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Targets of the QseM Antiactivator in *Mesorhizobium loti*

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Abstract

Quorum Sensing (QS) is a system used by bacteria to coordinate gene expression in response to population density using secreted diffusible signalling molecules, known as autoinducers. Many QS systems are similar to the model LuxR/I system originally discovered in *Vibrio fischeri*, where constitutive expression of the autoinducer synthase *luxI* produces acyl homoserine lactone molecules (AHLs) known as autoinducers at low levels. Once the population density reaches a threshold level, the regulator LuxR recognises and responds to the AHLs, activating downstream gene expression. These systems may also involve an antiactivator, that acts on the LuxR protein to prevent premature activation of the system by low AHL levels.

Mesorhizobium loti strain R7A contains a mobile 502-kb symbiosis island known as ICEM/Sym^{R7A} which can transfer to nonsymbiotic mesorhizobia in both the laboratory and the environment. The excision and transfer of ICEM/Sym^{R7A} is directly controlled through QS via the actions of the the regulator TraR that acts in conjunction with AHL made by the autoinducer synthase TraI1. TraR activity in turn is controlled by the antiactivator QseM, through direct interaction with the TraR+AHL signalling molecule complex to block promoter activation. In this work, RT-qPCR was used to demonstrate that QseM had an effect on downstream TraR-regulated gene expression. Strong expression of the ICEM/Sym^{R7A} excisionase gene *rdfS* or the TrbC protease gene *traF* is known to have an inhibitory effect on cell growth. These genes are regulated by QS through the intermediacy of the *msi172-msi171* gene product which is a single protein, FseA, that is produced by frame-shifting. A conjugation-based growth-inhibition assay involving introduction of a potentially lethal plasmid overexpressing target proteins into cells either overexpressing or not expressing QseM was developed to detect targets of QseM. The assay confirmed that TraR was a target of QseM and further suggested that FseA was a further target. RdfS and TraF were eliminated as targets. Bacterial two-hybrid analyses confirmed FseA as a target and narrowed

the interacting portion down to the Msi172 portion of the frame-shifted protein. Furthermore β -galactosidase assays showed that FseA was unable to activate the *rd/S* promoter in the presence of QseM. Overall, this work confirmed the role of QseM as an antiactivator within the ICEM/Sym^{R7A} transfer system regulatory network and revealed it has more than one target.

A 6-His tag was attached to QseM and a high concentration of protein was purified. Attempts at determining QseM interactants through Mass Spectrometry from a R7A Δ *qseM* lysate proved difficult despite distinct bands being seen. QseM was subjected to circular dichroism that inferred that QseM is composed solely of α -helices, as is TraM, an antiactivator that targets TraR from the *Agrobacterium tumefaciens* QS system.

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Abbreviations

°C	Degrees Celsius	min	Minute
6H	6-Histidine tag	MUG	4'methylumbelliferyl-β-D-galactoside
3-AT	3-amino-1,2,4-triazole	NS	Non-selective media
AHL	N-acyl-homoserine lactone	OD ₆₀₀	Optical density at 600 nm
bp	Base pair	ORF	Open reading frame
cDNA	Complementary DNA	PBS	Phosphate buffered saline
CFU	Colony-forming units	PCR	Polymerase chain reaction
DBD	DNA binding domain	PQS	<i>Pseudomonas</i> quinolone signaling
DMSO	Dimethyl sulfoxide	QS	Quorum sensing
DNA	Deoxyribonucleic acid	RCF	Relative centrifugal force
DTT	Dithiothreitol	RDM	Rhizobium defined medium
g	G-force	RFU	Relative florescence units
h	Hour	RNA	Ribonucleic acid
HGT	Horizontal gene transfer	rpm	Revolutions per minute
HSL	Homoserine lactone	RT-qPCR	Reverse transcriptase-quantitative PCR
ICE	Integrative conjugative element	S	Selective media
IPTG	Isopropyl β-D-1-thiogalactopyranoside	SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Kb	Kilo-base pairs	TAE	Tris-acetate
kDa	Kilo Dalton	tRNA	Transfer Ribonucleic acid
LB	Luria-Bertani media	TY	Tryptone yeast media
LBD	Ligand binding domain	UVCD	ultraviolet circular dichroism
MGE	Mobile genetic element	w/v	Weight-to-volume ratio

Table of Contents

Abstract.....	i
Acknowledgements	iii
Abbreviations	iv
Table of Contents	v
List of Figures.....	viii
List of Tables.....	ix
1 Introduction.....	1
1.1 Horizontal gene transfer as a means of adaptation.....	1
1.2 The <i>Mesorhizobium loti</i> strain R7A symbiosis island ICEM/Sym ^{R7A}	2
1.3 Quorum sensing.....	3
1.4 Mechanisms that prevent the premature induction of quorum sensing systems	5
1.5 Regulation of ICEM/Sym ^{R7A} transfer	10
1.6 Aims.....	18
2 Materials and Methods.....	19
2.1 Media	19
2.2 Bacterial strains and plasmids	20
2.2.1 Bi-parental spot matings	23
2.3 Enzymes and Chemicals	24
2.4 Storage of Strains	24
2.4.1 Frozen inocula.....	24
2.5 DNA Isolation	24
2.5.1 Commercial extraction kits	24
2.5.2 Alkaline Lysis Prep.....	25
2.5.3 PrepMan™ Ultra DNA preparations	25
2.5.4 Ethanol Precipitation	25
2.6 DNA Manipulations.....	26
2.6.1 Restriction enzyme digests.....	26
2.6.2 Subcloning of inserts	26
2.6.3 Agarose gel electrophoresis	26
2.6.4 Agarose gel extraction	26

2.6.5	Ligations	27
2.6.6	DNA sequencing	27
2.7	Polymerase chain reaction	27
2.7.1	Primers used for PCR, RT-qPCR and DNA sequencing	27
2.7.2	Standard PCR protocol	28
2.7.3	PCR product purification	29
2.7.4	Reverse transcriptase - quantitative PCR	29
2.8	Electroporation	29
2.9	β -galactosidase activity assays	30
2.9.1	Cultures for β -galactosidase assay	30
2.10	Protein expression and purification	30
2.10.1	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)	30
2.10.2	Purification and analysis of 6HQseM	31
2.10.3	Cell lysate preparation for detection of proteins interacting with QseM	31
2.10.4	Protein capture column set up	32
2.10.5	Mass spectrometry	32
2.11	Two-hybrid analysis	33
2.11.1	Creation of Msi172-Msi171 and Msi172-Msi171FS fusion combinations	33
2.12	AHL detection using <i>C. violaceum</i> , CV026	35
3	Results	36
3.1	QseM provides protection against the growth inhibitory phenotype caused by TraR over-expression	36
3.1.1	Bacterial Conjugation Assay exploring QseMs ability to protect against TraR induced growth-inhibition.	37
3.1.2	QseM does not regulate expression from the <i>traR</i> promoter	40
3.2	Production of excess QseM inhibits AHL production as quantified through the CV026 bioassay	41
3.3	QseM protects against growth-inhibitory effects of <i>msi172-msi171</i> over-expression.	43
3.3.1	Bacterial Conjugation Assay to investigate the ability of QseM to antagonise Msi172-Msi171 induced growth-inhibition	45
3.4	QseM does not interact with RdfS at the protein level	45

3.5	QseM does not protect against growth-inhibition resulting through TraF expression	47
3.6	Identification of QseM targets through bacterial two-hybrid analysis..	49
3.7	QseM binds a Msi172-171 frame-shifted protein to disrupt <i>rdfS</i> activation	55
3.8	Purification of QseM and exploratory experiments into its targets	58
3.8.1	QseM binds a protein present in a R7AΔ <i>qseM</i> cell lysate at a high affinity	59
3.8.2	Exploration into QseM folding	59
3.9	RT-qPCR analysis of the effect of QseM on the expression of other transfer genes and suspected targets for interaction.....	61
4	Discussion.....	65
4.1	QseM interacts with two distinct proteins within the quorum sensing system	65
4.2	Exclusion of genes and regions from interacting with QseM	70
4.3	Protein purification and folding.....	74
4.4	Concluding remarks	76
5	Appendix A	77
6	Bibliography	82

List of Figures

Figure 1. Cartoon depicting the mechanism of ICEMISym ^{R7A} transfer.....	3
Figure 2. Diagram showing the Las and Rhl QS and PQS signalling system network in <i>P. aeruginosa</i>	8
Figure 3. Growth inhibition as a result of over expression of Msi172-Msi171...15	
Figure 4. A model for regulation of QS and the excision of ICEMISym ^{R7A}	17
Figure 5. Example plasmid map for Two-hybrid assays.....	34
Figure 6. Bacterial Conjugation Assay plate for the introduction of pSRKGmtraR into various strains through spot mating.	39
Figure 7. CV026 bioassay for N-acyl homoserine lactone production.	42
Figure 8. Growth-inhibition through pSRKGm172171 induction via IPTG.	44
Figure 9. Resulting growth after 7 days incubation of spread spot matings after introducing pSRKGmrdfS or pSRKGm into R7A containing either pPROBE-KT or pNqseM.	46
Figure 10. Resulting growth of strains after 6 days in the presence (A) or absence (B) of 1 mM IPTG.....	48
Figure 11. Combined co-transformation experiment with spots of serial dilution on selective medium.	53
Figure 12. β -galactosidase activity from induced reporter genes in pSDZ172171FS variants in the presence or absence of pNqseM.	56
Figure 13. β -galactosidase activity from induced reporter genes in pSDZ172171 variants in the presence or absence of pNqseM.....	57
Figure 14. Sample of concentrated 6HQseM before mass spectrometry	58
Figure 15. Scan of SDS gel from protein purification using 6HQseM to capture possible interacting proteins.....	60

List of Tables

Table 1. Concentration of antibiotics used in this study	19
Table 2. Bacterial strains and plasmids used in this study.....	20
Table 3. Primers used in this study.....	27
Table 4. Variation of <i>lacZ</i> expression across different strains in the presence or absence of QseM across three biological replicates.	40
Table 5. Plate counts and ratio for Cotransformations, experiment one.	50
Table 6. Plate counts and ratio for Cotransformations, experiment two	50
Table 7. Plate counts and ratios for the combined experiment co-transformations.....	54
Table 8. The OD ₆₀₀ of each culture and resulting mRNA yield from each before cDNA synthesis.....	63
Table 9. Relative abundance of mRNA for genes implicated in the QS system from three replicate experiments (A, B and C) using different mRNA preparations in each sub table.....	64

1 Introduction

1.1 Horizontal gene transfer as a means of adaptation

Prokaryote evolution has been driven over time by the non-vertical transfer of genetic material between species, termed horizontal gene transfer (HGT). This mode of transfer has enabled the rapid dissemination of genes that have strategic importance between organisms, increasing fitness or virulence and enabling new combinations of genes to be trialled. HGT allows bacteria to uptake DNA from the surrounding area and effectively 'sampling it' as a means of adaptive evolution. Mechanisms of distribution of genetic material vary and include conjugation (direct cell to cell contact), DNA transformation and phage transduction (1–4).

HGT via conjugation is promoted by Mobile Genetic Elements (MGE), which are clusters of genes that are able to move within bacterial populations and likely, in many cases, between species. For their continued survival and existence, MGEs not only have to confer a long-term advantage to the host organism but also be able to offset any negative short-term effects of carriage (3).

A subset of MGE's are the Integrative and Conjugative Elements (ICEs), which achieve vertical carriage by integrating themselves into host genomes often in such a way as not to interrupt the host's original genes (5–7). These elements can exist not only integrated into the host chromosome but also in a transient circular, plasmid-like form. ICEs often delay host binary fission when in the excised circular form through hijacking of the host's own cell machinery, and re-integrate into both the donor and recipient bacterial chromosome to ensure self-preservation (7). Evolutionarily speaking, capture and devolution of a MGE is the best way for a bacterium to ensure long-term advantage, however many strains maintain the MGE at significantly increased metabolic cost.

The fitness advantages conferred by a MGE are often complex, with different elements providing different combinations of genes. This allows for a more refined classification of MGEs, based on their main gene set and shared homology despite hugely divergent bacterial hosts. Symbiosis islands are one of

the least innocuous classifications as they allow a symbiotic relationship between bacterium and plant, the ICEMISym^{R7A} of *Mesorhizobium loti* strain R7A falls into this category. ICEMISym^{R7A} is responsible for the nodulation of roots of *Lotus* species and nitrogen fixation in return for carbon sources (8). Other examples of ICEs are resistance and pathogenicity islands, with the SXT element of *Vibrio cholerae* conferring antibiotic resistance for the host cell (9), the High Pathogenicity Island 1 of *Yersinia pestis* (10) and catabolic islands such as the *clc* element of *Pseudomonas* strain B13 which contains genes required for chlorocatechol degradation (5, 11).

1.2 The *Mesorhizobium loti* strain R7A symbiosis island ICEMISym^{R7A}

Mesorhizobium loti is a gram-negative α -proteobacterium which is free-living within the soil, capable of invading roots of assorted leguminous plants, forming a symbiotic relationship, and maintaining a continuous colony within the root (8, 12). This symbiosis involves nodulation of the plant roots and stimulates the fixation of atmospheric nitrogen (N₂) by *M. loti*, for use by the plant as ammonia. This process has the potential to reduce the agricultural sector's reliance on nitrogen-based fertilisers (urea), decreasing the downstream side effects such as toxic run-off and water or soil acidification (13).

Mesorhizobium loti strain R7A contains a symbiosis island ICEMISym^{R7A} that is transmissible to non-symbiotic *mesorhizobium*, both naturally in the environment and in the lab (8, 14). ICEMISym^{R7A} is 502-kb in size and encodes 414 genes that include genes for excision and transfer, quorum sensing, nodulation and nitrogen fixation (8, 15). ICEMISym^{R7A} always integrates into the host genome at the 3' end of the host strain's phenylalanine tRNA (phe-tRNA) gene. Upon integration the full phe-tRNA gene is reconstructed at the 5' integrative junction as ICEMISym^{R7A} contains the missing bp and a 17-bp segment of the 3' end of the phe-tRNA gene is duplicated at the other integrative junction. ICEMISym^{R7A} transfer begins with excision and recircularisation (Figure 1), and the host phe-tRNA gene is restored upon excision of the ICE. A mating pore is formed and a single strand of the circularised DNA is fed through

to the recipient via a rolling circle mechanism. The synthesis of the second strand then occurs in both host and recipient, before (re)-integration of the ICE into the host and recipient phe-tRNA gene.

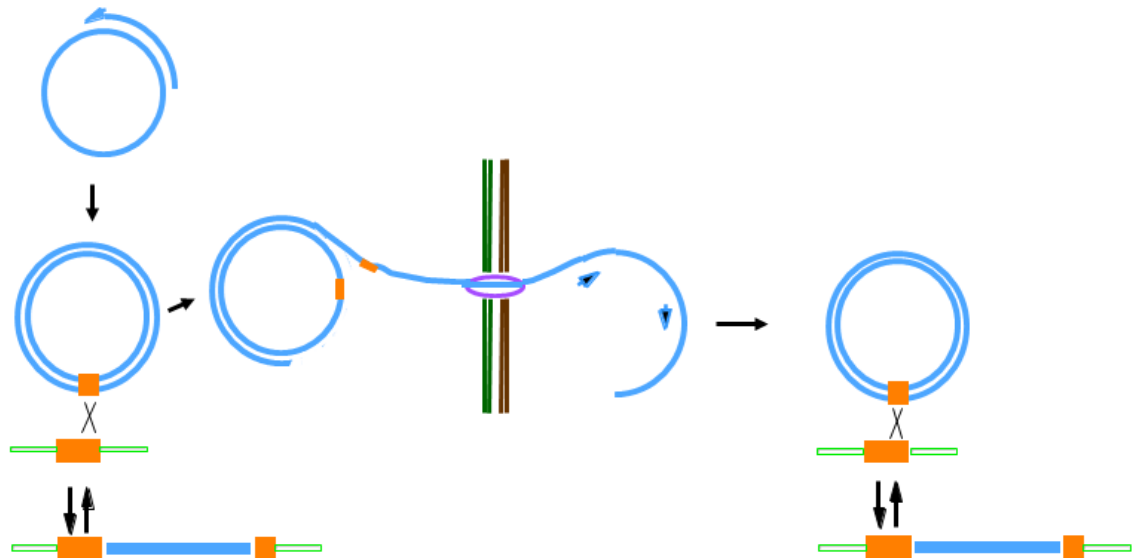


Figure 1. Cartoon depicting the mechanism of ICEM/Sym^{R7A} transfer.

The double-stranded DNA of ICEM/Sym^{R7A} is excised and circularised. A single strand of the circularised DNA is fed through the mating pore. Once the single-strand is fully transferred and circularised, synthesis of the second strand occurs before (re)-integration into the host and recipient phe-tRNA gene. (Derived from Ramsay, 16)

1.3 Quorum sensing

ICEM/Sym^{R7A} transfer and the transfer of many other MGE is regulated in part by quorum sensing (QS). QS is a means of cell to cell communication and a critical aspect for coordination of gene expression (17). At the basic level, QS is defined as the production and secretion of diffusible signalling molecules, named autoinducers, into the surrounding environment. As population size increases, the threshold for autoinducers is exceeded and selective activation of gene expression occurs. Thus QS allows the control of individual cell gene expression through a population-based system (18–22). This method of control is particularly useful in situations where the regulated genes encode virulence factors and premature expression may have a direct effect on the microorganism's ability to live within a host (19, 23). This method of regulation allows the carriage and selective expression of metabolically costly genes, as

expression only occurs when a build-up of cells (and therefore autoinducer) occurs, such as in biofilms, infection sites or areas of symbiosis (23).

The first described occurrence of QS was in the Hawaiian squid *Euprymna scolopes* where *Vibrio fischeri* colonised the light organ, upon reaching the threshold *V. fischeri* produce light (24, 25). This symbiotic relationship in the light organ provides the *V. fischeri* with high quantities of nutrients and a suitable space to reach a greater cell density and therefore activate the bioluminescence genes. In return, *E. scolopes* uses this light to disguise itself from predators during the day by reducing its shadow, expelling all but a few bacterium of the original colony once night falls, turning off expression of the bioluminescence genes in order to hide.

The system controlling the activation of this bioluminescence (luciferase) operon comprises two proteins LuxI and LuxR. LuxI is the autoinducer synthase, which produces the acyl-homoserine lactone (AHL) autoinducer 3-oxo-C6-homoserine lactone, while LuxR is the cytoplasmic autoinducer receptor and DNA-binding transcriptional activator. Upon production, AHL freely diffuses in and out of the cell and increases in concentration as cell density increases. Once the critical threshold concentration has been met, the AHL is readily bound by LuxR, this complex then activates the transcription of the luciferase operon. This activation also contains a positive induction loop for the production of AHL, as *luxI* is encoded within the luciferase operon, ensuring that autoinducer is flooded into the cytoplasm and the population as a whole is switched to produce light (26, 27). This phenomenon is what led to the AHL being termed an autoinducer.

In the thirty years since the initial characterisation of the LuxR/I system, many homologous systems, in both sequence and role, have been identified. Many Gram-negative bacteria rely on an AHL-based signalling system. Some of these systems include additional components such as antiactivators, indicating higher levels of regulation complexity. Each I protein in the various different systems synthesises specific AHLs, with each system's specific R protein containing the correct ligand binding domain (LBD) for that particular AHL, reducing the likelihood of aberrant activation of the QS system by another species.

Conversely some QS systems have redundancies built in to deal with co-infection scenarios where two or more AHLs may be present in the infection site or biofilm (22, 28–30), while others have been reported to repress production of certain immune cells to potentially aid infection (31). Wang *et al.* (32) reported that both *Escherichia coli* and *Salmonella* have a LuxR homologue, SdiA, however both lack a LuxI homologue and do not produce AHLs. Michael *et al.* (33) reported that the purpose of SdiA in *Salmonella* is not to sense an autoinducer produced in cell, but rather those of other bacterial species, a form of cross-talk.

The flip side of co-infectivity is that some species have the ability to disrupt signalling in QS systems by degrading those AHL autoinducers expressed by other bacteria. Specifically, *Bacillus* a Gram-positive soil bacterium, produces a lactonase enzyme that hydrolyses the lactone ring in AHLs, causing a structural change and likely interfering with the signalling pathway (34). Competitive inhibition of a bacterium's QS ligands has also been reported as an effective way to interrupt or halt infection (28).

As QS systems on the whole are very sensitive to both quantity and stereochemistry of their cognate AHL, some recent research has focused on creating synthetic AHLs in the forms of large scale libraries and testing these against various genes in QS systems with the aim of finding possible agonists/antagonists. Mattmann *et al.* (35) reported that synthetic AHLs may provide a method for targeting other species' QS systems as a means to stop infection of hosts through competitive binding. Current large scale library assay experiments have determined several excellent candidates but they are often very dissimilar between studies (35, 36). This research provides a new approach for targeted antibiotic or anti-QS drugs to target pathogens.

1.4 Mechanisms that prevent the premature induction of quorum sensing systems

Many additional regulators have recently been characterised in QS systems where they play a key role by preventing early autoinduction in response to low amounts of AHL, thus maintaining the correct expression rate. Examples include antiactivators that target the various R proteins blocking activation of gene

expression for the QS system they exist in. The mode of action varies from system to system, some bind the R protein near the LBD or linker and the resulting conformational change blocks the DNA-binding domain (DBD), while others bind the R protein and increase its sensitivity to proteolysis (37).

