with high similarity/identity to bacterial proteins of postulated function (shown in table 7.3)

Table 7-2. *S. meliloti* genes showing decreased expression in the Rm1021 Δ*bacA* mutant, compared to the Rm1021 parent

Gene[a]	Fold change in expression (-)	M value[b]	Function/Annotation[c]
***smc02255*[#] (*qxtA*)**	1.96	-0.98	**Putative quinol oxidase subunit I transmembrane protein**
***smc02173*[#] (*frcS*)**	1.98	-0.99	**Conserved hypothetical protein (*frcS*)**
smc01711	2.12	-1.06	Hypothetical protein
***smc02167*[#] (*frcK*)**	2.24	-1.12	**Fructose transport system kinase**
***smc02171*[#] (*frcB*)**	2.44	-1.22	**Fructose ABC-type transport system, periplasmic component**
smc02689	3.14	-1.57	**Probable aldehyde dehydrogenase**
***sma1296* (*adhA1*)**	3.96	-1.98	**Alcohol dehydrogenase, Zn-dependent class III**

[a] sma, smb, and smc indicate location of genes on *S. meliloti* megaplasmids pSymA, pSymB and the chromosome, respectively
[b] Genes displaying M-values (log2 ratio) ≥0.9 were regarded as induced
[c] Gene function/annotations are taken from http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/
[#] Gene thought to be part of operon
Bold text highlights genes encoding proteins with known function/annotation or genes encoding hypothetical proteins with high similarity/identity to bacterial proteins of postulated function (shown in table 7.3)

Table 7-3. Up-regulated genes in the *S. meliloti* Δ*bacA* mutant encoding hypothetical proteins showing significant identity and similarity to bacterial proteins of postulated function.

Gene showing increased expression	% identity and similarity of the *S. meliloti* proteins to bacterial proteins of postulated function[a]
smb21123 (+ 2.14)	90% identity and 95% similarity to endoribonuclease L-PSP from *Sinorhizobium medicae* WSM419
smb20133 (+ 2.08)	93% identity and 97% similarity to cobalamin biosynthesis protein (CobW) from *S. medicae* WSM419
sma2061 (+ 2.08)	82% identity and 91% similarity to non-specific serine/threonine protein kinase from *Rhizobium leguminosarum* bv. *trifolii* WSM1325
smb20521 (+ 2.04)	57 % identity and 72 % similarity to multimeric flavodoxin WrbA from *Ralstonia eutropha* H16

[a] shows the bacterial protein with the highest percentage of similarity and identity to the *S. meliloti* hypothetical protein

7.2.6. Two dimensional gel analysis of free-living Rm1021 parent and the Δ*bacA* mutant

Preliminary 2D gel analysis was performed on cellular proteins extracted from free-living Rm1021 parent and Δ*bacA* mutant cells. This analysis was performed by the University of Aberdeen proteomics facility (http://www.abdn.ac.uk/ims /proteomics/ services.shtml). Prior to analysis the cells were grown to late exponential phase (OD_{600} ~3.00), harvested and the cell pellets treated with lysis buffer, after which the cell supernatants were collected and frozen at -20 ºC, until use by the proteomics facility. Small format 2D gel electrophoresis was performed and the proteins were visualized using commassie blue straining (Fig. 7-8).

