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Cloning and Characterization of SAS1738, a Hypothetical Exported Protein from Community-Associated Strain of Staphylococcus Aureus

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CLONING AND CHARACTERIZATION OF SAS1738, A HYPOTHETICAL EXPORTED PROTEIN FROM COMMUNITY-ASSOCIATED STRAIN OF *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) is a group of *S. aureus* strains that has acquired resistance to a class of beta lactam antibiotics and is the major cause of hospital associated infections. Their discovery goes back to 1960 when the first cases were identified. Recently community associated MRSA infections have emerged and are caused by strains that are independent of those from the hospital environment, related only because they carry some of the same antibiotic resistance genes. Community associated infections (CA) are more severe, producing pus filled lesions that are painful and capable of invasion of deep tissues. Virulence factors comprised of exported proteins are associated with the invasiveness of CA strains. Most of these proteins are hypothetical in nature with unknown function.

The aim of this study is to identify and characterize potential virulence factor proteins that may be involved in the infection pathway of CA-MRSA. This study focuses on a unique gene that encodes an exported protein, SAS1738, found on the chromosome of the CA strain MSSA476. The protein SAS1738 was chosen because it is unique to CA strains and has homology to some proteins identified in other *S. aureus* strains known for their virulence and host immune evasion. The goal of this work is to characterize SAS1738 and to determine its role in the infection pathway of the organism. The gene of interest has been successfully cloned, expressed, and tested for toxicity in *Caenorhabditis elegans*, a nematode. The toxicity tests showed that SAS1738 is inhibitory to the growth and development of *C. elegans*. The actual mode of action of this protein in *C. elegans* is yet to be established. However, location of

SAS1738 using a GFP fusion showed that the highest concentration of the fusion protein was in the gut of the worms. The purified protein when tested in a killing assay against *C. elegans*, resulted in the death of the worms at an average time point of 8 min after treatment.

Microbiological assay results showed that the purified SAS1738 possessed antibacterial activity towards *Micrococcus luteus* and *Proteus vulgaris*. This suggests that SAS1738 may play a dual role of antagonizing the commensal flora of the human skin such as *Micrococcus luteus* and also induce a toxic effect on the human cells as suggested by its toxic effect on *C. elegans*. Determination of the role of this protein in the infection cycle of CA-MRSA will lead to a better understanding of the pathogenicity of the organism and possible development of new treatment strategies.

PREFACE

The aim of this dissertation is to highlight the importance of studying the as yet uncharacterized hypothetical proteins in community associated (CA) *Staphylococcus aureus*. This work particularly focuses on SAS1738, one of the many as yet uncharacterized proteins produced and secreted by this group of bacteria. Based on the assays performed using both prokaryotic and eukaryotic systems SAS1738 is suggested to possess a dual function of inhibiting other normal flora of the skin and also play a role during the infection with a CA strain of *Staphylococcus aureus.*

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INTRODUCTION

Part I

Staphylococcus aureus is a Gram positive bacterium that is often implicated in bacterial infections. It is an opportunistic pathogen that permanently colonizes about 20% and transiently colonizes about 60% of the population. The infections caused by these pathogens, after they manage to cross the barrier created by the epidermal or the mucosal layer by means of virulence factors, are wide-ranging. Diseases caused by *S. aureus* may involve skin, soft tissues, bone (osteomyelitis and septic arthritis), heart (endocarditis) and lungs (pneumonia). Numerous extracellular proteins produced by different strains make it difficult to evaluate the pathogenic mechanism of this bacterium because not all of these proteins are known or characterized. Though most of these proteins do not appear to be toxins, studies have shown that some of them enhance virulence (Abramson 1973) and thus contribute to the increased invasiveness of *S. aureus* by evading the host immune system. Some of the known toxins of *S. aureus* are enterotoxins, exfoliative toxins, toxic shock syndrome toxin, and other exotoxins. Food poisoning, toxic shock syndrome and scalded skin syndrome are some of the most common infections caused by secreted toxins. In addition, these bacteria also secrete proteases, lipases,

nucleases, phosphatases, phospholipases, coagulase and staphylokinase all of which play roles during a Staphylococcal infection.