The QS system in *Agrobacterium tumefaciens* that controls the transfer of the Ti plasmid is well characterised and has been further dissected to reveal an anti-activator at the heart of control. The *tra* operon is tightly controlled at a transcriptional level through the regulation of *traR* expression, and TraR activity by the antiactivator TraM (19). TraM is an 11.4 kDa protein composed of two antiparallel α -helices, which exists in the cytoplasm as a homodimer that folds into a coiled-coil structure (38). TraR is a 25 kDa protein, composed of α -helices and β -sheets with two distinct binding domains connected with short linker (39). TraR also exists as a dimer in the cytoplasm, with the N-terminal domain completely engulfing the cognate AHL within a hydrophobic pocket, while the C-terminal domain consists of the DNA-binding helix-turn-helix motif (39).

Early autoinduction of this system is prevented through the expression of TraM, with a TraM dimer interacting with a TraR dimer. This process forces a conformational change in TraR, enabled by the linker strand, that forces the two DBDs apart and possibly forcing out the signal AHL from the LBD (40). Due to the change in conformation, the DBDs of TraR are no longer exposed correctly and the activation of the promoter regions of the *tra* regulon cannot occur (19, 41–43). Spontaneous point mutations of *traM* resulted in a mutant that was not able to exert an antiactivation function, leading to over-production of the AHL QS signal and constitutive transfer of the Ti plasmid (44).

Basal levels of production of TraM are sufficient to maintain populations at a state of no induction at low culture densities (19), however during plant infection when opine signalling molecules are more abundant, activation of the *tra* operon occurs producing more TraR. This in turn promotes the positive feedback loop activating the *tra* operon again, ultimately producing more TraR than controllable, leading to activation of downstream transfer genes (43).

In organisms that contain multiple QS systems, the complexity is increased. *Pseudomonas aeruginosa* is an opportunistic pathogen that commonly causes infections in immunocompromised individuals and people with cystic fibrosis. It contains two different complete QS systems; the Las system, LasR/I controlling virulence factor expression and the Rhl system, RhlR/I, controlling production of rhamnolipids and secondary metabolites, and in addition a third R protein QscR that lacks a corresponding I gene forms an incomplete system (45, 46). LasR positively regulates expression of *rhlR* by acting as a transcriptional activator, while RhlR's activity is negatively regulated post-translationally through 3-oxo-C12-HSL produced by LasI blocking binding of RhlR's cognate AHL, C4-HSL, thereby inhibiting *rhl* gene expression until sufficient C4-HSL is present to outcompete levels of 3-oxo-C12-HSL. This system is thought to be in place to delay induction of genes controlled by RhlR (47). LasR also activates QscR through 3-oxo-C12-HSL production. Despite the mode of action for QscR remaining incomplete, there is speculation pointing towards a possible switch to turn off virulence in *P. aeruginosa* (46, 48–50). See Figure 2 to aid the explanation.

Transcription of LasR in *P. aeruginosa* is controlled by the cyclic AMP receptor protein (CRP) that binds the CRP-binding consensus sequence (CCS) found directly upstream of the promoter, as well as upstream of *P. aeruginosa* CRP homolog Vfr. Vfr is required to bind the CCS upstream of *lasR* to activate expression (51). Park *et al.* (49) recently created a truncated version of QscR by removing the AHL binding domain. This truncated protein competitively binds the promoter region of its target gene compared to QscR*wt*, resulting in no transcription of the target gene. While Lequette *et al.* (46) reported that a *qscR* mutant had massive upregulation of virulence factors and another 400 other genes.

RsaL has been identified as a major negative regulator in *P. aeruginosa* and plays a pivotal role in regulation of pathogenicity. It is encoded downstream of *lasR*, is transcribed antisense relative to *lasR*, and translates to an 11 kD protein. Overexpression of RsaL reduced *lasB* expression and decreased elastase activity, and it was determined that RsaL achieves this by specifically repressing

transcription of *lasI* by competitively binding the promoter region, therefore dramatically decreasing 3-oxo-C12-HSL production (52, 53). More recent work determined that this is achieved through RsaL competitively binding the *rsaL-lasI* bidirectional promoter, thereby preventing the LasR-dependent activation of both promoters (18, 53–55).

Figure 2. Diagram showing the Las and Rhl QS and PQS signalling system network in *P. aeruginosa*.

Microarray work conducted by Rampioni using a $\Delta lasI \Delta rsaL$ mutant strain revealed that when strains were complemented with the RsaL expression vector pPSRsaL and 5 mM 3-oxo-C12-HSL in media, 120 genes were repressed and 10 genes were activated by RsaL expression independent of RsaL's effect on

3-oxo-C12-HSL (53). Many of the repressed genes encode antibiotic resistance or virulence factors including genes involved in pyocyanin production and hydrogen cyanide biosynthesis. It was determined that their promoters contain the same binding site as found in the *rsaL-lasI* bidirectional promoter (53). RsaL also repressed three characterised and three putative transcriptional regulators, indicating many genes could be controlled indirectly. Not all genes repressed by RsaL are activated by LasR or RhlR, indicating RsaL may also control genes outside of the QS system (53).

QslA was recently identified as a master antiactivator of the QS systems within *P. aeruginosa* that exerts control over the Rhl and Las systems as well as the pseudomonas quinolone signal (PQS) pathway. QslA is a 11.8 kDa protein that shares similarity with TraM through the conserved structural motifs, α -helices but not sequence, with QslA containing portions that overlap with the interacting regions of TraM (55). QslA primarily acts to suppress QS triggered gene expression by blocking promoter activation through direct protein-protein interaction with LasR, thus controlling the Rhl and Las QS pathways. This also reduces levels of cognate AHL for each system and has an effect on the PQS pathway (55, 56). Expression of *qslA* is constitutive and unaffected by the various stages of QS, further solidifying its antiactivation role.

The protein-protein interaction occurs when a QslA dimer comes into contact with a single LasR+3-oxo-C12-HSL complex binding in the LasR LBD blocking the LasR dimerization interface, which is required to aid in the positioning of the DBD on the promoter region. Recent work has identified the residues on QslA between the LasR and QslA interface region, that when substituted with alanine cause a dramatic decrease in levels of antiactivation as well as QslA dimerization, indicating that these residues are required for successful protein-protein interactions and therefore QS system control (56).

A null mutation of *qslA* resulted in enhanced expression of QS and PQS genes, enhanced virulence factor production, such as elastase (20 – 200%) and pyocyanin (150 – 280%), and also increased bacterial virulence in the *Caenorhabditis elegans* animal model. Complementation of a $\Delta qslA$ mutant with *in trans* expressed QslA drastically reduced the transcriptional expression of

rhII, *lasI* and *pqsA*, the production of QS regulated virulence factors and the pathogenicity of bacterial infection in *C. elegans* (55). Addition of extra AHLs to a $\Delta qslA$ mutant has a limited effect on gene activation, likely because of the complex arrangement of promoters. Strains that are deficient for QslA do not show enhanced *lasI* expression likely due to a negative feedback mechanism involving RsaL as *lasI* contains a promoter specific repressor site for RsaL (55, 56).

$\Delta qslA$ strains also experienced a reduction in AHL production required to trigger a QS response, up to 9 times less, than wild type, indicating QslA's role in providing a hurdle to prevent premature induction of gene expression. The overall complexity of three systems working in concert suggest that a number of factors are activated when the quorum is reached within *P. aeruginosa* and the system is far harder in artificially activate (56).

Building on this already complex system, other work involving DNA-affinity chromatography has identified more repressors of the multiple systems within *P. aeruginosa*. Putative transcription factor PA3699 was characterised as a TetR-like protein that binds directly to the *lasR* promoter region *in vitro*. When PA3699 is induced in *P. aeruginosa* PAO1 cultures, *lasR* promoter activity is decreased and the production of LasR-dependent virulence factors is reduced. This may represent another antiactivator or possibly a repressor of the system (57).

1.5 Regulation of ICEM/Sym^{R7A} transfer

In *M. loti*, the system controlling ICEM/Sym^{R7A} transfer includes homologues of TraR and TraI that regulate transfer of the Ti plasmid in *Agrobacterium*. The AHL 3-oxo-C6-HSL produced by TraI1 complexes with TraR which then activates the expression of the *tral1* and *tral2-msi172-msi171* operons through DNA binding (Figure 4, 58). The function of *tral2*, a second *tral*-like gene, is as yet unknown beyond ensuring the downstream *msi172-msi171* genes are activated in response to QS.

As the TraR+3-oxo-C6-HSL complex activates the expression of both *traI1* and *traI2*, a positive feedback loop is maintained. This ensures that once a threshold is met within, for example, an infection thread or culture, the surrounding cells are flooded with excess autoinducer, changing gene expression within the whole population. Expression from the *traI1* and *traI2* promoters can be induced by the presence of plasmid-expressed *traR*, but not by the addition of extra AHL's only. The latter only induces expression in strains that are deficient of *qseM* (58). In all cases this expression leads to the activation of the *traI2* operon and its downstream genes *msi172* and *msi171*, now known to encode one protein produced by frame-shifting, and the activation of *rdjS* and the excision of the island (Figure 4, 59).

As noted above, although *M. loti* contains two copies of the AHL synthases within ICEMISym^{R7A}, *traI1* and *traI2*, mutational analyses identified *traI1* as the functional AHL synthase despite its distant genomic positioning (58). However deletion of a region of *traI2*, that included its promoter, eliminated ICEMISym^{R7A} excision and caused *msi172-msi171* to not be expressed. Complementation of this region with a plasmid carrying the deleted portion, failed to restore both excision and downstream gene expression inferring these downstream genes, *msi172-msi171*, are required in the excision activation pathway and that they are transcribed from the *traI2* promoter (58).

Recent work has identified a gene within ICEMISym^{R7A} named *qseM* (previously *msi170*), that likely functions as the antiactivator for this system. QseM is a 10 kDa protein that is encoded by the *qseM* located downstream of the *traI2-msi172-msi171* operon, in reverse orientation. This arrangement is likely to counteract read-through occurrences, inferring that this system is very tightly regulated. *qseM*'s genomic positioning adjacent to not only its target but also its own repressor *qseC* further reinforces a tightly regulated system. Interestingly this layout is strikingly similar to that of *traM* in several (but not all) *Agrobacterium* Ti and Ri plasmids(60).

R7AΔqseM mutants can be characterised by higher levels of AHL production, due to a deregulated signalling system; and increased rates of expression of the *traI2* operon, which results in downstream gene activation resulting in 100%

excision of ICEM/Sym^{R7A} with the effect being seen at both stationary and exponential phases (60). These observations suggest that QseM interacts with TraR, much like TraM/TraR of the Ti plasmid, to maintain expression levels of TraR and AHL at a basal state, and to prevent the premature activation of the QS genes by restricting expression at all times. However, its mode of action was incompletely characterised. Previously it was determined that ICEM/Sym^{R7A} does not contain a *traM* sequence homologue, pointing perhaps to QseM having an analogous function to TraM within the system and providing a the missing link in the control of ICEM/Sym^{R7A} transfer. A more in depth analysis of island related genes by Ramsay (16) produced an array of TraM, or TraM-like gene homologues within a number of different genomic islands, some within *mesorhizobium* family, while others are found in more commonly known genomic islands. TraM present on the Ti plasmid is responsible for the control of the TraR mediated QS response.

QseM's antiactivation role is to control expression of QS genes through interaction with the TraR+3-oxo-C6-HSL complex, through currently unknown means, and either encouraging proteolysis of TraR or creating a conformational change that blocks the DBD. The sequestering of the signalling complex occurs at such a high rate, it is postulated that few copies of TraR are present in the cytoplasm even at quorum and it is likely that regulation of QseM is modulated by QseC as a result of this event as well.

Regulation of *qseM* occurs through *msi169*, renamed *qseC*, which encodes a XRE transcriptional regulator, with BLASTP and other bioinformatics searches identifying a number of homologs on various ICESym clusters and other QS regulated plasmids. XRE genes are often found downstream of, and divergently transcribed to, *traM*, on these other elements and appear at least partially responsible for control in all cases (60). Deletion of *qseC* results in reduced levels of excision and AHL production, likely through an increase in QseM, while a double deletion of both *qseM* and *qseC*, including the intergenic region between genes, results in AHL overproduction and excision levels near those seen when TraR is constitutively expressed. Both phenotypes can be complemented with a plasmid expressing either QseM or QseM and QseC. This

indicates that QseC binds the promoter of *qseM*, to negatively regulate production and subsequently excision and QS, as XRE family genes are DNA binding (16). This intergenic region contains the promoters for both *qseM* and *qseC*, QseC binds over either separately, or both at once to block transcription from that site modulating the activity of itself and QseM (60). This is strikingly similar to the *rsaL-lasI* bidirectional promoter region in the Las system of *P. aeruginosa* mentioned earlier.

Previous studies by Ramsay determined that *M. loti* produced mainly 3-oxo-hexanoyl-HSL (3-oxo-C6-HSL) and a range of other AHL with differing chains and backbones, C6-, C8- and 3-oxo-C8-AHL, in very small quantities (58). Complementation of R7A Δ *traR* with a plasmid constitutively expressing QseM retards AHL production and excision to levels below those found in wild-type R7A, suggesting TraR as a target for QseM (58). This reduction was also seen when the plasmid constitutively expressing QseM was introduced to R7A. Ron Dy, (61) demonstrated through a bacterial two-hybrid analysis that QseM and TraR interact, but only in the presence of the systems autoinducer, 3-oxo-C6-HSL, reinforcing this hypothesis. Ramsay *et al.* (58) also reported that constitutive expression of QseM in a R7A Δ *traR* background reduced excision levels below that of R7A and R7A Δ *traR* alone, this was unexpected and suggests QseM has another target within the excision activation pathway possibly activated through TraR expression.

The placement of genes within ICEMISym^{R7A} varies considerably when compared to more completely defined examples like the Ti plasmid. On Ti, *traI* exists directly upstream of the *trb* family of genes responsible for conjugation in *A. tumefaciens*, while on ICEMISym^{R7A} in *M. loti*, *traI1*'s genomic position is in relative isolation and close to 9-kb away from the *trb* genes. Similarly on Ti, *traM* is divergently encoded near TraR with other known genes between them. Between *traR* and *qseM* on ICEMISym^{R7A} 3 genes exist; *tral2* and *msi172-msi171*, now known to encode a singular transcriptional regulator that modulates the excision of ICEMISym^{R7A}. This indicates another possible site for interaction with QseM, due to its close proximity and role.

Previous bioinformatics studies conducted by Ramsay (16) on Msi172-Msi171 found homology between R000034 protein, of Tn4371 in *Cupriavidus oxalaticus* and Msi172-Msi171 when Msi172-Msi171 existed as a single protein. Homologs of genes activated by Msi172-Msi171 were also uncovered on Tn4371, such as *rdfS* and *rlxS*, while a second region containing the *trbBCDEJLFGI* operon which encoded a Type 4 secretion system, albeit modified in ICEMISym^{R7A} was also located. R000034 was also found to be conserved on a number of elements closely related to Tn4371 suggesting a common ancestor (16, 62).

msi172-msi171, previously the two genes downstream of *tral2* in the operon, are responsible for activation of the *rdfS* operon by binding the promoter region directly upstream, a mutation in either gene results in abolishment of excision of ICEMISym^{R7A}. Ramsay (59) reported that the genes required for excision of ICEMISym^{R7A} were *rdfs* and *intS*, as mutants for each excised ICEMISym^{R7A} at lower frequencies. However the additional requirement for Msi172-Msi171 has been proven, as an in frame mutation of either gene resulted in the abolishment of excision of ICEMISym^{R7A}, with only R7AΔ*msi171* able to be complemented with plasmid produced Msi171, indicating that its required for *intS* and *rdfs* expression, and possibly activates a promoter region (58, 63). Exploration of Msi172-Msi171 through plasmid introduction shows that constitutive expression of both genes is able to activate the excision pathway and results in excision in 100 % of cells tested (58) and inhibition of culture growth on plates of R7A, R7ANS which lacks ICEMISym^{R7A} was unaffected by the introduction of the plasmid (Figure 3; 62). This growth-inhibition created by Msi172-Msi171 expression was first thought to be a direct result of the expression; however, once the site of interaction was determined to be the *rdfS* promoter, the growth inhibitory phenotype was determined to be likely the result of RdfS or TraF expression.

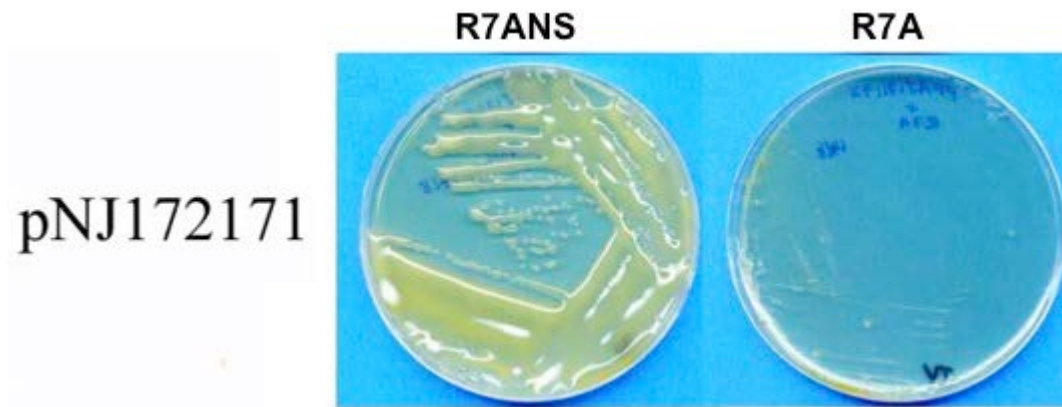


Figure 3. Growth inhibition as a result of over expression of Msi172-Msi171.

Introducing the plasmid pNJ172171, which constitutively produces Msi172-Msi171 into *M. loti* R7A causes a growth inhibitory phenotype as seen on the right, while when pNJ172171 is introduced into R7ANS, growth is unaffected as it contains no ICEM/Sym^{R7A}. This growth-inhibition seen here is very similar as described throughout the results section of this thesis.

Previous work, conducted by myself, screening a series of in-frame deletion mutants for a strain that was able to grow in the presence of constitutively expressed Msi172-Msi171, proved inconclusive. Professor Mike Hynes continued this project and located a polar pFUS based mutant in the two gene ORF, *rdfS-traF*, that was able to grow despite excess Msi172-Msi171 (Unpublished Data). This not only affirmed Msi172-Msi171 as a requirement for the activation of excision but also indicated a likely promoter region for interaction. Recent work by Laura Tester has confirmed Msi172-Msi171 does activate the *rdfS* promoter region even in the R7ANS background, absent of other ICEM/Sym^{R7A} genes, as well as identifying a frameshift region which is responsible for a differentially translated protein, ~4.5% of the time (Unpublished Data).

At the beginning of this project our understanding of the QS system in *M. loti* was as the population density builds to a threshold, levels of 3-oxo-C6-HSL and TraR within culture or infection site increase. Once the threshold is met, TraR+ 3-oxo-C6-HSL complexes then activate the promoters for *traR*, *traI1* and the *traI2-msi172msi171* genes, promoting the positive feedback loop and activating

downstream genes' expression. QseM acts as an antiactivator within this system; controlling levels of TraR+3-oxo-C6-HSL to suppress activation until ideal conditions or to turn off the QS triggered gene expression once levels of 3-oxo-C6-HSL drop. QseM expression is modulated by QseC, which dimerises and blocks the promoter region of *qseM* while also controlling its own expression.

Artificial expression of *traR*, *traI1* or *msi172msi171* produces a false positive response for cell density, stimulating the positive feedback loop, triggering the activation of excision pathway which causes the growth-inhibition phenotype. The over expression of QseM understandably has the opposite effect, lowering excision levels to almost below measureable. Basal expression levels of TraR maintain low levels of expression of TraI and TraI2 but the system is ultimately controlled by QseM when cell density is low (Figure 4).

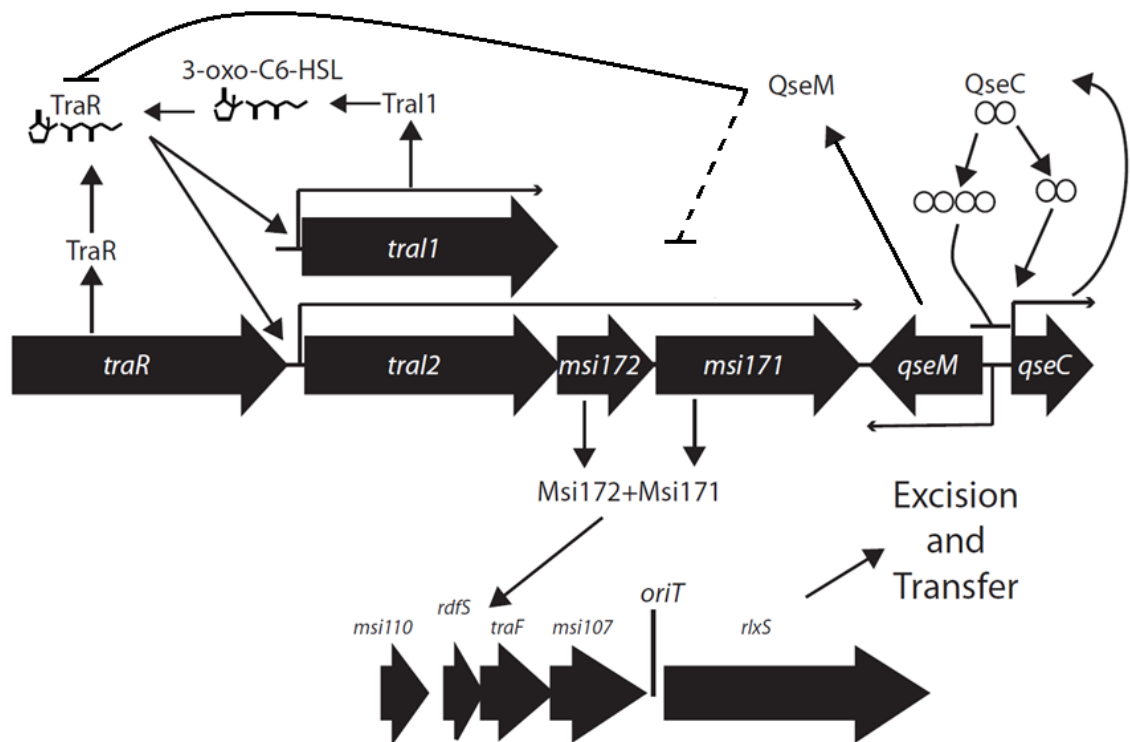


Figure 4. A model for regulation of QS and the excision of ICEMISym^{R7A}.