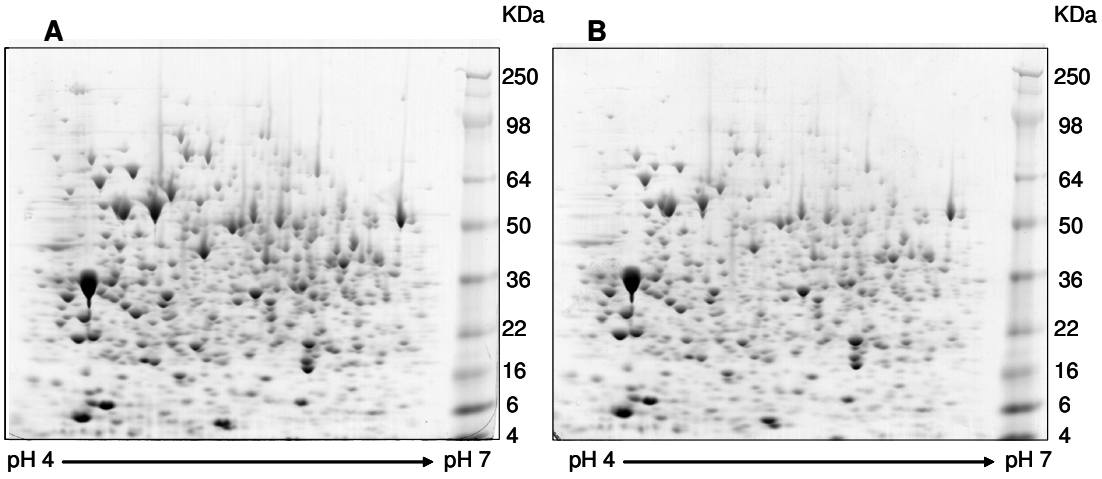


Figure 7-8. 2D gels showing cellular proteins from the *S. meliloti* Rm1021 parent (A) and the Rm1021 Δ*bacA* mutant (B) in the pH range 4-7. Proteins were visualised by coomassie blue. Work performed by the University of Aberdeen proteomics facility.

Although no major changes could be detected by eye, these 2D-gels next require analysis by computer software e.g. Progenesis software which can detect and quantify any changes in the protein spots with high sensitivity.

7.3. Discussion

Work in this chapter determined that not all the free-living phenotypes displayed by the *S. meliloti* Δ*bacA* mutant can be accounted for by the VLCFA modification, thus suggesting that disruption of *bacA* may result in other cellular changes in *S. meliloti*.

Preliminary microarray analysis revealed a small number of genes are up and down-regulated upon disruption of *bacA*. However, further work is required to confirm these changes are real.

7.3.1. The increased sensitivity of the *S. meliloti* Δ*bacA* mutant to detergents and the DNA damaging agent mitomycin C appears to be due to the altered VLCFA

An altered sensitivity to detergents in bacterial cells is most often an indication of an alteration in the cell envelope, in particular bacterial mutants with defects in their LPS display increased sensitivity to DOC (Campbell *et al.*, 2002; Lagares *et al.*, 1992). Thus, it was not surprising that stress assays revealed the VLCFA modification accounted for the majority of the sensitivity displayed in the *S. meliloti* Δ*bacA* mutant to SDS and DOC. However, it is interesting to note that disruption of *bacA* in the *S. meliloti acpXL* mutant background, which completely lacks the VLCFA modification, did slightly increase sensitivity of the cells to DOC and SDS. Thus, this may be consistent with an additional cellular alteration in the *S. meliloti* Δ*bacA* mutant, which plays a minor role in sensitivity to detergents.

The increased sensitivity of the *S. meliloti ΔbacA* mutant to the DNA damaging agent mitomycin C also appeared to be largely due to the VLCFA alteration, although as for the detergent phenotype, disruption of *bacA* did increase the sensitivity of the Rm1021 *acpXL* mutant slightly. Work in chapter 3 determined that the *S. meliloti* Δ*bacA* mutant did not display an increased sensitivity to the other DNA damaging agents tested, methylglyoxal and methyl methanesulfonate, thus suggesting the increased sensitivity of the *S. meliloti* Δ*bacA* mutant to mitomycin C is unlikely to be due to an alteration in DNA or a DNA repair process. To the best of my knowledge there appears to be no literature detailing how mitomycin C enters bacterial cells. In the current literature the majority of bacterial mutants which have been isolated showing increased sensitivity to mitomycin C are those defective in DNA repair proteins such as *E. coli recA* and *uvrB* mutants (Lusetti *et al.*, 2003; Vidal *et al.*, 2006). However, one study details two *E. coli* mutants which have an altered LPS and display an increased sensitivity to mitomycin C (Coleman & Leive,

1979). Thus, this is consistent with the findings in this work that the altered LPS VLCFA results in an increased sensitivity to mitomycin C.

7.3.2. The increased sensitivity of the *S. meliloti* Δ*bacA* mutant to Crystal Violet is independent of the VLCFA modification

The increased sensitivity of the *S. meliloti* Δ*bacA* mutant to the hydrophobic dye Crystal Violet appears to be independent of the altered VLCFA modification, thus suggesting that there may be other alterations in the *S. meliloti* Δ*bacA* mutant that account for this phenotype.

The toxicity of the Crystal Violet is thought to be due to its ability to enter bacterial cells, where it binds ribosomes which then function less efficiently in protein synthesis (Gustafsson *et al.*, 1973). Previous work has shown that in *E. coli*, alterations in the LPS, particularly the carbohydrate content, and alterations in the peptidoglycan lead to an increased uptake of Crystal Violet (Gustafsson *et al.*, 1973). Since it has previously been shown that the *S. meliloti* Δ*bacA* mutant does not have a dramatic alteration in the carbohydrate composition of its LPS (Ferguson *et al.*, 2002), it may be possible that the *S. meliloti* Δ*bacA* mutant has alterations in the peptidoglycan component of the cell envelope. Consistent with this, *E. coli* treated with agents affecting the cell wall e.g. lysozyme, display an increased uptake of crystal violet (Gustafsson *et al.*, 1973). Indeed, past research has shown that the peptidoglycan layer, which is the shape determining component of the bacterial cell, is also involved in the barrier function of gram-negative bacteria (Burman *et al.*, 1972).