Modified from J. Ramsay, personal communication. QseM interacts with the TraR+3-oxo-C6-HSL complex to disrupt activation of *tral1* and *tral2* operons restricting downstream activation. Msi172-Msi171 activates the *rdfs* promoter region and begins the activation of genes required for the excision and transfer of the island. Production of QseM is modulated through binding of QseC to the intergenic region between *qseM* and *qseC*, which contains promoters for both genes. Dashed line represents an area of possible interaction explored within this thesis.

1.6 Aims

The aims of this study were to identify target(s) of QseM within the genes or proteins involved in the regulation of the excision and transfer of ICEMISym^{R7A}, and to determine the mode of action, for any identified target(s).

The previous work carried out on this topic has built a solid foundation to begin the explanation of the QS system and the excision activation pathway. QseM has been identified as a potential master regulator of this system as it interacts with TraR, near the beginning of the QS system, however QseM likely interacts with another yet unidentified protein as its control is still exerted in the absence of *traR*. This project seeks to confirm this unknown interactant for QseM while also seeking to confirm or exclude other possible interaction sites.

To achieve this various genes and regions implicated in parts of the excision and transfer system were selected based on their ability to inhibit growth in R7A and mutant variations. Using these genes on inducible expression vectors was assayed for in the presence or absence of QseM. Regions of interest were attached to a reporter gene and assayed for β -gal activity in a variety of different backgrounds, and in the presence or absence of QseM expression to determine whether or not there was a protective effect.

RT-qPCR was used to examine the mRNA levels of interest genes in the presence or absence of QseM, while the addition of a 6H tag to the N terminal region of QseM enabled purification of both QseM itself and possible interacting proteins.

2 Materials and Methods

2.1 Media

Escherichia coli strains were grown at 37°C on Luria-Bertani (LB) agar (64) or in LB broth with shaking (200 rpm) as well as on Tryptone yeast (TY) agar (65) plates and in broth for bi-parental spot matings. *E. coli* strain ST18 was grown with the addition of 50 µg/mL 5-aminolevulinic acid to media. Mesorhizobial strains were grown at 28°C on TY agar plates, or in TY broths with shaking (160 rpm) or on rhizobium defined medium (RDM) (66) agar plates supplemented with 0.4% glucose as the sole carbon source (GRDM). *Chromobacterium violaceum* CV026 was grown at 28°C on LB agar or in LB broths with shaking at 160 rpm. Antibiotic supplements (Table 1) and vitamins [thiamine (1 µg/mL), nicotinate (1 µg/mL) and biotin (20 ng/mL)] were added to GRDM as required.

Table 1. Concentration of antibiotics used in this study

Antibiotic	Concentration (µg/ml)	
	<i>E. coli</i>	<i>Mesorhizobium</i>
Kanamycin (Km)	50	-
Gentamicin (Gm)	25	50
Neomycin (Nm)	-	100
Tetracycline (Tc)	10	2
Ampicillin (Ap)	50	-
Chloramphenicol (Cm)	34	-

2.2 Bacterial strains and plasmids

Bacterial strains and plasmids used are listed in Table 2.

Table 2. Bacterial strains and plasmids used in this study.

Strain/plasmid	Description	Reference
<i>Mesorhizobium</i>		
R7A	Field reisolat of ICMP 3153; wild-type symbiotic strain	(14)
R7ANS	Non-symbiotic derivative of R7A; lacks ICEMISym ^{R7A}	(59)
R7A*	Spontaneous mutant of R7A that produces increased amounts of 3-oxo-C6-HSL compared to R7A	(16)
R7AΔ <i>qseM</i>	<i>qseM</i> in-frame markerless deletion mutant of R7A	(60)
R7AΔ <i>qseM</i> Δ <i>traR</i>	<i>qseM</i> in-frame markerless deletion mutant made in R7AΔ <i>traR</i> background	(60)
R7AΔ <i>traR</i>	<i>traR</i> in-frame markerless deletion mutant of R7A	(58)
R7AΔ <i>rdfs</i>	<i>msi109</i> in-frame deletion mutant made using pJQ200SK. Suspected to be from R7A* background	(16)
<i>E. coli</i>		
S17-1	Tp ^R Sm ^R <i>recA thi pro hsdR-M⁺recA::RP4-2-Tc::Mu Km::Tn7</i>	(67)
ST18	S17-1 derivative auxotrophic for 5-aminolevulinic acid	(68)
BL21(DE3)(pLYS)	F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻m_B⁻)</i> λ(DE3) pLysS, Cm ^R	(69)
BacterioMatch® II Validation Reporter Competent Cells	Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 hisB supE44 thi-1 recA1 gyrA96 relA1 lac</i>	Agilent Technologies Catalog #200192

XL1 – Blue MRF’ Kan	$\Delta(mcrA)183$ $\Delta(mcrCB-hsdSMR-mrr)173$ <i>endA1 supE44 thi-1 recA1 gyrA96 relA1</i> <i>lac</i> [F’ <i>proAB lacI^qZ</i> $\Delta M15$ Tn5 (Kan ^R)]	Agilent Technologies Catalog #200248
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C. violaceum

CV026	<i>cviI::mini-Tn5</i> derivative of ATCC 31532, Km ^R , AHL ⁻	(70)
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Plasmids

pPROBE-KT	pVS1/p15A replicon promoterless <i>gfp</i> ; Nm ^R	containing (71)
pNqseM	pPROBE-KT derivative expressing driven <i>qseM</i>	<i>nptII</i> - (60)
pSRKGm	pBBR1MCS-5 based vector for inducible expression of cloned genes Gm ^R	(72)
pSRKGmtraR	<i>traR</i> cloned downstream of inducible promoter in pSRKGm	IPTG- Mike Hynes, this lab
pSRKGm172171	<i>msi172-msi171</i> cloned downstream of IPTG-inducible promoter in pSRKGm	IPTG- Mike Hynes, this lab
pSRKGmrdfS	<i>rdfS</i> cloned downstream of inducible promoter in pSRKGm	IPTG- Mike Hynes, this lab
pSRKGmtraF	<i>traF</i> cloned downstream of inducible promoter in pSRKGm	IPTG- Mike Hynes, this lab
pQe80-6HqseM	pQe80oriT carrying the <i>qseM</i> gene fused downstream of the 6-Histidine tag	This study
pN6HqseM	pPROBE derivative expressing driven 6HqseM	<i>nptII</i> - This study
pNJ172171	<i>msi172msi171</i> cloned by PCR into pFAJ1708	(63)
pFJX	pFAJ1700 containing promoterless gene	<i>lacZ</i> (73)

pFJXtraR	pFJX carrying the 743-bp region (60) upstream of the <i>traR</i> start codon fused to lacZ, amplified using traRpro5 and traRpro3 and cloned as a BamHI fragment	
pSDZrdfspro	carrying <i>rdiS</i> promoter-lacZ fusion, contains inducible <i>lac</i> promoter	Josh Ramsay, this lab
pSD172171	pSDZrdfspro containing <i>msi172msi171</i> -6H downstream of <i>lac</i> promoter	Josh Ramsay, this lab
pSD172171FS	pSDZrdfspro containing <i>msi172msi171</i> -6H FRAMESHIFTED downstream of <i>lac</i> promoter	Josh Ramsay, this lab
pF172171	pFAJ1708 containing <i>msi172</i> and <i>msi171</i> . <i>msi171</i> is C-terminally 6-H tag Tc ^R	Laura Tester, this lab
pFΔT229	pFAJ1708 containing engineered frame-shift of <i>msi172</i> and <i>msi171</i> . <i>msi171</i> is C-terminally 6-H tagged Tc ^R	Laura Tester, this lab
pBT	Bacteriomatch II bait cloning vector, p15A oriT, Cm ^R , <i>lac-UV5</i> promoter, λcl ORF	Agilent Technologies
pBTL	pBT carrying a (Gly ₄ -Ser) ₃ linker (74) between λcl and cloning cassette, p15A oriT, Cm ^R , <i>lac-UV5</i> promoter, λcl ORF	
pTRG	Bacteriomatch II target cloning vector carrying RNAP-α fragment, <i>lpp/lac-UV5</i> promoter, ColE1 oriT, Tc ^R	Agilent Technologies
pBTLGF2	Bacteriomatch II positive control plasmid, p15A oriT, Cm ^R , <i>lac-UV5</i> promoter, λcl-LGF2 fusion	Agilent Technologies
pBTqseM	pBT carrying a cl-QseM fusion gene, (60) <i>qseM</i> cloned as a NotI fragment	
pBTLtraR	pBTL carrying a cl-(Gly ₄ -Ser) ₃ -TraR fusion gene, <i>traR</i> cloned as an EcoRI-NotI fragment	(60)
pBTL172171	pBTL carrying a cl-(Gly ₄ -Ser) ₃ - <i>msi172msi171</i> fusion, cloned as MfeI and SpeI fragment	This Study

pBTLfseA	pBTL carrying a $\text{cI}-(\text{Gly}_4\text{-Ser})_3\text{-msi172msi171}$ permanent frameshift fusion, cloned as MfeI and XhoI fragment	This study
pTRG-Gal11 ^P	Bacteriomatch II positive control plasmid, <i>lpp/lac-UV5</i> promoter RNAP- α -Gal11 ^P fusion, ColE1 oriT, Tc ^R	Agilent Technologies
pTRGqseM	pTRG carrying an RNAP- α - <i>qseM</i> fusion, <i>qseM</i> cloned as a NotI-EcoRI fragment.	(60)
pTRGtraR	pTRG carrying an RNAP- α - <i>traR</i> fusion, cloned as a NotI fragment	(60)
pTRG172171	pTRG carrying an RNAP- α - <i>msi172-msi171</i> fusion, cloned as MfeI and SpeI fragment	This study
pTRGfseA	pTRG carrying an RNAP- α - <i>msi172-msi171</i> permanent frameshift fusion, cloned as MfeI and XhoI fragment	This study
pTRGmsi171	pTRG carrying an RNAP- α - <i>msi171</i> fusion, from PCR product	(61)
pTRGmsi172	pTRG carrying an RNAP- α - <i>msi172</i> fusion, from PCR product	(61)

2.2.1 Bi-parental spot matings

Introduction of plasmids from *E.coli* ST18 into *M. loti* strains was achieved through bi-parental spot matings. *E.coli* ST18 was used as it contains an integrated plasmid that enables formation of a conjugal bridge and subsequent transfer of DNA from donor to recipient strain. As *E.coli* ST18 is also auxotrophic for 5-aminolevulinic acid no growth occurs unless the media is supplemented with it (68).

Both *E.coli* and *M.loti* strains were grown to stationary phase in TY. 250 μl of each culture was centrifuged at 8000 g for 1 min before being resuspended in 100 μl of TY and combined. Once vortexed, cells were centrifuged again and resuspended in 100 μl of TY, where 50 μl was spotted onto TY agar and incubated at 28 °C overnight. The spot was then subjected to a serial dilution series and 100 μl spread onto media containing the correct selective antibiotics.

2.3 Enzymes and Chemicals

Enzymes used in this study were purchased from Roche Molecular Biochemicals or New England Biolabs. All chemicals were analytical grade and dissolved in water unless otherwise specified. Antibiotics were provided by Sigma and made up to the concentrations listed in Table 1. Gm, Km, Nm and Am were all dissolved in MilliQ® water, whilst Tc and Cm were dissolved in 100% methanol and 100% ethanol respectively. Solutions were filter-sterilised through a 0.45 µm syringe filter (Sartorius) unless dissolved in alcohol.

2.4 Storage of Strains

Strains to be stored were grown to stationary phase in LB for *E. coli* or GRDM for *M. loti*. Culture (700 µl) was mixed with 80 µl of dimethylsulfoxide (DMSO) or 300 µl of 50% glycerol in cryovials (Cryo.S) and stored at -70°C.

2.4.1 Frozen inocula

For RT-qPCR assays using *M. loti* broth cultures, frozen inocula were used for convenience and to produce reproducible culture growth. Inocula were produced from 5-ml TY broths grown from single colonies over a period of 64 h (stationary phase). Culture (700 µl) was thoroughly mixed with 300 µl of 50% glycerol and immediately stored at -70°C for future use. All inocula were frozen in duplicate.

2.5 DNA Isolation

2.5.1 Commercial extraction kits

Plasmid DNA used for cloning, template DNA or enzyme digests was isolated from 5-ml broth cultures of *E. coli* using the Roche High Pure Plasmid isolation Kit (#11754785001) or a Zymo Zippy plasmid miniprep kit (#D4020). Instructions from the kits were followed with an additional follow-up spin (15,871 g) with no wash buffer added. Furthermore, 30 µl of filter-sterile water was added for the elution step.

2.5.2 Alkaline Lysis Prep

Plasmid DNA was extracted using a method based on Feliciello and Chinali (75). Five ml overnight cultures of *E. coli* grown in LB at 37°C were harvested via centrifugation. Pellets were resuspended in 250 µl of lysis solution (0.2M NaOH, 1 % w/v SDS, inverted 8 times and incubated at room temp for 1 min. 350 µl of neutralising solution (3M KAc, 5% w/v formic acid) was then added and mixed via inversion. Lysate was then centrifuged at 15,871 g for 10 min, before supernatant was removed to a new tube. DNA was precipitated with the addition of 600 µl of isopropanol. Samples were then centrifuged again at 15,871 g for 5 min after which supernatant was discarded and DNA pellets were then washed in 1 ml of 70% ethanol and another 5 minute spin. Samples were then air dried at 37°C and resuspended in 50 µl of filter sterile MilliQ water.

2.5.3 PrepMan™ Ultra DNA preparations

Crude DNA for use in colony PCR was extracted from bacterial pellets harvested from 1 ml of overnight broth culture inoculated from single colonies. This pellet was resuspended in 100 µl of PrepMan™ Ultra reagent (Applied Biosystems) in microfuge tubes (Axygen), which were sealed with autoclave tape, placed in a boiling water bath for 10 min, cooled on ice for 2 min and centrifuged at 15,871 g for 5 min. The supernatant was transferred to a clean microfuge tube and either frozen for future use or used immediately in a PCR reaction (section 0) then subsequently frozen.

2.5.4 Ethanol Precipitation

DNA samples were precipitated by adding 3 volumes of 100% ethanol and 0.1 volumes of sodium acetate (3 M, pH 5.2). Visualisation of precipitated DNA was aided by adding 1 µl of pellet paint (Novagen). Samples were centrifuged for 10 min at 15,871 g. The supernatant was discarded and the pellet was rinsed with 70% ethanol and centrifuged for 5 min at 15,871 g. The supernatant was once more removed and the pellet was air dried at 37°C for approximately 15 min. The pellet was resuspended in 30 µl of sterile water.

2.6 DNA Manipulations

2.6.1 Restriction enzyme digests

Vector DNA preparations and insert DNA PCR preparations were digested for at least 1.5 h at 37 °C, using an appropriate buffer for both restriction enzymes where possible. When a conflict of buffers existed, a single digest was carried out followed by an ethanol precipitation and digestion with the second enzyme and buffer. Vector DNA was dephosphorylated by adding 2 µl of calf alkaline phosphatase (Roche) and incubating for 1 h at 37°C.

2.6.2 Subcloning of inserts

Inserts were prepared for ligation through PCR, using gene specific primers that contained specific or broad restriction enzymes cut sites. The PCR product insert was cleaned with a Roche High Pure PCR Product Purification Kit and digested with the required enzymes.

2.6.3 Agarose gel electrophoresis

To visualise DNA fragments on gels, 5 µl of PCR products were mixed with 20% v/v tracking dye (1 mL of 10 mg/mL bromophenol blue, 1 mL MilliQ® water and 1 mL glycerol). The marker was a mix of λ DNA HindIII digests and bacteriophage Φ X174 DNA HaeIII digests (Finnzymes). Electrophoresis was carried out on a 1% agarose gel in 1x Tris- acetate (TAE) buffer (40 mM Tris pH 8, 20 mM acetic acid, 1 mM EDTA pH 8, 1 µg/ml ethidium bromide) at 90 to 100 volts for approximately 1 h. Images of gels were captured with a Kodak Gel Logic 200 imaging system under ultraviolet (UV) light.

2.6.4 Agarose gel extraction

Bands were excised from the gel using a sterile scalpel and placed on a UV transilluminator. DNA was separated from the gel slice using a Roche High Pure PCR Product Purification Kit (Roche, cat #1732688) following the manufacturer's instructions.

2.6.5 Ligations

Ligation mixtures had a final volume of 20 µl consisting of the following: 3 µl of vector DNA (300 ng/ml, 10 µl of insert (30 ng/ml), 2 µl of 10x ligation buffer (Roche), 1 µl of T4 DNA ligase (Roche) and 4 µl of filter sterile MilliQ water. Ligations were incubated overnight at 12°C.

2.6.6 DNA sequencing

Purified plasmid DNA samples and PCR products were sequenced by the Allan Wilson Centre Genome Service (ABI3730 Genetic Analyzer, Applied Biosystems; Massey University, Palmerston North, NZ). The plasmids constructed in this study were all sequenced.

2.7 Polymerase chain reaction

PCR was used to amplify plasmid insert DNA for electroporation and sequencing, and insert DNA for cloning and sequencing. RT-qPCR was used to quantify the relative abundance of specific RNA molecules within a culture at a giving time point.

2.7.1 Primers used for PCR, RT-qPCR and DNA sequencing

Primers were designed ensuring similar annealing temperatures and purchased from Invitrogen. They were resuspended as 100 nM/ml stocks.

Table 3. Primers used in this study.

Name	Sequence 5' - 3'
Qe_80msi170BamHI_fwd	TCACGGATCCAAACGCAAAGTCCAAGACGAA
Qe_80msi170_HindIII_rev	TCACAAGCTTTCAGCGCGCGAACAGTTCT
Probe_msi170_asp718_fwd	ATTAGGTACCATTAAGAGGAGAAATTAAGTATGAGA
Probe_msi170_ecori_rev	ATTAGAATTCTCAGCGCGCGAACAGTTCT
pBTL-172-171_fwd	ATATCAATTGGCGCTATGATAGGTAATGA

pBTL-172-171_rev	ATTACTCGAGTTAGTGGTGATGATGGT
pTRG-171-171_fwd	ATATCAATTGGCGCTATGATAGGTAATGA
pTRG-172-171_rev	ATTA <u>ACTAGT</u> TTAGTGGTGATGATGGTG

RT-qPCR

gyrAQPCR ^F	CCAATCTGCTGGTCAACGGCT
gyrAQPCR ^R	CGGTCGAATAGGCGCTGTAGA
msi170_QPCR_5	CAATGAGCACTTCACCATCTATATGA
msi170_QPCR_3	TATCCAGTCGTAACCATCCAGT
traRQPCR ^{V2F}	GCATTTCAACGCGACAGCAACA
traRQPCR ^{V2R}	ATAATCTCGAGCGCCTGCTCAT
tral2_v2_QPCR	AAACCGCACTATCTTGTGTTGT
tral2_V2_QPCR_3	GCAACAGCAATACTCCCACT
tral1_qpcr_5	GAGGCGAAATGGAAACAGACA
tral1_qpcr_3	CCACAGCCTCACCCAGCA
msi170_QPCR_5	TATCCAGTCGTAACCATCCAGT
msi170_QPCR_3	AACGACTATGCCGGCATCGA
rdfS_5_QPCR	GGCAGTCCGTTTCTGAACA
rdfS_3_QPCR	TCGATGTGGTAGCGGACATA

2.7.2 Standard PCR protocol

The Phusion High Fidelity PCR system (Finnzymes) was used for amplification of genomic or plasmid DNA. The reaction consisted of 10 µL of 5X Phusion High-Fidelity buffer, 1 µL of each primer (1pM/µl), 1 µL of dNTP mixture (provided with kit), 0.5 µL of Phusion High-Fidelity Polymerase, 0.5 to 5 µL of DNA template suspension. MilliQ® water was used to bring the reaction volume to 50 µL. Cocktails were used to ensure consistency. Thermal cycling was

performed using a Hybaid PCR Express thermal cycler. PCR cycling conditions were as follows: 1 x 98°C (60 s); 30 x 98°C (10 s), 57°C (15 s), 72°C (15 s/kb); 1 x 72°C (5 min).

2.7.3 PCR product purification

PCR products were purified using the Roche High Pure PCR product purification kit (Roche, cat #1732688) or ethanol precipitation for small amounts (<30 ng/ml) of product.

2.7.4 Reverse transcriptase - quantitative PCR

Assay flasks were inoculated using 180 µl of previously prepared frozen inoculum (2.4.1). RNA was extracted from 8 ml of *M. loti* culture following 24 h growth in TY at 28°C (OD₆₀₀ of 0.8–1.2). RNA was stabilized using RNeasy Protect Bacteria (Qiagen) and extracted using Protocol 4 from the RNeasy Protect Bacteria Reagent handbook (Qiagen). DNAase treatment was carried out using the TURBO DNA-free kit (Ambion). cDNA synthesis was carried out using random hexamers (Qiagen) and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. RT-qPCR was performed on an ABI ViiA 7 machine using Fast SYBR Green Master Mix (Applied Biosystems) and analysed using ViiA 7 software version 1.2. Cycling conditions were 20 s at 95°C then forty cycles of 3 s at 95°C and 30 s at 60°C. Relative quantification was determined using primers specific for *gyrA* as a housekeeping gene and efficiency correction was used for the calculation of final values

2.8 Electroporation

2 µl of plasmid DNA or 6 µL of ligation product was dispensed into 40 µL of thawed *E. coli* ST18 electrocompetent cells. The mixture was transferred to a chilled 1 mm cuvette (Biorad) and electroporated at 1800 V using a Biorad GenePulserXcell. The culture was immediately suspended in 1 mL LB and incubated at 37°C with shaking at 200 rpm for 1 h, then plated on LB agar with appropriate antibiotics.

2.9 β -galactosidase activity assays

The monitoring of β -galactosidase produced from reporter-gene fusions was determined using 4'-Methylumbelliferyl- β -D-glucuronide. Ten-microliter aliquots of each sample culture were frozen at -80°C for 10 min and then thawed at room temperature. Next, 100- μL reaction buffer (PBS, 400 $\mu\text{g}/\text{mL}$ lysozyme, 250 $\mu\text{g}/\text{ml}$ 4'-Methylumbelliferyl- β -D-glucuronide) was added and samples were immediately monitored in an Infinite Tecan M200 microplate reader with the following parameters; excitation 360 nm, emission 450 nm, gain manual 85, eight reads per well, measured every min for 30 min. RFUs produced/s were calculated from a period of linear increase in fluorescence normalized to the OD_{600} of the sample.

2.9.1 Cultures for β -galactosidase assay

Strains were grown in 5ml, TY broths, containing 1 mM IPTG to; 24, 48 or 72 h, dependant on the assay type. Aliquots of 200 μl of each sample were stored in 96-well plates (BD Falcon) and frozen at -70°C for future assays.

2.10 Protein expression and purification

The plasmid pQe80-6HqseM used within the protein expression experiments was created by amplifying the *qseM* gene from pNqseM with primers containing appropriate cut sites for pQe80. The *qseM* region now with attached 6H tag was then cut out and ligated back into pPROBE-KT to produce pN6HqseM with two clones being tested in section 3.2 for proper function of QseM, while pQe80-6HqseM was sent for sequencing.

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

Proteins were separated by SDS-PAGE using the mini-PROTEAN Tetra System (BIORAD). The buffer system was based on Laemmli (76). The composition of gels and buffers are listed in Appendix A. A ratio of 3:1 protein: dithiothreitol (DTT) and loading dye (total volume 16 μl) was boiled for 5 min, briefly cooled at room temperature and loaded into a discontinuous gel composed of a 4%

acrylamide stacking gel and a 15% acrylamide resolving gel. Electrophoresis was carried out at 125 V for 30 min and 90 V for up to 3 h. Gels were stained for 30 min with Coomassie brilliant blue staining solution (0.025% Coomassie Brilliant blue R 250, 40% methanol and 7% acetic acid). Gels were destained using MilliQ water on a platform shaker until the majority of background staining was removed. Gels were imaged with an Epson Perfection 1650 scanner.

2.10.2 Purification and analysis of 6HQseM

Two 5-ml overnight LB cultures of *E. coli* strain BL21(DE3)(pLYS) carrying plasmid pQe80-6HQseM were used to seed two 400 ml broths supplemented with Ap and 1 mM IPTG, which were then incubated at 28°C overnight. Cells were collected by centrifugation and resuspended in 50 ml lysis buffer [50 ml Wash buffer, 1 x Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche)]. The cell suspension was then processed twice through an E1061 constant cell disruption system (Mitsubishi Electric) at 6900 kPa to lyse cells. Insoluble material was removed by centrifugation at 15 000 g for 30 min. Clarified lysate was passed through a PolyPrep® Chromotography column (BIORAD) containing 400 ml of Ni-NTA resin (Qiagen), pre-equilibrated with wash buffer [50 mM NaH₂PO₄·2H₂O, pH 8.0, 100 mM NaCl, 20 mM imidazole, 20% glycerol]. Bound protein was washed overnight with 500 ml of wash buffer, and eluted with 10 ml of elution buffer [50 mM sodium phosphate buffer, pH 9.2, 100 mM NaCl, 250 mM imidazole, 20% glycerol] into ten approximate 1 ml fractions. The presence of purified 6HQseM was confirmed by analysis on a 15% acrylamide SDS-PAGE gel and by mass spectrometry of the trypsin-digested band. Fractions containing purified 6HQseM were combined and concentrated in a centrifugal filter unit (Amicon Ultra 10K, 0.5 ml) to a concentration of ~1 mg /ml (estimated using the Pierce BCA Protein Assay Kit) and stored in elution buffer at 4°C.

2.10.3 Cell lysate preparation for detection of proteins interacting with QseM

Two 5-ml broths of R7AΔ*qseM* grown for 24 h were used to seed two 200 ml TY broths. The cultures were grown for 72 h with shaking and centrifuged at 8000

rpm for 15 min. Pellets were resuspended in lysis buffer, then the cell suspension was processed twice through an E1061 constant cell disruption system (Mitsubishi Electric) at 6900 kPa to lyse cells. Insoluble material was removed by centrifugation at 15 000 g for 30 min. Thirty μ l of 100 mM 3-oxo-C6-HSL was added before a final clarification centrifugation (7,740 g) to encourage any AHL-dependant protein to remain dissolved. Ni-NTA resin (400 μ l) was added post-clarification to capture any Ni-binding protein present that could indicate a false positive. Once incubated for 3 to 5 min, a final centrifugation (7,740 g) was carried out to remove the Ni-NTA resin. The lysate was used immediately as described in section 2.10.4.

2.10.4 Protein capture column set up

Two columns were used for capturing proteins. One column contained 6HQseM bound to Ni-NTA resin, achieved by incubating 6HQseM with Ni-NTA resin overnight at 4°C with shaking, the other column contained NTA resin only. Columns were set up at the same time with Ni-NTA mixtures being added to and circulated through a column (BIO-RAD Poly-Prep®) containing wash buffer until the mixture settled. R7A Δ *qseM* lysate was passed through the column followed by 20 ml of wash buffer and run until almost dry. Two ml of elution buffer was added and forced through with 3 fractions being caught for each column. Presence of re-purified 6HQseM and possible target protein patterns were visualised on a 15% acrylamide SDS-PAGE gel (2.10.1) and by mass spectrometry of the trypsin-digested lanes.

2.10.5 Mass spectrometry

Bands or lanes from SDS gels were sent for mass spectroscopy by the Centre for Protein Research in the Department of Biochemistry, University of Otago using a LTQ-Orbitrap hybrid mass spectrometer. A database for comparison, created by Dr. Josh Ramsay, contained known ICEMISym^{R7A} protein sequences in all possible frames. The trypsin digested chunks were referenced against each provided sequence and the likelihood that chunk was from that protein.

2.11 Two-hybrid analysis

Experiments to detect protein–protein interaction of QseM and possible targets were performed using the Bacteriomatch II system (Agilent Technologies), according to the manufacturer’s instructions, with the following modifications. Screening media contained 13.5% w/v Na₂HPO₄, 0.6% w/v KH₂PO₄, 0.1% w/v NaCl and 0.2% w/v NH₄Cl and M9 media additives [0.19% w/v Yeast Synthetic Drop-out Medium Supplement (Sigma, Cat Y-1751-20G), 0.4% glucose, 0.1 mM adenine hemisulphate, 1 mM MgSO₄, 0.1 mM Thiamine HCl, 0.01 mM ZnSO₄, 0.1 mM CaCl₂, 0.05 mM IPTG]. Selective and non-selective media were supplemented with 14 mg/ml Tc and 10 mg/ml Cm. Selective media contained 5 mM 3-AT. Electrocompetent ‘BacterioMatch II Validation Reporter Competent Cells’ were prepared and used in plasmid cotransformation assays, 50 ng of each plasmid along with 40 µl of cells were used per cotransformation. Transformation efficiency was estimated by dilution series on non-selective medium. Positive protein–protein interactions were detected by increased colony-forming units (cfu) on media containing 3-AT compared with empty plasmid controls. Cfus were normalized to transformation efficiency on medium lacking 3-AT.

2.11.1 Creation of Msi172-Msi171 and Msi172-Msi171FS fusion combinations

Genes *msi172msi171* or *msi172msi171FS* were amplified from plasmids provided by L. Tester, via PCR with two sets of primers that contained specific restriction sites for each vector (Table 3). Digests were carried out at 37 °C for 2 h unless otherwise stated. PCR products destined for pBTL were digested first with Mun1 then cleaned with an Ethanol precipitation, followed by a second digest with Xho1 and a second Ethanol precipitation, while PCR products for pTRG were double digested with Mun1 and Spe1 then cleaned via Ethanol Precipitation. pBTL vector preps were digested with EcoR1 and Xho1, while pTRG vector preps were digested with EcoR1 and Spe1. EcoR1 was used on vectors as it and Mun1 are cut site orthologs and leave the same sticky end, streamlining the digestion process. Cut vectors were then treated with alkaline phosphatase for 1 hour at 37 °C and were gel purified using the method in

section 2.6.4. Ligations were performed as per section 2.6.5, with 5 μ L being electroporated into XL1–Blue MRF competent cells for storage at -70 °C (Agilent Technologies).

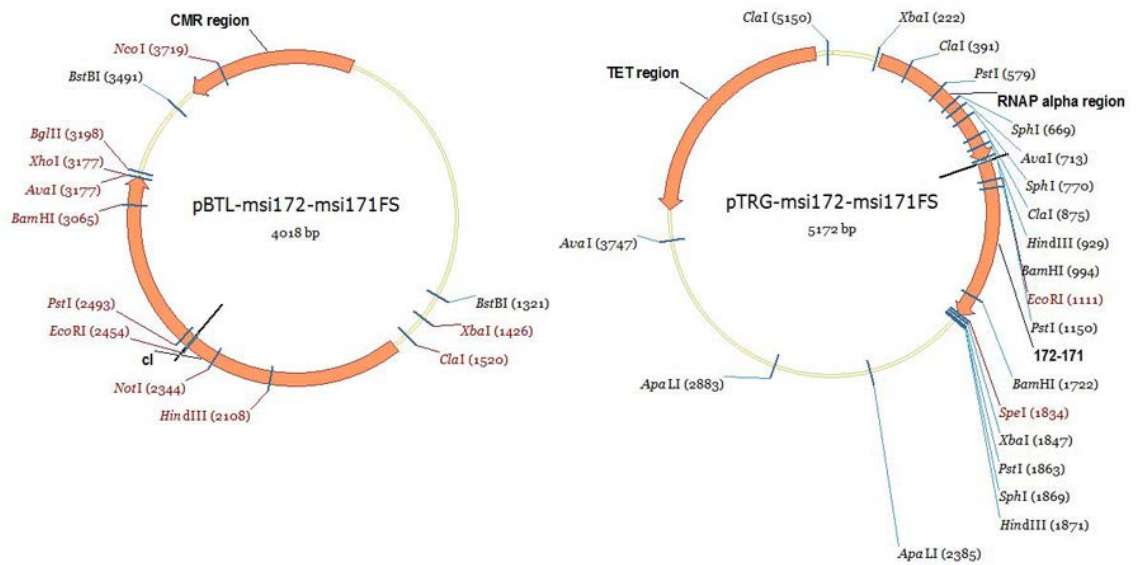


Figure 5. Example plasmid map for Two-hybrid assays.

pBTL and pTRG based plasmids for the use in two hybrid assays. In these plasmids *msi172msi171* or *msi172msi171FS* have been cloned downstream of the RNAP- α or the *cl* region. In both plasmids, the longer black line represents the beginning of the gene being carried, in this case *msi172msi171FS*.

2.12 AHL detection using *C. violaceum*, CV026

To detect short-chain AHL production, Nunc 25 x 25 cm bioassay plates containing 200 ml LB solidified with 1.5% agar were overlaid with 100 ml of warm (<40 °C) LB with 0.5% agar containing 5-ml of stationary phase culture of the CV026 reporter strain. One-cm diameter holes were cut 6 cm apart through both layers of agar. Assay strains were grown to late stationary phase (72 h) and the supernatant was harvested, filter-sterilised and 150 µl was aliquoted into a well, with the remainder being stored at -70 °C. The plate was incubated at 28°C for 24 h agar side down.

3 Results

As described in the Introduction (Section 1.5), QseM is a master negative regulator of ICEMISym^{R7A} excision. Genetic studies indicate it is likely to work through TraR to prevent activation of the *tral2-msi172-msi171* operon. However overexpression of QseM leads to a lower level of excision than found in a R7AΔ*traR* mutant, suggesting that QseM may also have additional targets. The aims of this study were to identify the target(s) of QseM within the genes or proteins involved in the regulation of the excision and transfer of ICEMISym^{R7A}, and to determine how QseM interacts with them, possibly via direct protein-protein interaction.

Two families of plasmid vectors were mainly used in this study, pPROBE-KT (71) and pSRKGm (72). pPROBE-KT is based on pVS1/p15A (77) and is the parent plasmid of pNqseM. pNqseM contains *qseM* cloned downstream of the constitutive *nptII* promoter, creating constitutive expression of QseM (60). The other vector, pSRKGm, is based on the vector pBBR1 (78) and has an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter as well as the *lacI^q* gene, providing an inducible expression system and allowing the safe introduction of potentially lethal genes. Several derivatives of pSRKGm were used, each containing different genes that are members of the ICEMISym^{R7A} excision and transfer system. These derivatives were constructed by Prof. M. Hynes while on research and study leave in this lab. The “growth-inhibitory” phenotype mentioned throughout this section is due to either the overexpression of the *msi172-msi171* genes (Figure 3), or the consequential overexpression of one or both of the *rdfS* and *traF* genes (M. Hynes, personal communication) that then prevent R7A cell division by unknown mechanisms.

3.1 QseM provides protection against the growth inhibitory phenotype caused by TraR over-expression

Previous genetic studies showed that artificial overexpression of TraR or Msi172-Msi171 caused a growth-inhibitory phenotype and that pNqseM is able to complement a Δ*qseM* mutant, reducing excision of ICEMISym^{R7A} to below

wild type levels. It was hypothesized that QseM may repress the expression of the QS genes through sequestering the TraR+3-oxo-C6-HSL complex. It was decided to create strains containing pPROBE-KT or pNqseM and pSRKGm or pSRKGmtraR within different host backgrounds to test in a CV026 assay (Section 3.2). However during the initial strain creation, it was noticed that introducing pSRKGmtraR into strains resulted mainly in an absence of colony growth or production of very few, small, non-passageable colonies. However strains that contained pNqseM were less affected by this phenomenon. It was decided to suspend other experiments using these strains and focus on this growth-inhibitory phenotype instead, caused by leaky expression from the inducible promoter (Section 3.1.1).

3.1.1 Bacterial Conjugation Assay exploring QseMs ability to protect against TraR induced growth-inhibition.

From the difficulties experienced introducing pSRKGmtraR into R7A/pPROBE-KT, R7A/pNqseM and other strains described in the previous section, bacterial conjugation assays were used to investigate the ability of QseM to suppress the *traR*-mediated growth-inhibitory phenotype in a variety of strains. Initial testing saw each strain contain either pNqseM or pPROBE-KT with pSRKGmtraR introduced via spot mating into the strain. A serial dilution of the resuspended spot mating was carried out and 80 µl of each dilution was spotted across a 25 cm by 25 cm bioassay plate of GRDM containing appropriate concentrations of Nm, Gm and no IPTG, ensuring there was no induction of the promoter. This method allowed a large scale comparison of any protective effect of QseM across a number of strains at once, visualised as variations in growth amount. As well as the wild-type strain R7A, strain R7A* was to be used in later experiments. This strain is a mutant of R7A that over-produces 3-oxo-C6-HSL and is more sensitive to the growth-inhibition caused by overexpression of TraR which is why it is assayed here. The site of the mutation in R7A* is unknown (16).

A trend across all strains assayed was that the presence of pNqseM permitted growth down to a lower dilution. This occurred in strains that contained a

functional *qseM* and also those that did not; importantly, the strain *R7AΔqseMΔtraR* that lacks genomic *qseM* or *traR* also showed this effect (Figure 6). Conversely strains containing the control plasmid pPROBE-KT had reduced levels of growth across all dilutions, especially *R7AΔqseM*/PROBE-KT/pSRKGmtraR and *R7A**/pPROBE-KT/pSRKGmtraR, producing only several non-mucoid colonies on the 10^{-1} dilution that could not be subcultured. *R7A*/pPROBE-KT/pSRKGmtraR and *R7AΔqseMΔtraR*/pPROBE-KT/pSRKGmtraR both produced slightly more growth at lower dilutions than the previously mentioned strains, indicating less TraR expression in these strains as predicted from their genotypes.

All strains containing pNqseM produced numerous single colonies right down to 10^{-3} dilution when pSRKGmtraR was introduced. *R7A*/pNqseM/pSRKGmtraR, *R7AΔqseM*/pNqseM/pSRKGmtraR and *R7AΔqseMΔtraR*/pNqseM/pSRKGmtraR all produced several single colonies at 10^{-4} , indicating excess QseM conferred a protective effect against leaky TraR expression and possible activation of downstream genes (Figure 6). This result suggests that QseM and TraR may interact at the protein level, as in *R7AΔqseMΔtraR*/pNqseM/pSRKGmtraR where both genes are uncoupled from their native promoters, the protective effect still occurred. The protective effect could be explained by QseM interacting directly with TraR or with downstream genes or their products activated by TraR expression, such as the *traI1* operon or *rdfS* operon, possibly removing gene products. The leaky expression and subsequent growth-inhibition experienced in this experiment provided a foundation for future experiments as the phenotype could be selected for with ease.

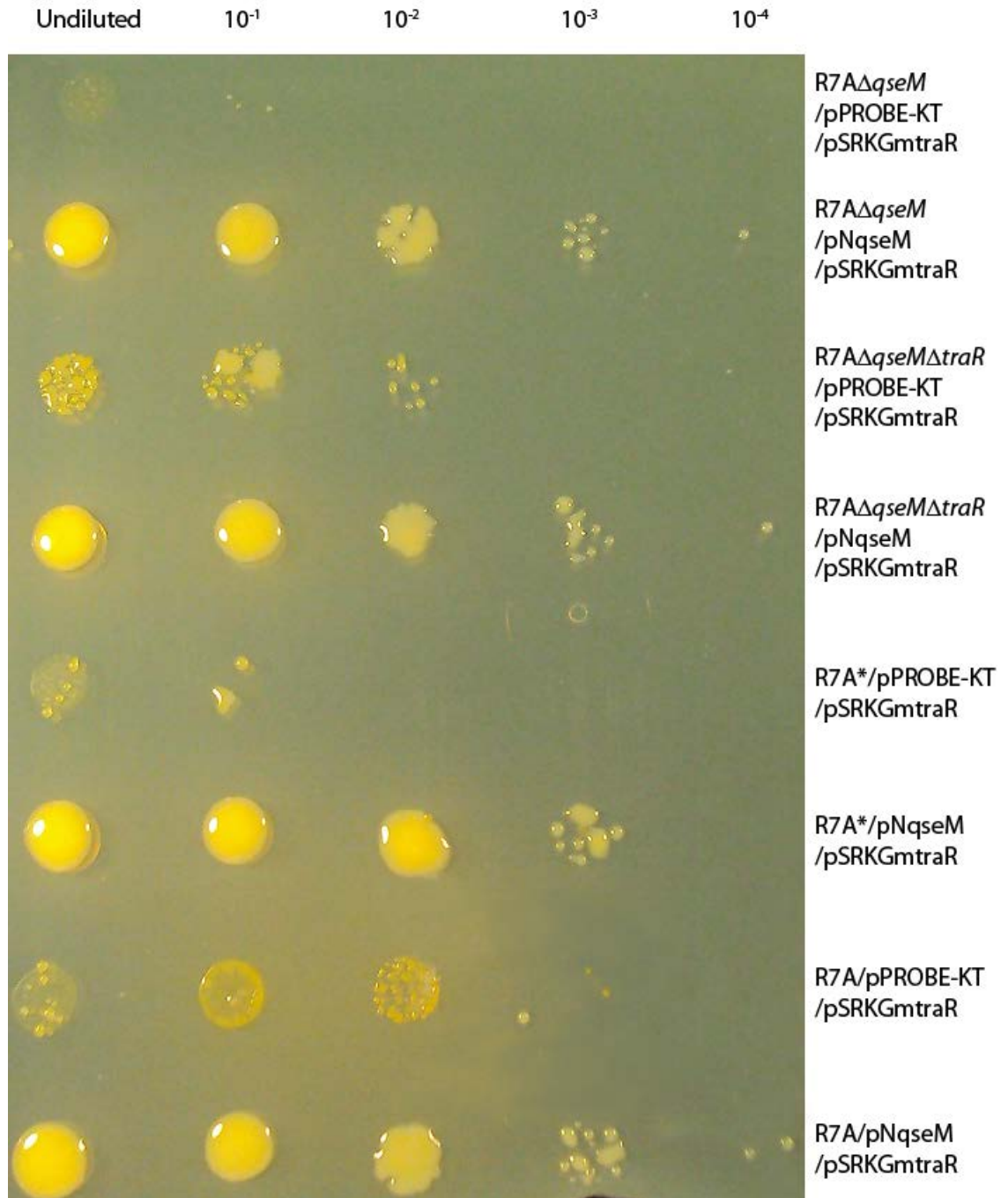


Figure 6. Bacterial Conjugation Assay plate for the introduction of pSRKGmtraR into various strains through spot mating.