7.3.3 The increased resistance of the *S. meliloti* Δ*bacA* mutant to the aminoglycoside antibiotic gentamycin is only partly due to the VLCFA modification

The bactericidal action of aminoglycoside antibiotics is known to principally occur by inhibition of protein synthesis, the antibiotic binds to the 30S ribosomal subunit, whereby the ribosome becomes unavailable for translation, resulting in cell death

(Kotra *et al.*, 2000). Upon entry into cells, aminoglycosides first bind to the negatively charged outer membrane of gram negative bacteria by electrostatic interactions, before diffusing though the outer membrane channels and entering the periplasmic space (Hancock *et al.*, 1991). However, transport across the inner membrane requires metabolic energy from electron transport (Hancock *et al.*, 1991). Accordingly, cell envelope alterations and defects in electron transport have both been shown to reduce aminoglycoside uptake in bacteria (Taber *et al.*, 1987). Thus, it is likely the altered VLCFA modification in the *S. meliloti* Δ*bacA* mutant effects the movement of gentamycin through the outer membrane, largely accounting for increased resistance in the mutant relative to the Rm1021 parent strain. However, disruption of *bacA* appears to result in an additional increased resistance to gentamycin, maybe caused by a lipid A independent alteration, perhaps in the periplasmic space or inner membrane.

7.3.4. Microarray analysis revealed a small number of genes showed altered expression in the free-living *S. meliloti* Δ*bacA* mutant, relative to the Rm1021 parent

It should be noted that the microarray data presented in this chapter is only preliminary data, since none of these changes have yet been confirmed by quantitative reverse-transcriptase-PCR (qRT-PCR). Additionally, it should be noted that several of the genes with altered expression are thought to be part of operons (as indicated in tables 7-1 and 7-2), yet in each case (with the exception of the fructose ABC-type transport system), no other genes in these potential operons were identified as been altered in expression. Only a small number of genes were altered in their expression in free-living *S. meliloti* upon disruption of the *bacA* gene. Several of the genes altered in their expression had related functions e.g. respiration/metabolism and transport systems. It was interesting to note both the up and down-regulation of ABC transport systems in the *S. meliloti* Δ*bacA* mutant, relative to the Rm1021 parent. Since one proposed function of the BacA protein is peptide uptake in the host, the up-regulation of ABC transport systems under free-living conditions in the *S. meliloti bacA* mutant may suggest a role for BacA in the

uptake of some metabolite important for free-living growth. Up-regulation of the putative sugar transporter (*smb20672*) would be consistent with this hypothesis. For instance if BacA were involved in the uptake of certain amino acids, utilized by the cell as a carbon source, then upon loss of BacA it would make sense to induce transport systems involved in uptake of an alternative carbon source i.e. sugar uptake. The second transport gene to be up-regulated was a periplasmic solute binding protein forming part of a putative myo-inositol ABC transporter (*smb20712/ibpA*). Indeed, *S. meliloti* has been shown to utilise myo-inositol as a carbon source (Galbraith *et al.*, 1998). So up-regulation of this gene in the Rm1021 Δ*bacA* mutant is also consistent with BacA being important for uptake of a metabolite during free-living growth which may be an important carbon source. The down-regulation of 3 genes encoding proteins forming part of a fructose ABC-type transport system (Lambert *et al.*, 2001), may have simply been as a consequence of up-regulation of the ABC transport systems, likewise down-regulation of this transport system could have induced their expression. However, this fructose ABC transport system has been characterised (Lambert *et al.*, 2001) and it should be noted it has been found not to be essential for the legume symbiosis.

Very recently, microarray analysis has been performed on the free-living *S. meliloti acpXL* mutant grown under the same conditions and at the same phase of growth as the *bacA* mutant (A.F. Haag, unpublished data). Although this data has yet to be analysed, it would be most interesting to compare the changes in gene expression in the *S. meliloti* Δ*bacA* and *acpXL* mutants, since this may enable one to determine which alterations, if any are occurring in the *S. meliloti* Δ*bacA* mutant as a consequence of the VLCFA alteration. This would enable possible changes in gene expression which occur independently of the altered VLCFA, to be examined more closely.