Each spot mating was resuspended in TY broth before a serial dilution was carried out on the resuspended spot mating. 80 µl aliquots were spotted onto the plate in a grid pattern enabling direct visual comparison between strains containing *qseM* or not. The growth-inhibitory effect caused by leaky *traR* expression can be seen, while the protective effect of QseM is clearly visible when compared against strains that do not contain a functional *qseM* gene. Strain names are listed on the right. Small, yellow and

hard-looking colonies seen in many strains lacking pNqseM were unable to be subcultured. No growth was seen beyond the 10^{-4} dilution.

3.1.2 QseM does not regulate expression from the *traR* promoter

Previous RT-qPCR experiments conducted by Josh Ramsay indicated that QseM might regulate expression from the *traR* promoter. To test this hypothesis, a previously made construct pFJXtraR, which contains the *traR* promoter region upstream of *lacZ*, was introduced into R7A, R7ANS and R7A Δ qseM alongside a negative control. Plasmids pNqseM and pPROBE-KT were then introduced and the resulting strains were grown to log phase and assayed for expression levels from the *traR* promoter. It was hypothesised that QseM may bind the *traR* promoter to block TraR production, so within this model there should be a decrease in expression of *lacZ* if this interaction does take place.

The resulting β -galactosidase activities (RFU/sec/OD₆₀₀; Section 2.9) listed in Table 4 showed that this is not the case. Expression levels across all strains were very similar with no clear indication of expression of the *traR* promoter obtained. This cannot be taken as confirmation that QseM interacts with the *traR* promoter. Further experimentation would be necessary to complete the picture.

Table 4. Variation of *lacZ* expression across different strains in the presence or absence of QseM across three biological replicates.

Strain	RFU/sec/OD ₆₀₀	Standard deviation
R7A/pFJX	6.22	0.56
R7A/pFJXtraR	5.07	0.27
R7A Δ qseM/pFJX	4.82	0.23
R7A Δ qseM/pFJXtraR	4.80	0.67
R7ANS/pFJXtraR/pPROBE-KT	6.12	1.48
R7ANS/pFJXtraR/pNqseM	5.26	0.37

3.2 Production of excess QseM inhibits AHL production as quantified through the CV026 bioassay

The aim of the experiment described in this section was to determine whether over-expression of QseM prevents AHL production that occurs in wild-type cultures and cultures overexpressing TraR. *Chromobacterium violaceum* strain CV026 was used as an indicator of short-chain AHL production. In the presence of AHLs, including 3-oxo-C6-HSL produced by TraI1 encoded on ICEMISym^{R7A}, CV026 produces the purple pigment violacein. The *M. loti* strains tested were hypothesised to produce AHLs at natural levels; or artificially high levels through the expression of a plasmid-borne *traR* gene. Control strains that were known not to produce AHLs were also included.

Initial experiments showed that the R7AΔ*qseM* mutant induced a large purple halo that was dependent on TraR, as the R7AΔ*qseM*Δ*traR* mutant did not induce violacein production (Figure 7). The R7AΔ*qseM* mutant was complemented by pN*qseM* and by two plasmids that produced a 6H tagged version of QseM (Section 3.8), indicating these plasmids all constitutively produced active forms of QseM, while the control strain containing the empty vector, pPROBE-KT, produced similar amounts of violacein as R7AΔ*qseM* (Figure 7). The differences in halo sizes (Figure 7) are semi-quantitative as the amounts of AHLs produced were not measured.

A reverse trend can be seen for the R7AΔ*traR* mutant, with no AHL produced due to an important segment of the feedback loop, TraR, missing. When R7AΔ*traR* was complemented with an uninduced pSRKGm*traR* plasmid, a large halo of violacein was seen. The production of this halo reflects the leaky expression seen, and previously described in Section 3.1. Interestingly the halo size was similar to that of the R7AΔ*qseM* mutant, suggesting that sufficient TraR was produced through leaky expression from pSRKGm*traR* to give full expression of target operons.

R7AΔ*rdiS* was assayed as it would likely be used in a future experiment and there was suspicion about its parent strain being R7A* (see above; 6), which produces naturally elevated levels of AHL through an unknown mutation. As

shown in Figure 7, AHLs were present to a similar level as in R7A* (16) and much greater than those seen in R7A. This result excluded R7A Δ *rdfS* from any further experiments.

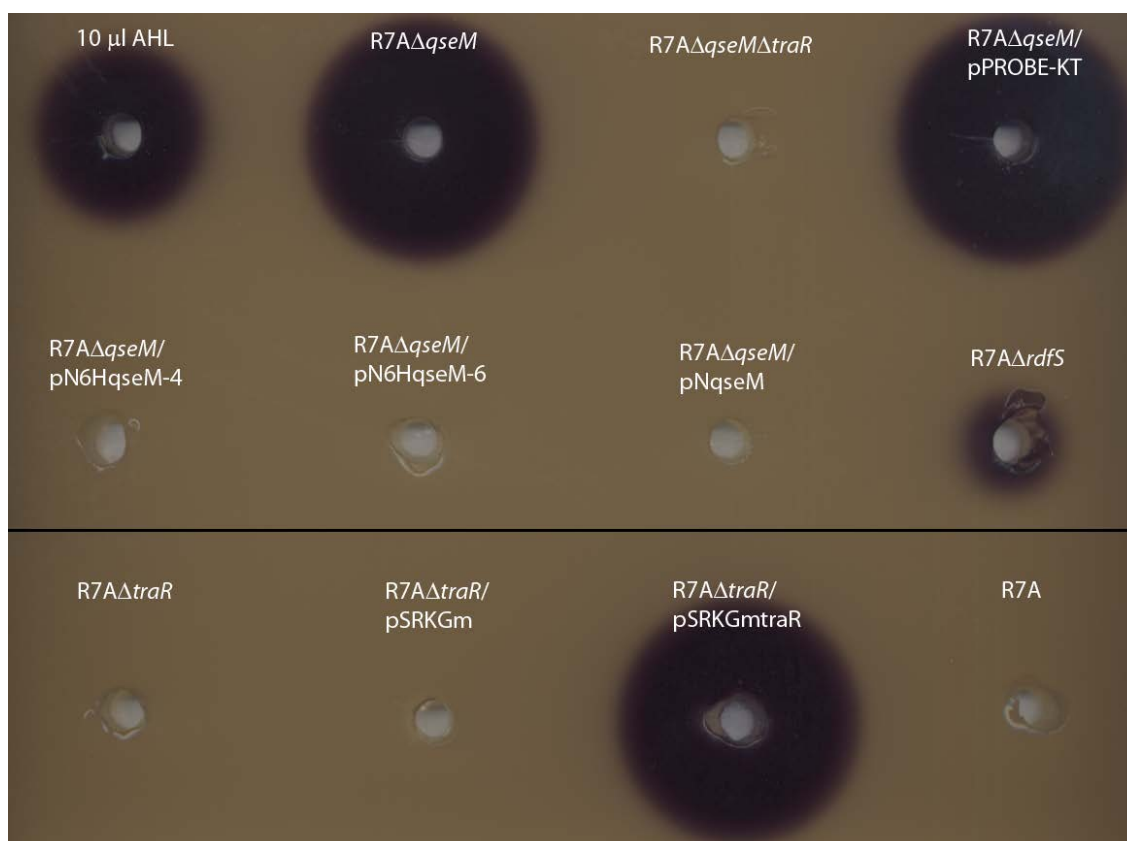


Figure 7. CV026 bioassay for N-acyl homoserine lactone production.

All mutants assayed were created in the R7A background (though it is likely that the Δ *rdfS* mutant was unknowingly made in the R7A* background, see text). The synthetic AHL control of 10 μ M 3-oxo-C6-HSL is plated in the top left corner, while R7A is in the bottom right. Culture supernatant (150 μ l) was added to the wells and the plate incubated overnight, agar side down. The presence of AHLs is shown by the halo of the purple pigment of violacein around the well. Strain names are listed above wells.

3.3 QseM protects against growth-inhibitory effects of *msi172-msi171* over-expression.

To explore another possible interaction site of QseM within the QS system an inducible plasmid, pSRKGm172171, was introduced by spot-mating to strains R7A/pPROBE-KT and R7A/pNqseM. The genes *msi172-msi171* exist downstream of *traR* in the *traI2* operon and the operon's promoter is directly activated through TraR binding (58). We hypothesised that if QseM interacted directly with Msi172-Msi171 then a similar protective effect would be seen as in section 3.1.1. If no interaction occurs, an absence of culture growth will be seen as the growth-inhibitory phenotype is not controlled.

Unlike previous experiments using this family of plasmids, no difference in spread plate colony formation occurred as a result of the introduction of pSRKGm172171. This indicates either leaky expression from this plasmid was less than the *traR*-containing variant or QseM was able to control any expression. This result enabled further experiments involving the growth of broths induced with IPTG. Initial testing used 1 mM IPTG to ensure maximum expression from the promoter, and resulted in complete inhibition of strains R7A/pPROBE-KT/pSRKGm172171 and R7A/pNqseM/pSRKGm172171. With confirmation the inducible promoter worked and that its induction produced the growth-inhibition phenotype, an assay with a concentration gradient of IPTG was carried out in triplicate. Figure 8 shows the effect of the varying concentrations of IPTG on the growth of the cultures tested.

Both the strains R7A/pPROBE-KT/pSRKGm and R7A/pNqseM/pSRKGm (Grey lines) that contained no gene downstream of the inducible promoter were unaffected at all concentrations of IPTG and consistently grew to similar ODs across all biological replicates (Figure 8). The error bars (SD) on the graph likely reflect natural variation between premade 5-ml TY broths and different biological replicates. R7A/pPROBE-KT/pSRKGm172171 was consistently inhibited at 0.1 mM IPTG, and had already begun to be inhibited at 0.05 mM IPTG; whereas R7A/pNqseM/pSRKGm172171 was barely if at all affected at 0.1 mM IPTG (Figure 8).

R7A/pNqseM/pSRKGm172171 culture growth was completely inhibited when broths were induced with 0.5 mM IPTG while, as mentioned above, at 0.1mM and below culture densities were well within the parameters for both negative control strains. Concentrations between 0.5 and 0.1 mM were not tested so actual concentration that completely inhibits R7A/pNqseM/pSRKGm172171 is unknown. The requirement of higher IPTG levels in broth suggests a requirement of more Msi172-Msi171 to inhibit growth and therefore that QseM and Msi172-Msi171 interact, through a yet unknown mechanism.

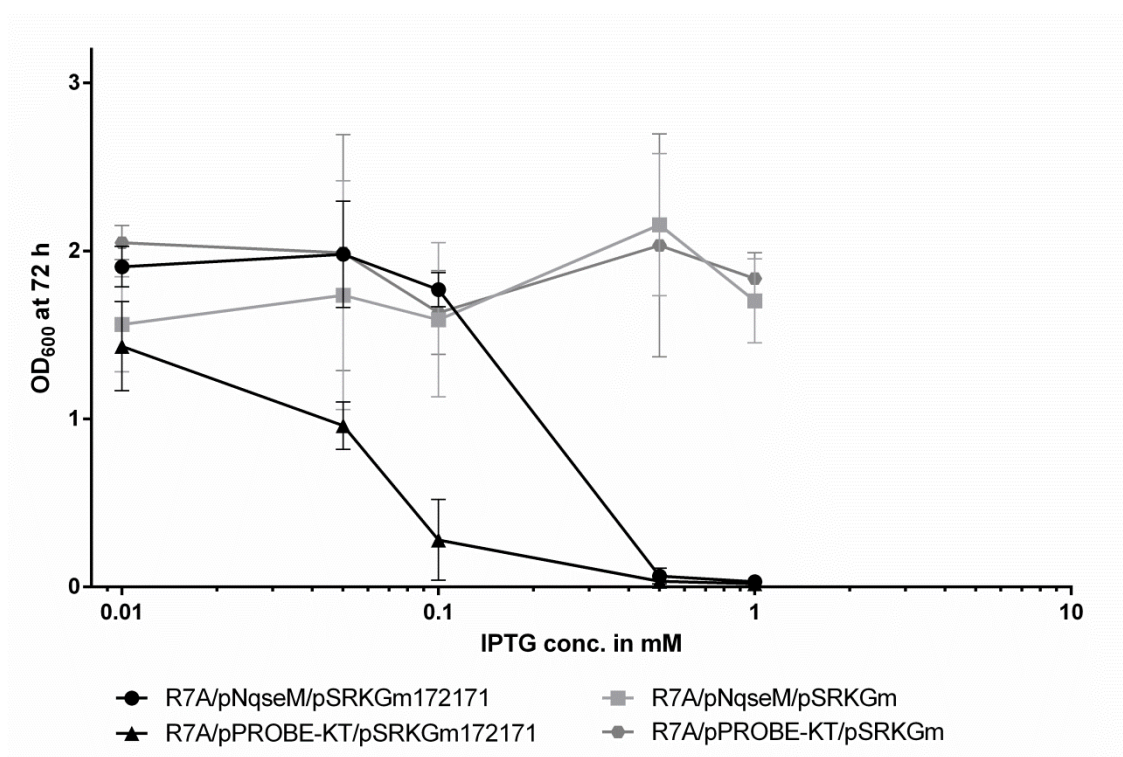


Figure 8. Growth-inhibition through pSRKGm172171 induction via IPTG.

Error bars are representative of 3 biological replicates and are shown as standard deviation. IPTG was present in media from inoculation. Samples were taken at 72 hours and measured in 1 ml or 1:10 aliquots where the OD was too high. The presence of QseM confers growth to a higher concentration of IPTG and therefore Msi172-Msi171. Strain names are listed in legend.

3.3.1 Bacterial Conjugation Assay to investigate the ability of QseM to antagonise Msi172-Msi171 induced growth-inhibition

With the evidence provided by a previous experiment (Section 3.1.1) introducing TraR into strains with a background of either pPROBE-KT or pNqseM, a similar experiment was designed to introduce a plasmid, pNJ172171, constitutively expressing Msi172-Msi171. We hypothesized that constitutively expressed QseM may be able to overcome the growth-inhibition bought on by the introduction of pNJ172171.

However all spot mating to create strains that contained pNJ172171 failed to produce viable colony growth, barring R7A/pNqseM/pNJ172171 that had 15 colonies on the undiluted spread plate which is not enough to be a positive result. Strains that contained the empty vector control plasmid pFAJ1708 produced an average 263 colonies at the 10^{-2} dilution. This result suggests that the constitutive expression of Msi172-Msi171 from pNJ172171 was higher than the level QseM was able to protect against.

3.4 QseM does not interact with RdfS at the protein level.

Previous experiments introducing both *traR* (Section 3.1.1) and *msi172-msi171* (Section 3.3) concluded that the presence of excess QseM is protective against expression of these genes. An idea was postulated that QseM and RdfS may interact as *rdiS* expression is activated by Msi172-Msi171. To test this hypothesis, attempts were made to introduce a pSRKGm vector containing *rdiS* through spot mating to strains R7A/pPROBE-KT and R7A/pNqseM. However, after 7 days incubation, both sets of serial dilutions for R7A/pPROBE-KT/pSRKGmrdiS and R7A/pNqseM/pSRKGmrdiS produced minimal colony growth, likely escape colonies; unlike the pSRKGm empty vector control strains. Importantly the GRDM plates used contained no IPTG, indicating that the leaky expression seen with the pSRKGm family of plasmids appears to allow production of enough RdfS to inhibit growth. Figure 9 graphically compares the growth of all strains and clearly shows the difference in growth between the two strains that contain the pSRKGmrdiS vector and the two strains that do not. It can be concluded that QseM and RdfS likely do not interact at the protein level

as levels of expression from the leaky promoter is enough to result in near complete inhibition.

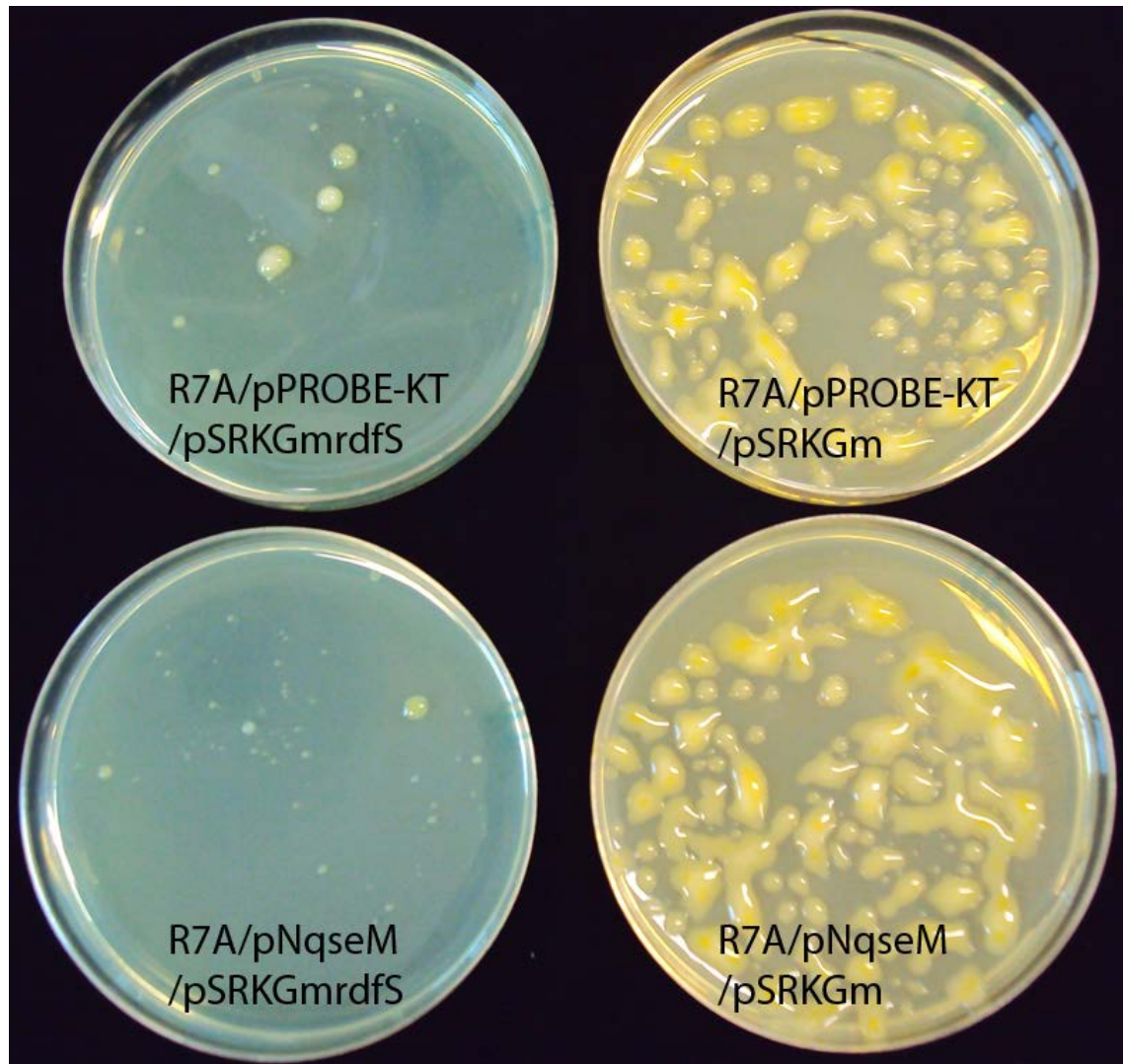


Figure 9. Resulting growth after 7 days incubation of spread spot matings after introducing pSRKGmrdfS or pSRKGm into R7A containing either pPROBE-KT or pNqseM.

The presence of pSRKGmrdfS produced the growth-inhibitory effect even in the presence of constitutively expressed *qseM*. Strain names are listed on the plates. All plates contained no IPTG and were spread with 100 μ l of the 10^{-2} dilution of the spot-mating serial dilution.

3.5 QseM does not protect against growth-inhibition resulting through TraF expression

Experiments in Section 3.4 suggested that QseM was unable to interact with RdfS on the protein level, as no protective effect against the growth-inhibitory phenotype was seen. However the *traF* gene immediately downstream of *rdfS* presents another possible site of interaction. Previous work by Prof. Michael Hynes showed that when either *rdfS* or *traF* is over-expressed, the growth-inhibition phenotype is induced.

To test the hypothesis that QseM may interact with TraF, pSRKGmtraF or pSRKGm plasmids were introduced into strains R7A/pPROBE-KT or R7A/pNqseM through spot mating. The spot mating was then serially diluted and spread without IPTG or spotted onto appropriate media with or without 1mM IPTG to activate the inducible promoter. Plate counts were carried out 6 days post spreading and showed only a small difference in CFU between strains that contained pNqseM and those with pPROBE-KT on media without IPTG, indicating leaky expression was either minimal or at a controllable level. Figure 10 shows the levels of growth of identical strain dilutions in the presence or absence of IPTG. Those strains containing pSRKGmtraF were completely inhibited when spotted onto media containing 1 mM IPTG, while on media that contained no IPTG, there was only minor growth inhibition; R7A/pPROBE-KT/pSRKGmtraF produced single colony growth at 10^{-2} while R7A/pNqseM/pSRKGmtraF produced single colonies at 10^{-3} .

These results suggested that QseM was unlikely to directly interact with TraF, as constitutively expressed QseM was not able to overcome the growth-inhibitory phenotype brought on by *traF* overexpression. However the leaky expression seen on other variations of the pSRKGm plasmids appears less severe with pSRKGmtraF, as only a slight difference in colony numbers was seen between the R7A/pPROBE-KT and R7A/pNqseM parent strains. The presence of pNqseM confers growth to a lower dilution compared with pPROBE-KT, this may be a result of a more restricted QS system.

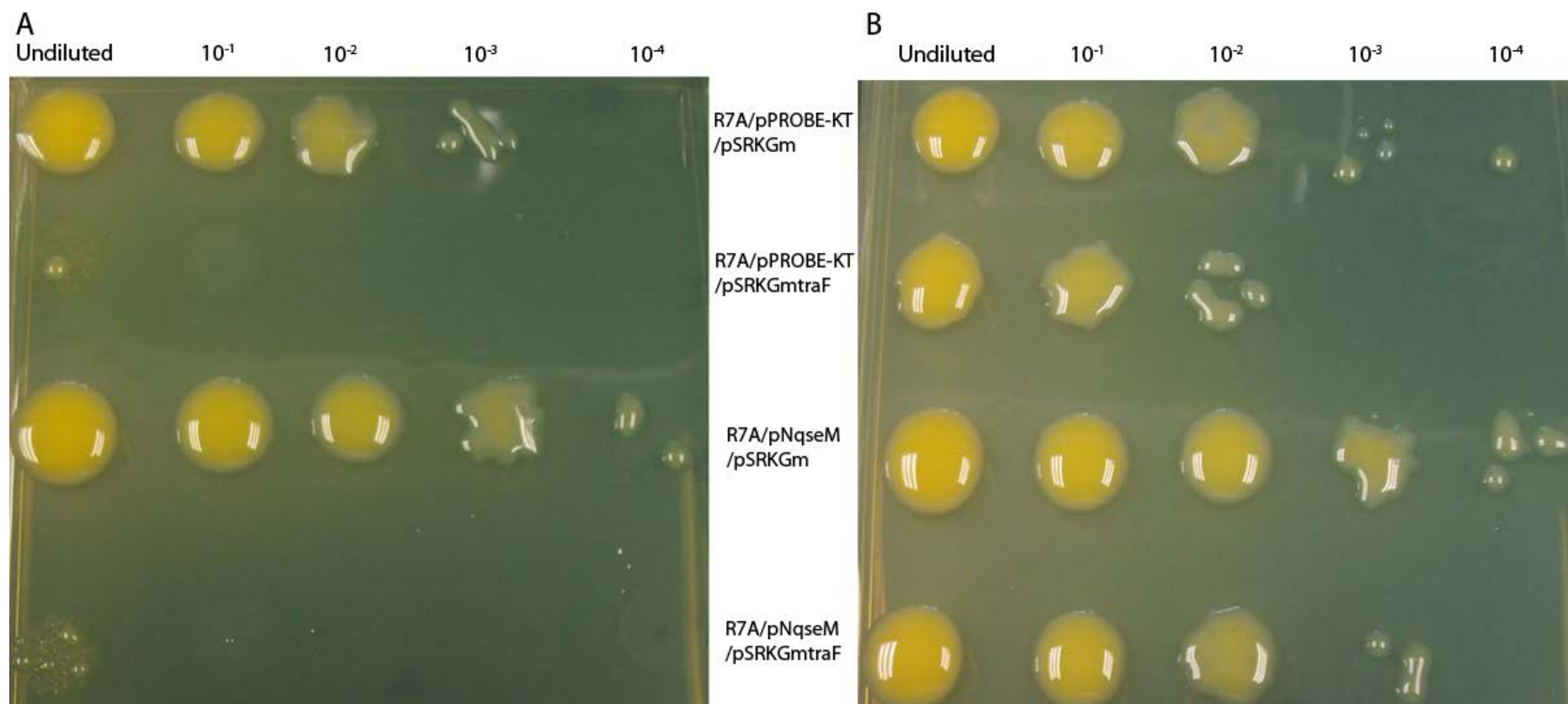


Figure 10. Resulting growth of strains after 6 days in the presence (A) or absence (B) of 1 mM IPTG

Strains containing pSRKGmtraF were inhibited completely with only minimal escape colonies in the undiluted spot. Strains grown without IPTG were only affected by growth-inhibition at lower concentrations. Strain names are listed between serial dilutions.

3.6 Identification of QseM targets through bacterial two-hybrid analysis

During the course of this work, L. Tester and J. Ramsay (this laboratory) found that the active product of *msi172-msi171* is a single protein FseA, produced by a +1 translational frameshift occurring at a low frequency (~4.5% of translation events). Two genetic constructs with either *msi172msi171*wt or Msi172 and Msi171 reading frames fused to produce *fseA* were made. To test the hypothesis that QseM interacts with Msi172Msi171 or FseA, the *E. coli*-based two-hybrid system Bacteriomatch II (Agilent Technologies) was used. In this system, the protein or fragments of it, are fused downstream of the α -subunit of RNA polymerase or the λ cl protein, dependent on the vector plasmid. If a protein-protein interaction occurs, transcription of the yeast HIS3 gene begins, allowing histidine prototrophy and conferring the ability to grow in the presence of 3-AT. The genes *qseM*, *msi172msi171*, *fseA* (engineered frame-shifted version of *msi172msi171*), and later *msi172* and *msi171* were fused to vectors pBTL or pTRG. Plasmids were created either myself (Containing *msi172msi171* or *fseA*: Section 2.11.1) or previous lab members R. Dy or J. Ramsay. Co-transformations consisted of 50 ng of each plasmid combined with 40 μ l of Electrocompetent 'BacterioMatch II Validation Reporter Competent Cells' and were grown on both selective, containing 3-AT and non-selective media for 48 hours at 28°C. This method allows standardisation and direct comparisons between CFU.

A strong interaction was seen between pBTL*qseM* and pTRG*fseA* co-transformation as indicated by large numbers of colonies on selective media containing 3-amino-1,2,4-triazole (3-AT). However, the opposite plasmids pBTL*fseA* and pTRG*qseM* produced no colonies on the 3-AT containing selective media, indicating no positive interaction. No growth was seen with all co-transformations containing pTRG*qseM*. This result is consistent with previous work conducted by this lab, suggesting a possible problem with the pTRG*qseM* plasmid fusion. As expected, the control co-transformations produced very few colonies on selective media (Table 5).

Table 5. Plate counts and ratio for Cotransformations, experiment one.

	pTRGGall ^p pBTLGF2	pTRG pBTL	pTRG pBTLfseA	pTRG pBTLqseM	pTRGfseA pBTL	pTRGfseA pBTLfseA	pTRGfseA pBTLqseM	pTRGqseM pBTL	pTRGqseM pBTLfseA	pTRGqseM pBTLqseM
NS rate	3.67x10 ⁶	2.53x10 ⁵	5.58x10 ⁵	2.56x10 ⁵	7.68x10 ⁶	7.46x10 ⁶	5.21x10 ⁶	1.9x10 ⁶	2.94x10 ⁶	2.21x10 ⁶
S rate	4.8x10 ³	0	0	0	7 x10 ¹	5x10 ¹	4.3x10 ⁵	5x10 ¹	1x10 ¹	2x10 ¹
Ratio	1.31x10⁻³	0	0	0	9.11x10 ⁻⁶	6.70x10 ⁻⁶	8.25x10⁻²	2.63x10 ⁻⁵	3.40x10 ⁻⁶	9.05x10 ⁻⁶

Note: A Positive interaction is indicated by a higher ratio and is bolded in the above table. NS cfu is the colony forming units on non-selective medium while S cfu is the colony forming units on selective medium.

Table 6. Plate counts and ratio for Cotransformations, experiment two

	pTRGGall ^p pBTLGF2	pTRG pBTL	pTRG pBTLqseM	pTRGfseA pBTL	pTRGfseA pBTLqseM	pTRG172171 pBTL	pTRG172171 pBTLqseM
NS rate	4.16x10 ⁶	6.7x10 ⁵	3.14x10 ⁵	3.96x10 ⁵	9.18x10 ⁶	6.62x10 ⁶	9.12x10 ⁶
S rate	8.2x10 ⁴	1x10 ¹	0	5x10 ¹	6.42x10 ⁵	1.2x10 ²	1.35x10 ⁶
Ratio	1.97x10⁻²	1.49x10 ⁻⁵	0	1.26x10 ⁻⁴	6.99x10⁻²	1.81x10 ⁻⁵	1.48x10⁻¹

Note: Positive interactions are indicated by higher ratios and are bolded in the above Table. NS cfu is the colony forming units on non-selective medium while S cfu is the colony forming units on selective medium.

In further repetitions, it was decided to remove pTRGqseM from reactions to reduce the chances of false positive results. The pBTLqseM and pTRGfseA co-transformation produced the result of a positive interaction, being growth of colonies on selective media (Table 6). Interestingly, this result was also produced by the pBTLqseM and pTRG172171 co-transformation, which contains no engineered frameshift. The pBTLqseM and pTRG172171 co-transformation occurred at a 2 fold higher rate than the reaction of pBTLqseM and pTRGfseA (Table 6).

The interaction between QseM and Msi172-171 or FseA led to the investigation for interaction between the individual proteins QseM and Msi172 or Msi171. Previous work (61) found no interaction between Msi171 and Msi172 (which are now known to be one protein); however, interaction between QseM and Msi172 or Msi171 was not investigated at that time. The plate count showed a strongly positive reaction between pBTLqseM and pTRG172, with large numbers of colonies on the selective medium at the dilutions used. The pBTLqseM and pTRG171 transformation produced a negative result with very low numbers of colonies present on the selective medium (3 on the undiluted plate). This result confirmed that QseM and Msi172 interact at the protein level and strongly suggests a possible binding site for QseM on the Msi172-Msi171 or FseA complex. This result also confirmed the protective ability of QseM to prevent the growth-inhibition phenotype caused by excess Msi172-Msi171 and provided insight in to the mode of interaction.

To confirm the results of previous experiments mentioned in this section, another series of co-transformations was carried out to allow direct comparison between experiments (Figure 11 and Table 7). A slight difference was seen at the undiluted level of the pTRG171 and pBTLqseM co-transformation with a small amount of growth (139 colonies on the undiluted plate); however this result can likely be excluded as a false positive, as the positive reactions produced spots of greater density and had a higher CFU on the selective media than the positive control reaction provided by Agilent Technologies (Figure 11 and Table 7).

Reactions between QseM and Msi172-Msi171 or FseA produced very similar ratios, 0.21 and 0.242 respectively, suggesting a similar affinity for binding with

QseM, however the ratio between QseM and only Msi172 produced a rate of 0.437, twice that of the full Msi172-Msi171 protein. This difference can be visually quantified in Figure 11 with the pTRG172 and pBTLqseM cotransformation having a denser spot at the 10^{-3} dilution compared to the other two positive reactions and the positive control.

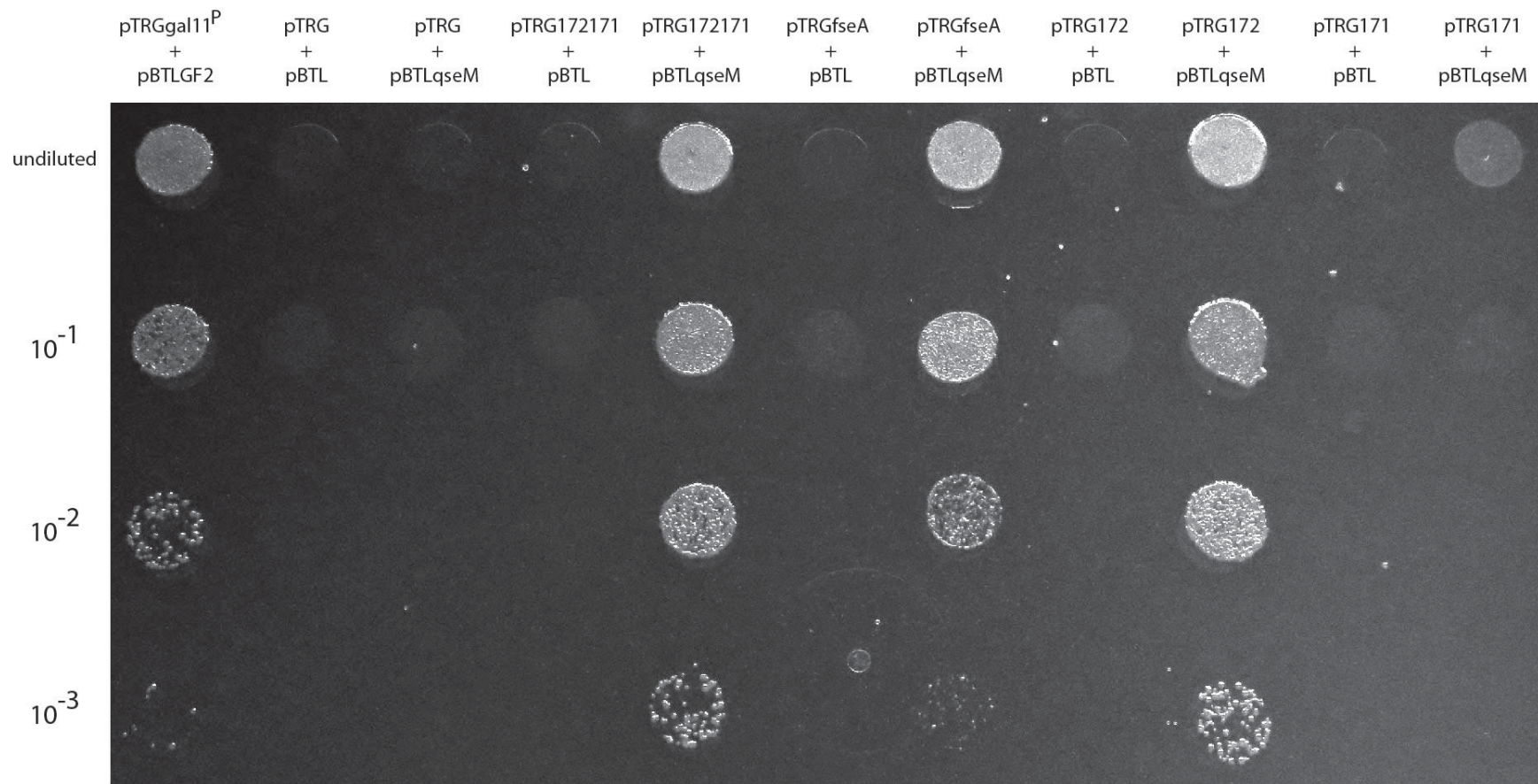


Figure 11. Combined co-transformation experiment with spots of serial dilution on selective medium.

This figure combines separate experiments to allow direct comparison. Growth indicates a protein-protein interaction. Plate counts were carried out concurrently (Table 7). Co-transformation plasmids listed above corresponding column. A protein-protein interaction was confirmed between QseM and Msi172-Msi171, the interacting portion was narrowed down do the Msi172 region of Msi172-Msi171 or FseA.

Table 7. Plate counts and ratios for the combined experiment co-transformations.

	pTRGGall ^p pBTgf2	pTRG pBTL	pTRG pBTLqseM	pTRG172171 pBTL	pTRG172171 pBTLqseM	pTRGfseA pBTL	pTRGfseA pBTLqseM	pTRG172 pBTL	pTRG172 pBTLqseM	pTRG171 pBTL	pTRG171 pBTLqseM
NS cfu	1.57 x10 ⁶	1.05 x10 ⁶	8.3 x10 ⁵	6.12 x10 ⁶	1.096 x10 ⁷	2.10 x10 ⁷	2.11 x10 ⁷	3.04 x10 ⁷	1.24 x10 ⁷	9.42 x10 ⁶	1.78 x10 ⁷
S cfu	5.28 x10 ³	6 x10 ¹	4 x10 ¹	3.2 x10 ²	2.65 x10 ⁶	4.8 x10 ²	4.44 x10 ⁶	9.6 x10 ²	5.44 x10 ⁶	4 x10 ¹	1.39 x10 ³
Ratio	3.35 x10⁻³	5.71 x10 ⁻⁵	4.82 x10 ⁻⁵	5.23 x10 ⁻⁵	2.42 x10⁻¹	2.29 x10 ⁻⁵	2.1 x10⁻¹	3.15x10 ⁻⁵	4.37 x10⁻¹	4.25 x10 ⁻⁶	7.79 x10 ⁻⁵

Note: A positive interaction is indicated by a larger ratio. NS cfu is the colony forming units on non-selective media while S cfu is the colony forming units on selective media. Positive interactions are bolded in the Table.

3.7 QseM binds a Msi172-171 frame-shifted protein to disrupt *rdfS* activation

Another lab member (L. Tester) previously showed that the *msi172-msi171* frameshift product, FseA activates the *rdfS* promoter. It is known RdfS is required for the excision and transfer of the ICEM/Sym^{R7A} and that it is also one of the proteins that produces the growth-inhibition phenotype seen throughout this project. We hypothesised that QseM would disrupt transcriptional activation of the *rdfS* operon as previous experiments demonstrated that QseM and Msi172Msi171, FseA and Msi172 interact strongly (Sections 3.3 and 3.6). A plasmid pSD172171 was constructed by J. Ramsay that contained the *msi172msi171* genes in front of the IPTG-inducible *lac* promoter of vector pSDz and, in reverse orientation, the *rdfS* promoter region upstream of the promoterless *lacZ* gene. This allowed the testing of the interaction between QseM and Msi172-Msi171 without plasmid read-through issues that could create false positives. A similar plasmid that contained the engineered frameshifted *msi172-msi171* gene producing FseA in place of wild-type *msi172-msi171* was also made (J. Ramsay) and used along with the empty vector. A R7ANS background was used to limit interference from other ICEM/Sym^{R7A} factors as it's cured of the island.

The plasmids were introduced to the strains R7ANS/pPROBE-KT or R7ANS/pNqseM through spot mating. The resultant strains were sub-cultured and grown in 5-ml broths with IPTG for 24 hours. The ODs were recorded and 200 μ l aliquots of the cultures were frozen at -70°C until use in β -galactosidase assays using the plate reader. This assay design enabled comparison of relative fluorescence units per second per OD₆₀₀ unit (Figure 12 and Figure 13). The activity of β -galactosidase for R7ANS/pPROBE-KT/pSD172171FS was extremely high at 204 RFU/OD₆₀₀/s, whereas the activity of R7ANS/pNqseM/pSD172171FS was 100-fold lower at 2 RFU/OD₆₀₀/s (Figure 12). This indicated that QseM was able to interact with FseA and reduce the activation of the *rdfS* promoter region, decreasing expression. Other strains assayed had comparable trends in expression levels, with much smaller differences in expression. R7ANS/pPROBE-KT/pSD1721716H had a near three-fold higher expression (0.88 RFU/OD₆₀₀/s)

than R7ANS/pNqseM/pSD1721716H (0.31 RFU/OD₆₀₀/s; Figure 13). The difference between the R7ANS/pSDZrdfS control strains was almost negligible.

While the difference between both pSDZ1721716H strains was not large, it was significant when analysed with a t-test ($p < 0.0016$). Across all strains tested, the presence of plasmid pNqseM caused a reduction in β -galactosidase activity. This was likely to be through the repression of Msi172Msi1716H activation on the *rdfS* promoter region, which strongly indicates the interaction between QseM and Msi172171 or the frame-shifted product, even when genes are uncoupled from native promoters.

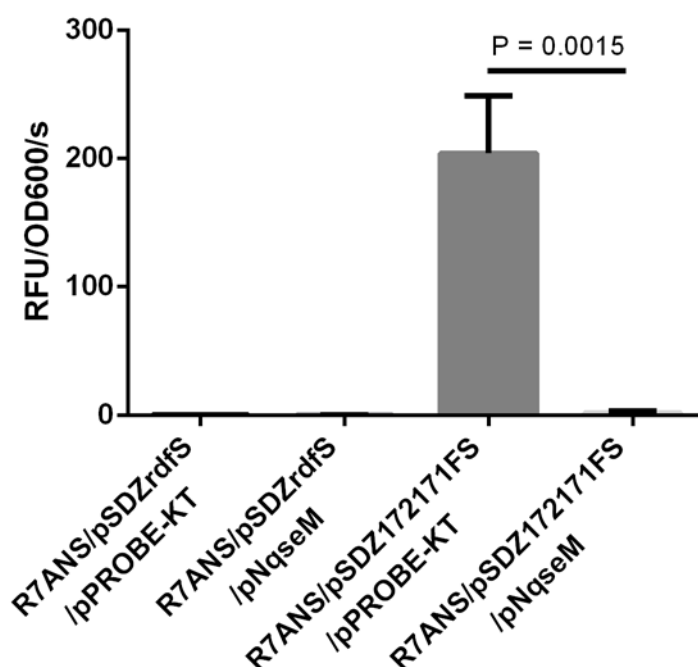


Figure 12. β -galactosidase activity from induced reporter genes in pSDZ172171FS variants in the presence or absence of pNqseM.

Strain names are below columns with three biological replicates included in each column. Error bars indicate standard deviation. Activity from R7ANS/pSDZ172171FS/pPROBE-KT was much higher than other strains leading to the other strains being barely visible, while the presence of QseM clearly reduces this expression 100 fold. Values for these other strains are shown on an expanded scale in Figure 13.