Chapter 8: Concluding Remarks

Sinorhizobium meliloti is a beneficial legume symbiont able to survive long term within plant cells and is closely related to *Brucella abortus*, a chronic mammalian pathogen (Galibert *et al*., 2001; Halling *et al*., 2005; Ugalde, 1999). Parallels exist between strategies employed by both bacteria to survive within their host cells. One such parallel is the requirement for the inner membrane BacA protein, which is essential for the persistent infection of *S. meliloti* and *B. abortus* within their respective hosts (Ferguson *et al*., 2004; LeVier *et al*., 2000; Roop *et al*., 2002). Free-living *S. meliloti and B. abortus bacA* mutants display an increased resistance to the glycopeptide bleomycin (Ferguson *et al*., 2002; Ichige & Walker, 1997; LeVier *et al*., 2000). Based on this phenotype and the close homology of BacA to the *E. coli* SbmA protein (Glazebrook *et al*., 1993; Ichige & Walker, 1997), a putative peptide transporter, it was hypothesized that BacA could be involved in peptide uptake. Subsequently it was also shown that BacA is necessary for the complete modification of the lipopolysaccaharide (LPS) with an unusual very-long-chain-fatty-acid (VLCFA) modification in free-living *S. meliloti* and *B. abortus* (Ferguson *et al*., 2004). Based on the distant similarity between BacA and the adrenoleukodystrophy family of eukaryotic proteins (Ferguson *et al*., 2004), a model was proposed whereby BacA could be involved in the transport of VLCFA out of the cytoplasm where they then can be used to modify the lipid A in the outer membrane.

In this work it was determined that the increased resistance of the *S. meliloti bacA* mutant to bleomycin and also to the truncated eukaryotic peptide Bac7(1-16), is independent of the VLCFA reduction. This finding supports a role for BacA having multiple non-overlapping functions. Flow cytometry studies with fluorescently labelled bleomycin and Bac7(1-16) revealed that although BacA is involved in bleomycin uptake, it is absolutely essential for the uptake of the truncated eukaryotic peptide Bac7. To date peptides with structural similarities to bleomycin or Bac7 have not been identified in root nodules. However transcriptome analysis of *Medicago truncatula* root nodules has revealed the presence of over 300 cysteine-rich peptides (Alunni *et al.*, 2007; Mergaert *et al.*, 2003; Mergaert *et al.*, 2006), which have been proposed to play a role in *S. meliloti* bacteroid development (Mergaert *et al.*, 2006). Since this work has shown BacA is involved in the uptake of structurally diverse peptides, it is possible that BacA may play a role in the uptake of

one of these peptides in the root nodule. Additionally, as the full length Bac7 peptide was originally isolated from bovine neutrophils (Frank *et al.*, 1990), it is plausible that *B. abortus* may encounter proline rich peptides within its mammalian host. Thus, the uptake of a peptide could also be important for signalling the transition from the acute to chronic state of *B. abortus* infection.

This study revealed that in free-living *S. meliloti* glutathione only appeared to be important for detoxification of the glycopeptide bleomycin when cells were exposed on solid media. However, since glutathione has been shown to play a vital role in the *S. meliloti* legume symbiosis (Harrison *et al.*, 2005), it may be possible that glutathione may play a role in the detoxification of host derived peptides taken up by BacA.

Since two symbiotically defective *S. meliloti bacA* site directed mutants with known reductions in their lipid A VLCFA contents were still capable of Bac7 uptake, this suggests that BacA function which leads to the VLCFA modification could also play a key role in host persistence. To investigate the importance of the VLCFA modification in host persistence, *S. meliloti* mutants were characterised in the plant host lacking either AcpXL (VLCFA acyl carrier protein) or LpxXL (VLCFA acyl transferase). Although it has previously been shown these mutants can persist in the host (Ferguson *et al.*, 2005), it was observed that both displayed major defects, relative to the parent strain. The *S. meliloti acpXL* mutant was delayed in release into the host cell and shown to prematurely senesce, whilst the *S. meliloti lpxXL* mutant was aberrantly shaped and swollen, relative to the parent strain. The precise role the VLCFA modification plays in host persistence remains to be investigated. However, since the VLCFA modification has the potential to span the entire bilayer of the outer membrane, it may play a role in protection against stresses encountered in the host environment such as low pH and reactive oxygen species (Hérouart *et al.*, 2002).

To learn more about the potential role of the BacA protein in VLCFA transport and where in the cell envelope this may occur, the role of two putative lipid trafficking proteins MsbA1 and MsbA2 in *S. meliloti* were investigated. Interestingly it was discovered that the *S. meliloti* MsbA2 protein is essential for the legume symbiosis, since the *msbA2* mutant was unable to enter host cells and induced a plant defence response more characteristic of a pathogen than a symbiont.