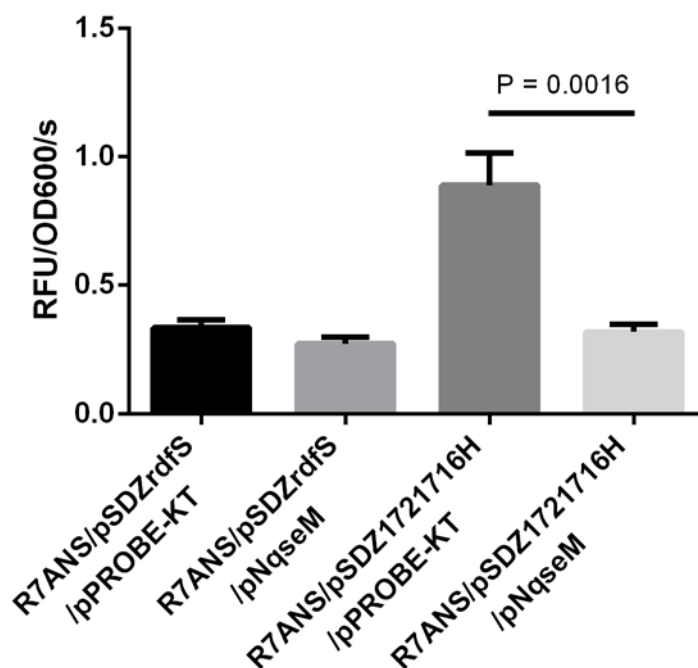


Figure 13. β -galactosidase activity from induced reporter genes in pSDZ172171 variants in the presence or absence of pNqseM.

Strain names are below columns with three biological replicates included in each column. Error bars indicate standard deviation. Presence of QseM decreases expression of *lacZ* in both the test strain and the controls strains, although the reduction was only significant between strains that contained pSDZ1721716H. Note the much decreased Y axis scale compared to Figure 12.

3.8 Purification of QseM and exploratory experiments into its targets

The plasmid pQe80-6HQseM was created (Section 2.10) to enable protein purification through a capture column. Before purification, a version of 6H-QseM was assayed in the AHL experiment in Section 3.2 to ensure that QseM still had functionality with a 6H tagged N-terminal region. Purification (Section 2.10.2) resulted in a final concentration of 929.93 $\mu\text{g/mL}$ of 6HQseM, with the isolated protein confirmed as correct through 15% SDS-PAGE gel and mass spectrometry (Figure 14).

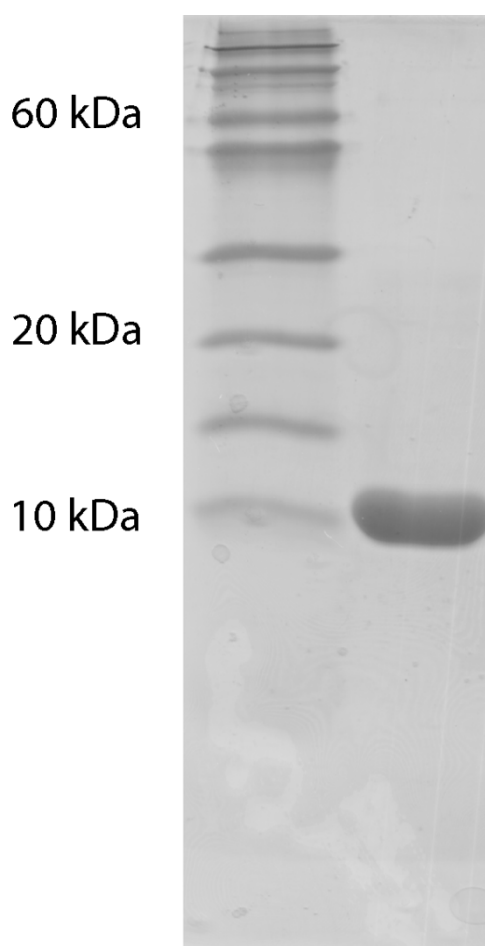


Figure 14. Sample of concentrated 6HQseM before mass spectrometry

The band exists at ~ 10 kDa as expected. Novex Sharp Standard Pre-stained ladder (Life Technologies) was used as reference. This band was removed and used in mass spectrometry.

3.8.1 QseM binds a protein present in a R7AΔ*qseM* cell lysate at a high affinity

6HQseM was used to capture possible interacting proteins from an R7AΔ*qseM* whole cell lysate (strain lacks a functional *qseM*) through attachment to the Ni-agarose in a purification column. The column was loaded with Ni-NTA resin bound with 6HQseM and the lysate was passed through. Elution of this column after a short wash with wash buffer (Appendix A) reisolated 6HQseM and quantities of other unknown proteins (Figure 15, lane 4). The lower half of Lane 4 was excised and then underwent mass spectrometry using a database provided by J. Ramsay that contained all proteins from ICEM/Sym^{R7A} in all possible frames. The result from this was inconclusive as the hypothesised interacting proteins scored low on this list after analysis (See Appendix A for download link).

3.8.2 Exploration into QseM folding

Purified 6HQseM was used in the biophysical technique of Ultra-Violet (UV) Circular Dichroism (CD). UV CD refers to the differential absorption of left and right circularly polarized light based on the secondary structure of the matter, which in this case are the protein and the salt in the buffer. Initial results were inconsistent due to a high salt concentration in the buffer, and changing to a lower salt buffer garnered a more accurate result. The structure of QseM was estimated as 86.81% α-helical in shape. The real percentage is likely higher as this value takes into account both the 6H tag and linker, as well as interference from the remaining salt in the buffer.

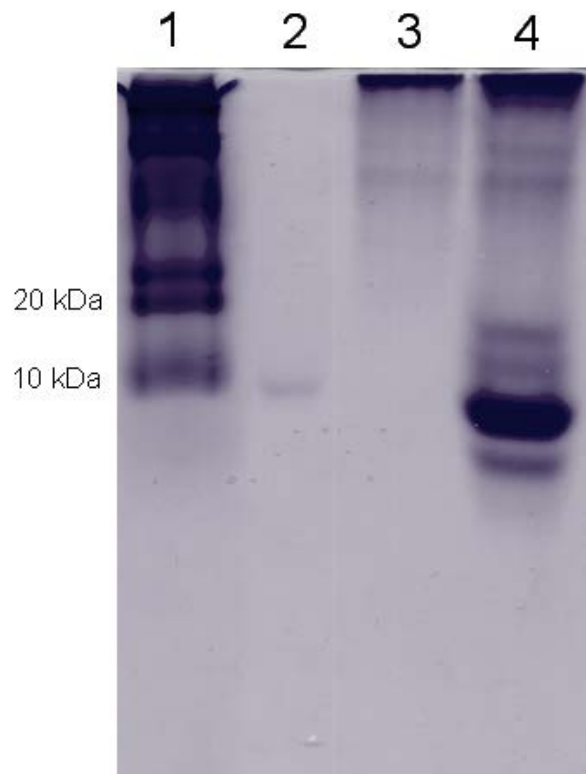


Figure 15. Scan of SDS gel from protein purification using 6HQseM to capture possible interacting proteins.

Lane (1) Novex Sharp Standard Pre-stained ladder, (2) Small sample of purified 6HQseM, (3) Whole Cell Lysate with unknown proteins, and (4) Re-purified 6HQseM with unknown proteins pulled from cell lysate. Complete destaining of the gel was not attempted as the samples contained within were to be sent for mass spectrometry.

3.9 RT-qPCR analysis of the effect of QseM on the expression of other transfer genes and suspected targets for interaction

qPCR is a technique that enables the detection and quantification of a specific sequence of DNA. This technique works through the basic principle of PCR with the major difference being the simultaneous quantification of amplified DNA in real time, as it accumulates after every cycle. The assay designed in this study looked at the different amounts of transcriptional activity across a select few genes of interest in various strain backgrounds. This is achieved through the creation of cDNA, using reverse transcriptase, from the mRNA being transcribed from the interest genes. As QseM is a known repressor of the QS system in *M. loti*, the selection of strains was based on their lack of the *qseM* gene. It should be noted that this work was carried out very early in the project and now since other options have come to light this experiment can be expanded and more genes assayed using a wider variety of strain backgrounds.

R7A/pPROBE-KT was used as the basal comparison strain that other strains were normalised against as it contains a functional *qseM* gene and the empty vector control plasmid for the other strains. R7AΔ*qseM* was selected as it lacks a functional *qseM* gene. The plasmids pPROBE-KT and pNqseM were introduced into R7AΔ*qseM*. A double mutant R7AΔ*qseM*Δ*traR* strain was also used as it lacked both *qseM* and the previously shown QseM target and QS activator TraR (Section 3.6: 61). The strains used were as follows: R7A/pPROBE-KT, R7AΔ*qseM*/pPROBE-KT, R7AΔ*qseM*/pNqseM, R7AΔ*qseM*Δ*traR*/pPROBE-KT and R7AΔ*qseM*Δ*traR*/pNqseM.

Frozen inocula were used to seed the experimental broths to encourage even growth across flasks and to attempt to normalise yields of mRNA. This was somewhat successful at creating cultures of a similar OD₆₀₀ but it ultimately failed with high levels of variation present in mRNA between each replicate and repetition of the experiment (Table 8). This variability was also prevalent within different technical repeats of RT-qPCR assays with levels of expression varying from almost nothing, to levels seen by J. Ramsay and above (Personal communication).

Normalisation of RT-qPCR was attempted by selecting one strain from each biological replicate with an OD₆₀₀ and mRNA levels most similar to other strains. This method helped to smooth out differences seen with abundance but ultimately a conclusion was reached here that higher or consistent OD₆₀₀ between biological replicates does not ensure similar mRNA levels. All mRNA concentrations reported in this work are significantly below values that have been collected previously (J. Ramsay, Personal communication).

In an effort to reduce the inconsistencies seen between and within replicates, primer binding efficiencies were carried out and it was determined that primers used within this work were excellent and produced a range from 1.85 to 1.92 with no significant levels of Primer dimer occurring. Each assay plate included the minus Reverse Transcriptase samples to measure the levels of DNA contamination; which were minimal, often only affecting one sample per repeat a small amount. Variation could be a result of trying to assay gene expression at log phase when activation of these particular genes is generally restricted to 5% of the population. Similarly this could also affect the levels of mRNA seen as the majority of cells are being processed not just those expressing our genes of interest at this given time point.

Table 9 demonstrates the significant variation seen between technical replicates but does not offer any insight to the cause. mRNA abundance within technical replicates is, generally, consistent; however between assays, using the same cDNA preparations, variation of an unexplained origin skewed the values dramatically. For example, previous work has shown levels of *rdfS* expression are higher in a strain deficient for *qseM* and that removal of *traR* reduces *rdfS* expression, therefore in a R7AΔ*qseM*Δ*traR* double mutant decreased expression should be seen. This work reports a very broad relative abundance range from 0.54 to 23.75. Complementation of R7AΔ*qseM*Δ*traR* with pN*qseM* reduced expression levels and decreased the range from 0.34 to 11.97. Genes implicated in *rdfS* activation were also similarly affected by the presence of *QseM*.

Table 8. The OD₆₀₀ of each culture and resulting mRNA yield from each before cDNA synthesis

Pre cDNA synthesis 1	OD ₆₀₀ at 24 h			mRNA ng/ml		
	Biological replicate			Biological replicate		
	1	2	3	1	2	3
R7A/pPROBE-KT	0.198	0.266	0.186	67.7	76.5	118.8
R7AΔ <i>qseM</i> /pPROBE-KT	0.316	0.327	0.283	72.8	44.8	89.6
R7AΔ <i>qseM</i> /pNqseM	0.239	0.232	0.321	48.5	58.7	81.8
R7AΔ <i>qseMΔtraR</i> /pPROBE-KT	0.135	0.288	0.459	40.4	57.5	68.7
R7AΔ <i>qseMΔtraR</i> /pNqseM	0.258	0.258	0.319	50	64	60.9

Pre cDNA synthesis 2	OD ₆₀₀ at 24 h			mRNA ng/ml		
	Biological replicate			Biological replicate		
	1	2	3	1	2	3
R7A/pPROBE-KT	0.342	0.384	0.399	71.8	58.3	80.2
R7AΔ <i>qseM</i> /pPROBE-KT	0.223	0.251	0.338	62.3	87.8	74
R7AΔ <i>qseM</i> /pNqseM	0.432	0.468	0.494	78.5	48.8	57.4
R7AΔ <i>qseMΔtraR</i> /pPROBE-KT	0.391	0.465	0.48	62.6	44.2	77.3
R7AΔ <i>qseMΔtraR</i> /pNqseM	0.396	0.378	0.495	65.7	40.6	66.9

Pre cDNA synthesis 3	OD ₆₀₀ at 24 h			mRNA ng/ml		
	Biological replicate			Biological replicate		
	1	2	3	1	2	3
R7A/pPROBE-KT	0.655	0.556	0.561	113.1	66.1	54.7
R7AΔ <i>qseM</i> /pPROBE-KT	0.566	0.485	0.602	53.4	50.5	75.5
R7AΔ <i>qseM</i> /pNqseM	0.372	0.542	0.341	118.5	85.1	98.7
R7AΔ <i>qseMΔtraR</i> /pPROBE-KT	0.67	0.577	0.743	149.4	55.6	32.4
R7AΔ <i>qseMΔtraR</i> /pNqseM	0.591	0.689	0.73	75.9	85	55.8

Note: Grey shaded values represent those samples that were used in RT-qPCR assays. In the bottom tables strains chosen to undergo cDNA synthesis were selected based on a similar OD₆₀₀ in the hope that they would have similar levels of mRNA and subsequently cDNA.

Table 9. Relative abundance of mRNA for genes implicated in the QS system from three replicate experiments (A, B and C) using different mRNA preparations in each sub table.

Each column under a gene name represents a technical replicate conducted on that mRNA. Values were normalised against R7A/pPROBE-KT.

A	OD ₆₀₀	<i>traR</i>			<i>tral2</i>			<i>tral1</i>			<i>rdjS</i>		
R7AΔ <i>qseM</i> /pPROBE-KT	0.316	0.98	6.36	1.05	2.6	13.33	3.85	2.12	0.19	1.74	2.03	2.71	12.22
R7AΔ <i>qseM</i> /pNqseM	0.239	1.07	8.04	1.26	1.22	15.62	1.99	1.08	4.62	0.72	1.11	2.91	3.82
R7AΔ <i>qseM</i> Δ <i>traR</i> /pPROBE-KT	0.135	ND	ND	ND	0.44	21.38	2.96	1.13	8.94	4.98	0.88	23.75	20.01
R7AΔ <i>qseM</i> Δ <i>traR</i> /pNqseM	0.258	ND	ND	ND	0.92	24.61	1.75	0.88	5.17	0.43	0.74	11.97	3.39
R7A/pPROBE-KT	0.198												

B	OD ₆₀₀	<i>traR</i>			<i>tral2</i>			<i>tral1</i>			<i>rdjS</i>		
R7AΔ <i>qseM</i> /pPROBE-KT	0.223	4.49	1.52	8.13	4.28	8.55	4.67	3.74	8.61	4.37	10.5	24.49	10.75
R7AΔ <i>qseM</i> /pNqseM	0.432	4.18	0.69	31.98	0.49	0.92	10.34	0.78	1.82	16.38	0.84	1.99	18.47
R7AΔ <i>qseM</i> Δ <i>traR</i> /pPROBE-KT	0.391	ND	ND	ND	0.63	0.69	1.12	0.67	0.89	1.32	0.54	0.98	1.13
R7AΔ <i>qseM</i> Δ <i>traR</i> /pNqseM	0.396	ND	ND	ND	0.82	0.2	1.86	0.81	0.22	2.34	0.83	0.34	3.33
R7A/pPROBE-KT	0.342												

C	OD ₆₀₀	<i>traR</i>			<i>tral2</i>			<i>tral1</i>			<i>rdjS</i>		
R7AΔ <i>qseM</i> /pPROBE-KT	0.566	0.82			11.69			3.17			4.45		
R7AΔ <i>qseM</i> /pNqseM	0.542	0.42			1.11			0.65			1.54		
R7AΔ <i>qseM</i> Δ <i>traR</i> /pPROBE-KT	0.577	ND			2.42			1.54			4.13		
R7AΔ <i>qseM</i> Δ <i>traR</i> /pNqseM	0.591	ND			3.42			1.61			3.42		
R7A/pPROBE-KT	0.556												

4 Discussion

This project sought to determine the target(s) of QseM within the genes or proteins involved in the regulation of the excision and transfer of ICEM/Sym^{R7A}, and to determine whether its mode of action was through protein-protein interaction or DNA binding. The first aim was achieved with TraR confirmed as an interactant through the ability of QseM to repress the growth inhibitory effect caused by excess TraR. This supported previous findings by R. Dy (this laboratory) who showed using a bacterial two-hybrid system that, in the presence of 3-oxo-C6-HSL, TraR and QseM interacted in *E. coli*. Msi172-Msi171 and the subsequent engineered frameshift product, FseA, were also identified as targets, as QseM was able to protect against the growth inhibitory phenotype caused by low levels of Msi172-Msi171 expression. Initial explorations into their mode of interaction via bacterial two-hybrid analysis confirmed a protein-protein interaction, with further assays identifying Msi172 as the portion of Msi172-Msi171 that contained a site for interaction for QseM.

Other proteins and regions in the excision and transfer pathway were excluded as targets for QseM; these were RdfS and TraF, the proteins directly responsible for the inhibitory effect when Msi172-Msi171 is over expressed, while the *traR* and *rdiS* promoter regions were also excluded as targets of QseM. This cemented the idea that QseM is a protein-binding protein.

4.1 QseM interacts with two distinct proteins within the quorum sensing system

Aims of this study included confirming where QseM was interacting within the QS system and to determine its mode of action. Previous bioinformatics work showed that *qseM* exists in a similar genomic position to *traM*, found on the *A. tumefaciens* Ti plasmid, suggesting that despite sharing no sequence homology, QseM likely functioned analogously to TraM. Therefore experiments were designed to determine whether QseM acted as an antiactivator of TraR. The observation that

QseM overexpression further reduced the low level of excision seen in a $\Delta traR$ mutant (60) also led us to explore other targets for QseM.

Previous two-hybrid work (61) suggested that TraR and QseM interact in the presence of 3-oxo-C6-HSL in *E. coli*. We sought to explore the effect of TraR on AHL expression levels using *M. loti* strains with different genetic backgrounds and carrying different combinations of plasmids. However it was found that when pSRKGmtraR was introduced into strains, the growth-inhibition phenotype normally seen when Msi172-Msi171 is overexpressed occurred (63), suggesting that the inducible promoter in pSRKGmtraR was leaky to some level in the absence of induction. Therefore it was not possible to construct the majority of intended strains containing this plasmid. However, the leaky expression phenotype provided an excellent foundation for other experiments. The bacterial conjugation assay (Figure 6) revealed that the presence of QseM provided a protective effect against TraR-induced growth-inhibition across all strains assayed, providing strong evidence that the interaction between QseM and TraR occurred in *M. loti*. Importantly, this protection phenomenon was seen in the $R7A\Delta qseM\Delta traR$ double mutant, where both genes are uncoupled from their native promoters, indicating that a protein-protein interaction likely occurred between QseM and TraR rather than QseM acting at the level of the *traR* promoter.

Ramsay et al. (58) identified that R7A does not produce detectable levels of AHLs through CV026 assay of stationary-phase culture supernatants, likely through QseM control. By assaying the levels of violacein produced by various strains in the CV026 assay, we determined that the lack of *traR* in both $R7A\Delta traR$ and $R7A\Delta qseM\Delta traR$ resulted in no pigment production and therefore no detectable production of 3-oxo-C6-HSL. The presence of constitutively expressed QseM repressed any 3-oxo-C6-HSL and pigment production in strains that were deficient for *qseM*. This included the N-terminally 6H tagged QseM variants constructed for use in protein purification experiments. The leaky expression from pSRKGmtraR was not completely controlled by native levels of QseM, as the $R7A\Delta traR$ /pSRKGmtraR mutant showed elevated levels of pigment production; however the broth grew satisfactorily enabling collection of supernatant. This indicates that QseM was able to control the growth inhibitory phenotype to some

degree (Figure 7). From this result, it can be inferred that the level of TraR needed within the QS system is low and that the leaky expression from pSRKGmtraR is above controllable levels, thus producing growth-inhibition. Previous work (58) reported a 1000-fold increase in 3-oxo-C6-HSL production and 100% excision when pJRtraR, expressing TraR from its native promoter, was introduced into strains. This confirms that anything other than low levels of TraR artificially activate the QS system no matter the cell density, activating the excision and transfer pathway and in some cases producing the inhibitory effect.

Several mutants existed within the laboratory culture collection that were suspected to have been made from the R7A* strain which produces elevated levels of AHL. Confirmation of the elevated expression levels in R7A Δ rdfS suggested it was derived from R7A*, excluding that strain from further work in this project. This result is interesting as the site of mutation or duplication in R7A* is unknown despite candidate regions within genes involved in QS having been sequenced (16). An opportunity exists here to sequence the whole genome in search of the mutation.

These results would benefit from more in-depth study into the interaction as *M. loti* TraR shares homology with *A. tumefaciens* TraR, yet QseM is dissimilar to *A. tumefaciens* TraM. Would addition of TraM to the system have an effect on TraR-mediated activation and the levels of AHL present in the supernatant, as occurs in *A. tumefaciens* (42, 43, 79)? Another experiment would be site-directed mutagenesis of regions in *traR* and *qseM* that encode likely areas for interaction or are critical in the secondary or tertiary structure of the proteins. Once created, these mutants could be tested through a function-based assay, selecting for the ability to either repress or activate the lethal phenotype dependant on the protein mutagenised or through bacterial two-hybrid assays selecting for the interaction. Error-prone PCR could be performed on *traR* and *qseM* to create mutants with different phenotypes which may alter the AHL expression pattern, possibly creating a TraR that does not require 3-oxo-C6-HSL for activation or a QseM that sequesters the complex at a much lower or higher rate. Ultimately the crystal structure of QseM in complex with TraR would allow a direct comparison between

the modes of action of QseM and TraM, as the crystal structure of the TraR-TraM complex has been determined (38).

Once TraR was identified as a target and the characterisation initiated, attention was turned to another possible site for QseM action, Msi172-Msi171. Previous work determined that overexpression of Msi172-Msi171 from a constitutive promoter resulted in a growth-inhibition phenotype independent of TraR expression (63) and that the *rdfS* operon was activated by Msi172-Msi171 at the transcriptional level (L. Tester, this lab, unpublished data). Using the same family of pSRKGm vectors, Msi172-Msi171 was introduced into R7A strains that contained constitutively expressed QseM or not. Leaky expression from this vector appeared to have a less severe effect, or QseM was able to control it more effectively, as the plasmid was introduced into the strains with minimal issues. Induction with 1 mM IPTG produced near-complete growth-inhibition of broth cultures, much like the plasmid constitutively expressing Msi171-Msi171, pNJ172171. Manipulation of Msi172-Msi171 expression using varying concentrations of IPTG determined that the presence of additional QseM, through constitutive expression, conferred a growth advantage at higher concentrations of IPTG and therefore in the presence of increased expression of Msi171-Msi171 (Figure 8). This provided suggestive evidence of protein-protein interactions between QseM and Msi172-Msi171 that would explain why QseM is protective against the growth-inhibitory phenotype caused by excess Msi172-Msi171. Interestingly constitutive QseM expression was not able to overcome the constitutive expression of Msi172-Msi171 from pNJ172171; this indicates that there is a threshold above which expression becomes unmanageable despite excess QseM. Currently what the cell does with a QseM+Msi172-Msi171 complex is unknown but this processing may be a limiting factor in the control of the growth inhibitory phenotype and subsequent gene transcription.

Bacterial two-hybrid analysis using *E. coli* as the host was used to confirm the interaction between QseM and Msi172-Msi171, with dense growth seen in the presence of 3-AT indicating an extremely strong interaction (Figure 11). Concurrent work by other lab members identified a frame-shifting region within Msi172-Msi171 that during transcription, creates a +1 shift (at a frequency of

around ~4.5%), leading to the production of a single transcriptionally active protein, FseA (L. Tester, J. Ramsay and W. Tate, unpublished data). This region was engineered to create a single reading frame corresponding to FseA. When this protein was assayed in a two-hybrid experiment, the same positive result for interaction was seen, complete with a similar ratio between NS and S plates. These positive results were taken a step further by analysing Msi172 and Msi171 individually against QseM. Previous two-hybrid work had determined that interaction between Msi172 and Msi171 did not occur, but this was conducted before *msi172* and *msi171* were known to encode a single protein through frameshifting. The co-transformation containing the Msi172 region and QseM produced substantial growth on selective media containing 3-AT, indicating a very strong positive reaction, whereas the Msi171 region and QseM co-transformation did not produce growth on selective media (Figure 11). As the QseM and Msi172 co-transformation produced a rate that was twice that of QseM and Msi172Msi171 or QseM and FseA this may indicate that there is reduced stoichiometric interference due to the lack of the Msi171 region. This result confirms that Msi172-Msi171 or FseA is another target of QseM, and also determined that Msi172 is the region of both that interacts with QseM.

As noted above, Msi172-Msi171 activates expression of the *rdfS* operon that when overexpressed produces a lethal phenotype that can be rescued by curing R7A of ICEM/Sym^{R7A} (16). We hypothesised that QseM will prevent the activation of the *rdfS* promoter, halting operon expression. When a plasmid expressing QseM and a second plasmid containing both *fseA* behind an inducible promoter and the *rdfS* promoter fused to *lacZ* were introduced into R7ANS, that lacks other ICEM/Sym^{R7A} genes, QseM repressed the activation of *rdfS* promoter (Figure 12). Expression created by the engineered frameshift version, FseA was much higher than regular Msi172-Msi171, reflecting the 4.5% frameshift level that occurs during translation of *msi172-msi171*, but normal frameshifting events on the pSDZ1721716H plasmid may still be occurring. However the presence of QseM in this background still had a significant ($p = 0.0016$) negative effect on activity (Figure 13). This confirms QseM's role in sequestering Msi172-Msi171 or FseA to control the QS system. It seems likely that QseM antagonises activity by sequestering Msi172-Msi171 or FseA, likely blocking DNA-binding regions, or by forcing a conformational change

as in both cases the presence of QseM reduced β -galactosidase activity down to that of the negative controls. Future work can be undertaken to investigate the ability of Msi172-Msi171 or FseA to bind the *rdfS* promoter region or to identify the region within Msi172-Msi171 that contains the DNA-binding domain.

Further work on this topic will seek to determine the region or residues of Msi172/FseA that interact with QseM and in particular if the region shares a common motif with TraR. Msi172-Msi171 has only recently been identified as being a single protein; however the sequence of the *msi172* and *msi171* open reading frames is conserved on a number of other ICEs that contain similar transfer systems to that of *M. loti*. Bioinformatics and computer-based modelling may offer insight and aid in the decryption of the folded structure of Msi172 and will help determine the regions required to complex with QseM. Once putative sites are identified, oligonucleotides can be manufactured and fused into a bacterial two hybrid system to test for positive interactions with QseM. Sequence analysis of these regions shows 4 residues in common between TraR and Msi172. This region and one other region have so far been trialled, but did not produce a positive result (J. Ramsay, unpublished data). A solution here is that an as yet unknown gene product or helper protein enables specific interaction between QseM and TraR or Msi172. This cannot be proven until the structure of the physical interaction is determined through crystallisation experiments.

4.2 Exclusion of genes and regions from interacting with QseM

With the successful identification of TraR, Msi172-Msi171 or FseA as targets of QseM, attention was turned to other regions that may be targeted. Previous work had identified that the *rdfS-traF* region was responsible for activating excision and induces a growth inhibitory phenotype when over-expressed. This is consistent with the observation that QseM binds Msi172-Msi171 and FseA to block activation of the *rdfS* promoter region. To exclude RdfS as an interactant, pSRKGmrdfS was introduced into R7A strains with or without QseM, in the absence of IPTG. It was suspected that this plasmid would also suffer from leaky expression though the extent of inhibition if any was unknown. Figure 9 shows that the level of leaky expression from the inducible promoter was enough to cause complete inhibition

of growth in strains containing pSRKGmrd*fS*, during the spot mating process, even in the presence of constitutive QseM expression. The inhibition seen in Figure 9 strongly suggests that QseM was unable to control Rdf*S* at the protein level. It can also be inferred that activation of the excision and the production of the growth inhibitory effect requires few molecules of Rdf*S*.

When *rd*fS** is knocked out leaving *tra*F** in-frame, the growth inhibitory phenotype still occurs when TraR is over-expressed (59). We hypothesised that if QseM was able to interact with Tra*F*, then constitutive expression of QseM would protect against the growth-inhibition caused by excess expression of Tra*F*. This hypothesis was proven incorrect, as we observed that when pSRKGmtra*F* plasmids were introduced into R7A strains containing constitutively expressed QseM and grown on media containing 1 mM IPTG, complete inhibition of growth occurred and no protective effect was seen (Figure 10). The caveat here is that concurrent plate counts in the absence of IPTG contained minor variations in CFU numbers, and that presence of QseM conferred growth to a marginally lower dilution when pSRKGmtra*F* was present, compared to when QseM was absent. This result was clearly not enough to show that QseM interacted with Tra*F* and may be a consequence of QseM manipulating levels of other QS proteins, such as TraR and FseA.

Strains that contained a pSRKGm empty vector produced very similar colony growth independent of IPTG levels, however the presence of pNqseM conferred growth to one factor dilution lower. Interestingly the difference in growth between the strains carrying pNqseM or pPROBE-KT was consistent whether or not they carried pSRKGmtra*F*. This suggests that pNqseM is conferring an overall fitness advantage that enables growth to lower dilutions regardless of any leaky expression from pSRKGmtra*F*. Performing a two-hybrid assay between QseM and Tra*F* would quickly clarify whether interaction occurs between these two proteins.

Before identification of Msi172-Msi171 as a target of QseM, it was hypothesised that QseM may bind the promoter region of the *tra*R** operon to block activation of *tra*R** expression, in addition to sequestering the TraR+3-oxo-C6-HSL complex, thus repressing the positive feedback loop further. Introduction of pJFXtra*R*, which contains the *tra*R** promoter region upstream of *lacZ*, into 3 strains containing or

lacking QseM determined that the presence of *qseM*, either as a genomic copy or on a plasmid, had no effect on β -galactosidase activity. The difference seen in activity between strains was small enough to be a result of natural variation between strains. However β -galactosidase expression levels from cultures containing plasmids with *traR* were not any higher than the negative control plasmids lacking *traR*. This suggests that there may be unknown issues that are hindering the activation of the promoter or that the promoter is very weakly and constitutively expressed. Activity that was higher in those plasmids that did not contain *traR* may be a function of plasmid promoter read-through. In order to determine this we would need to re-explore the design of each plasmid individually. The results suggest that either the *traR* promoter was not active under the conditions used and is subject to additional regulation, or the *traR* promoter region is not a target for QseM activity. Clearly expression from the *traR* promoter needs to be investigated further.

Published work from this laboratory (60) reported that in R7A Δ *qseM* mRNA levels of *traR* were up-regulated marginally while the transcripts of *traI1*, *traI2* and *rdiS* were increased markedly (2, 6, 7 and 26 fold respectively) relative to R7A when assayed using RT-qPCR. We hypothesised that if QseM interacted with TraR and other downstream genes, then complementation of the *qseM* mutant strains R7A Δ *qseM* and R7A Δ *qseM* Δ *traR* with pNqseM would reduce mRNA levels below R7A/pPROBE-KT, especially in the absence of genomic *traR*. Unfortunately reproducible data was not obtained, due to numerous problems in not only ensuring similar levels of growth between cultures, but also mRNA yields were very inconsistent even within biological replicates (Table 8).

As mentioned in the Section 2.4.1, frozen inocula were used to aid in the repetition and reproducibility of broth cultures, as previous experience had shown seeding larger broths from a single colony or fresh stationary-phase *M. loti* cultures produced inconsistent growth rates (John Sullivan, personal communication). Generally, use of the frozen inocula did give similar rates of growth between broths; however one or two broths per experiment would produce inadequate growth by 24 h and force a repetition of culturing. This phenomenon was not restricted to a particular strain or biological replicate and occurred seemingly at

random. Detergent contamination on glassware could have been a contributing factor and efforts were taken to ensure glassware was rinsed thoroughly before medium was added.

Inconsistent and low mRNA yields were problems in all extractions in preparation for cDNA synthesis and RT-qPCR and it was something that we were unable to rectify. Despite using a previously successful method and reagents from the same companies, mRNA concentrations were half, or less, than those obtained in previous experiments by others. Combined with variability between biological replicates that was independent of culture densities, results between individual RT-qPCR runs were not reproducible. Before the last cDNA synthesis was carried out, strains were selected from the 3 biological replicates on the basis of similar OD₆₀₀ values at 24 h. In doing this we hoped that mRNA levels would be similar across the five strains. This worked to a degree, as although variation between strains still existed, the range was much smaller (Table 8).

We remain perplexed as to why the yields reported here are much lower than those seen in (60) despite using the same method and reagents, and ensuring time above 0° was kept to a minimum. The addition of the pPROBE-KT or pNqseM vectors could have increased metabolic requirements of the cells and had a detrimental effect on growth compared to not having to maintain a plasmid. However as these plasmids were also used throughout the rest of the experiments in this thesis without any apparent detrimental effect, this seems unlikely. The addition or absence of half-strength antibiotics had no effect on growth rate or mRNA level.

What is hardest to understand is the difference in reported abundance as determined by RT-qPCR between technical replicates using the same cDNA preparation. The relative abundance varied substantially between individual preparations; therefore no conclusions can be drawn regarding the influence of QseM on gene expression. A possible limiting factor is that some (but not all) of the genes being assayed may only be being expressed in ~4.5% of the population at any one time due to the need to produce active FseA through frame-shifting, and the only way to harvest the mRNA from these cells is to harvest all cells. This dilutes the target mRNA within a sea of other mRNA, some of which may have

partial matches to the chosen primers. Even a large 20-fold increase in 5% of cells will only give a 2-fold increase in the overall population. Examining expression of these proteins at the single cell level using fluorescent tags and fluorescence microscopy would determine whether these proteins were co-localising as a result of interaction with QseM; or whether QseM was inducing degradation of the proteins over time, resulting in decreased fluorescence. Upregulation of genes in response to QS would also be traceable with this method.

4.3 Protein purification and folding

With the attachment of a 6H tag to the N terminal end of QseM shown to allow proper protein function, as seen in the CV026 assay (Figure 7), a large-scale purification was carried out successfully, yielding high quality and concentration of 6HQseM, as confirmed using mass spectrometry. The purified protein was then used in experiments designed to capture unknown proteins that likely interact with QseM, from a *R7AΔqseM* cell lysate, where expression of these proteins should be upregulated. Large amounts of captured protein were present in both 6HQseM-bound and blank columns. The column that contained 6HQseM prebound to the Ni-NTA, produced thick bands around the re-isolated 6HQseM protein. This was taken as an early indication that interacting proteins had been captured as these distinct bands were not present in the cell-lysate-only negative column (Figure 15). Unfortunately when the captured proteins were analysed with mass spectrometry and a purpose-created database, none of the suspected targets of QseM scored highly.

The database was created in this lab as the Centre for Protein Research did not have a suitable protein database for *M. loti*. As the database contained all known potential ICEM/Sym^{R7A} proteins in all possible frames, the lack of a strong positive result at any level was disheartening, as the 6HQseM bound lane that was analysed contained some very distinct bands that were not readily seen in the negative lane. The custom-made database was created in much the same way as others used for other model organisms, based off published sequences, both genomic and protein that can be found published online. It could be speculated that the proteins we are trying to isolate maybe extremely hydrophobic and simply did not elute from the

column when the technician believes they have. However due to the ease that large quantities of 6HQseM can be isolated, this experiment is repeatable, perhaps modified so that only the major, distinct bands are analysed by mass spectrometry, which would create a much narrower selection of digested protein segments. As we now also know that Msi172-Msi171 and TraR are other interactants, investment in a 6H-tagged version of these proteins or FseA may be a viable option to aid in the future crystallisation of QseM. Other groups (38, 39, 43, 44) have successfully catalysed crystallisation of TraR with the addition of AHLs, or TraM with the addition of TraR. This method may provide fruitful results when coupled to other 6H tagged proteins mentioned earlier.

The availability of large quantities of purified 6HQseM enabled other protein structural studies to be carried out and crystallisation to be trialled. A sample of 6HQseM was used in the biophysical technique circular dichroism and it was determined, after a buffer change to one with a much lower concentration of salts, that QseM was likely 86.81% α -helical in shape, similar to the published structure of *A. tumefaciens* TraM (43). This result was backed up by predicted folding patterns when modelled using computer algorithms. The actual shape is likely more α -helical as the value above takes into account the 6H tag attached to QseM as well as any interference created by salt in the buffer. An easy fix for the salt concentration would be a simple buffer change to water. However as we are unsure of QseM's ability to remain solubilised in water only, we did not attempt this. We were assured that it would be simple but we remain sceptical.

Exploratory experiments into crystallisation of QseM by another lab member have so far been unsuccessful, with 96 buffers tested but none promoting the crystallisation of QseM. There is scope here for more work perhaps using a 6H tagged TraR, Msi172-Msi171 or FseA to encourage crystals to form as in Chen et al. (40) where they determined the TraM-TraR complex. However given the relative arbitrariness of crystallisation studies nothing could eventuate without more in depth protein study.

4.4 Concluding remarks

In summary, this study confirmed the interaction between TraR and QseM and strongly suggested that this interaction does not involve the direct manipulation of the *traR* promoter region. Instead QseM acts through the TraR+3-oxo-C6-HSL complex to block downstream activation. Msi172-Msi171 was also positively identified as a target of QseM occurring through a protein-protein interaction, confirmed through bacterial two hybrid analysis. Characterisation was taken a step further with the Msi172 portion of the protein being shown as interacting directly with QseM. It is unknown at this stage if the same region is responsible for *rdfS* promoter activation; however QseM interacting with Msi172-Msi171 does block this activation. RdfS and TraF were both excluded as targets of QseM, as both proteins were, individually, able to induce a growth inhibitory phenotype that constitutive QseM expression was not able to protect against. Several other areas for possible interaction exist, such as the *tral1* and *tral2* promoter regions and they could be explored further using the pSDZ family of plasmids.

QseM proved to be a relatively easy protein to isolate when expressed in *E. coli*, producing a high concentration on the first attempt. The secondary structure was estimated to be ~86% α -helical in shape, which is in accordance with the reported shape for TraM, despite the lack of homology between the proteins. Fishing for interactant proteins at first appeared successful, but ultimately failed to deliver our confirmed interactant proteins when analysed using mass spectrometry.

5 Appendix A

Washing Buffer (1 L) pH 8.2

50 mM $\text{NaH}_2\text{PO}_4(2\text{H}_2\text{O})$ (MW 156.01) 15.601 g

100 mM NaCl (MW 58.44) 11.7 g

20 mM imidazole (MW 68.08) 2.72 g

20 % glycerol 400 ml

Adjust pH to 8.0 with NaOH

Lysis buffer (50 ml)

50 ml of Wash buffer

1 protease inhibitor cocktail tablet (Roche complete mini)

Elution buffer (200 ml)

50 mM $\text{NaH}_2\text{PO}_4(2\text{H}_2\text{O})$ (MW 156.01) 1.5601 g

100 mM NaCl (MW 58.44) 1.17 g

230 mM imidazole (MW 68.08) 3.12 g

20 % glycerol 40 ml

SDS PAGE buffers and gels

Loading dye

50 mM Tris-HCl pH 6.8,

2% SDS w/v,

2% β -mercaptoethanol,

12% glycerol w/v,

0.01% bromophenol blue.

SDS running buffer

25 mM Tris-HCl pH 8.3,

192 mM Glycine,

0.1% SDS w/v.

15 % resolving gel (20 ml)

6.76 ml 44.4% Acrylamide/1.2% Bis

7.5 ml 1M Tris/HCl pH 8.8
5.43 ml Distilled water
200 µl 10% SDS
100 µl 10% Ammonium Persulfate
10 µl TEMED

4% stacking gel (10 ml)

1 ml 44.4% Acrylamide/1.2% Bis
7.6 ml Distilled Water
1.25 ml 1M Tris/HCl pH 6.8
100 µl 10% SDS
50 µl 10% Ammonium Persulfate
5 µl TEMED

Mass Spectrometry results

Download available at: <http://bit.ly/1isjgvF>

Growth Media

Media was made up in distilled water unless otherwise stated, and autoclaved at 121 C (15 psi) for 15 min on a liquid cycle. When solid culture media was required, GIBCO-GRL bacteriological agar was added to broth to give a final concentration of 1.5% before autoclaving.

Growth media for *E. coli*

Luria-Bertani (LB) (64)

To 1000 mL water add:

10 g bacto-tryptone

5 g yeast extract

5 g NaCl

TY (65)

To 1000 mL water add:

5 g Bacto-tryptone

3 g yeast extract

2 mL CaCl₂ (0.65 mg/mL stock)

2YT (80)

To 1000 mL water add:

16 g bacto-tryptone

10 g yeast extract

5 g NaCl

SOB (80)

To 1000 mL water add:

20 g bacto-tryptone

5 g yeast extract

0.5 g NaCl

Add 10 ml of 250 mM KCl.

Growth media for Mesorhizobium

Rhizobium defined medium (RDM) (66)

To 700 mL of water add:

10 mL salts (25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g CaCl_2 , 1.5 g FeEDTA , 20 g NaCl per litre)

10 mL Bromothymol blue (2 mg/mL)

6 mL NH_4Cl (18 g/L)

1 mL Trace elements (3 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 40 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 50 mg H_3BO_3 , 40 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 4 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 1 mL CoCl_2 [0.2 g/L])

100 mg L-Histidine

10 g MES (S/RDM only)

For G/RDM: adjust pH to 6.5-7.0 with 2 M KOH

For S/RDM: adjust pH to 6.1-6.4 with solid KOH

Adjust volume to 1000 mL or for sucrose RDM adjust the volume to 750 mL. After the medium has been autoclaved, allow to cool and then add:

Carbon source

For RDM containing glucose as the sole carbon source (G/RDM): 20 mL of 20% (w/v) glucose; sterilised by autoclaving

For RDM containing sucrose as the sole carbon source: 250 mL of 20% (w/v) sucrose; sterilised by autoclaving

Phosphates

For glucose, glutamate, and sucrose RDM:

10 mL of stock solution containing 10% (w/v) K_2HPO_4 and 10% (w/v) KH_2PO_4

For succinate G/RDM:

4 mL of stock solution containing 10% (w/v) K_2HPO_4 and 10% (w/v) KH_2PO_4

Vitamins

1 mL of stock solution containing 50 mg nicotiamide, 50 mg thiamine HCl and 1 mL of Biotin (1 mg/ml) per 250 mL of water; filter sterilised. Antibiotics/colourmetric substrates/antifungals/ferrichrome/EDDHA/root and seed exudates/ FeEDTA added if required.

IPTG (24 mg/mL)

Dissolve 240 mg Isopropylthio- β -D-galactosidase (IPTG, Glycosynth, cat# 72045) in 10 mL water. Sterilise by filtration through a 0.45 μ m syringe filter and store -20 °C.

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