This was a very interesting finding since it highlights the delicate balance between the symbiosis and pathogenesis.

It was shown in this work that the increased sensitivity of the *S. meliloti bacA* mutant to the hydrophobic dye crystal violet is independent of the VLCFA alteration. This finding would suggest loss of the BacA protein may be resulting in another alteration to the cell envelope. Thus, it is possible there are other changes occurring in the *S. meliloti bacA* mutant yet to be determined, that may also account for the host persistence defect. Below a model for the proposed roles of BacA, the VLCFA modification and the MsbA2 protein in host persistence is presented (Fig. 8-1).

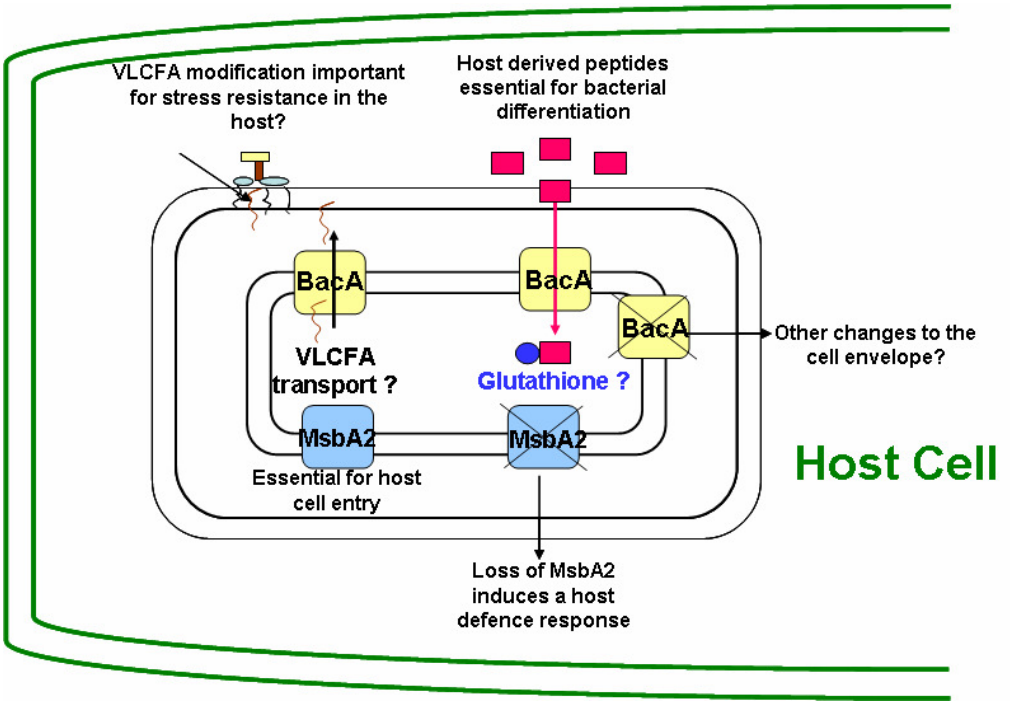


Figure 8-1. Model for the proposed roles of the BacA protein, the VLCFA modification and the MsbA2 protein in host persistence. Could the BacA protein be involved in the uptake of a host derived peptide essential for host persistence and/or could BacA be involved in the transport of VLCFA out of the cytoplasm, where they are then used to modify the lipid A, where they are important in stress resistance? If both proposed models for BacA function are correct, this would suggest BacA must be able to transport two very different molecules in different directions. The MsbA2 protein is essential for host cell entry and its loss results in a host defence response. There also remains the possibility that loss of BacA results in another change(s) to the cell which contribute to the host persistence defect.

Since *Brucella* are highly infectious by aerosol inhalation and research requires strict biosafety 3 level containment facilities and animal models, using a harmless symbiont to gain insights into the basis of *Brucella* infections is highly beneficial. In addition to *Brucella* species VLCFA modifications are present in other intracellular pathogens that cause chronic infections such as *Bartonella henselae*, and *Legionella pneumophila* (Bhat *et al.*, 1991) so insights gained from the *S. meliloti* system may prove invaluable. It has recently been shown that the *Mycobacterium tuberculosis* BacA-related protein (Rv1819c) plays an essential role in maintaining chronic infection in mice (Domenech *et al.*, 2008). Deletion of this gene has also been shown to result in an increased resistance to bleomycin and Bac7(1-16), relative to the parent strain. Thus, this finding suggests BacA-mediated peptide uptake may also play a key role in latent tuberculosis infections, which affect more than one-third of the world's population.

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Publications