Methicillin resistant *Staphylococcus aureus* (MRSA) is a group of *S. aureus* strains that has acquired resistance to a class of beta lactam antibiotics and is the major cause of many hospital associated (HA) infections. Their discovery goes back to 1960 when the first cases of methicillin resistant strains were identified after the introduction of methicillin. Since 1960, MRSA in hospitals has increased at a rapid rate. Clonal expansion and gene transfer within the already existing lineages have been implicated as the causes for the rapid increase in MRSA disease rates.

Recently, community associated (CA) MRSA infections have emerged and are caused by strains that are independent of those strains common in the hospital environment. CA and HA MRSA are related only because they carry many of the same antibiotic resistance genes. While the HA strains are resistant to a broad range of antibiotics, such as erythromycin, tetracycline and clindamycin, the CA strains are resistant only to erythromycin and the β-lactam group of antibiotics (CDC). Nonetheless, community associated (CA) infections are more severe, producing pus-filled lesions that are painful and capable of invasion of deep tissue. Virulence factors associated with CA strains mostly comprise exported proteins. Most of these proteins are hypothetical in nature with unknown functions. In addition to carrying a smaller number of antibiotic resistance genes compared to the HA strains, the CA strains harbor the staphylococcal chromosome *mec* type IV cassette (SCC*mec*). The type IV cassette is less complex than types I, II or III found in the HA strains and hence may contribute to the increased flexibility of the CA strains.

Genome organization in *S. aureus*

The *S. aureus* genome is broadly divided into core (78%) and accessory (22%) domains. The core segment carries genes that are acquired through vertical transfer while the accessory segment has horizontally transferred genes. The core domain comprises genes that are mostly conserved among different strains of *S. aureus*, and play essential roles in metabolic and housekeeping activities. Variations in genes such that more than ten protein coding sequences are affected are usually a result of horizontal gene transfer. The genes that make up this variation comprise the accessory genome and encode proteins with virulence and antibiotic resistance properties. Pathogenicity and genomic islands, transposons and bacteriophages, and SCC elements are all part of this accessory segment. The variation in accessory genomes causes size difference among different *S .aureus* genomes that range from 2,799,802 to 2,902, 619 bp (Chan, Sherman et al. 2006). Species specific genes that differentiate *S. aureus* from other species of *Staphylococcus* (Lindsay and Holden 2004) are found in the core genome. This includes coagulase, Protein A (Spa), alpha and gamma hemolysins (hla and hlg), capsular polysaccharide biosynthesis (cap), etc. Common infections caused by *S. aureus* rely on the species specific genes, whereas the genes that comprise the accessory genome are scattered throughout the core genome, are involved in toxin-mediated infections, and are not common to all *S. aureus* infections. While the stable core domain is maintained and conserved through vertical species-specific gene transfer, the accessory domain, transferred by horizontal gene transfer, is highly variable. The ability to easily acquire or lose new genes makes it a primary force behind the evolution of novel strains, with additional virulence and antibiotic resistance traits.

Elements of the core genome

Genomic islands

Genomic islands of the core genome are regions that, unlike the accessory genome, display minor variations in their genes. These variations in the gene sequences are not in association with point mutation or insertional elements (Hiramatsu, Watanabe et al. 2004; Holden, Feil et al. 2004), and their protein products are usually involved in the metabolic pathways of the organism, with an exception of SAR0285-SAR0291 islet of MRSA252. The genomic island of HA-MRSA strain MRSA252 is well studied and has 41 unique genes whose sequences have been deduced. One of the islets that spans genes SAR0285-SAR0291 lies next to a genetic locus that resembles a conserved region in *Mycobacterium tuberculosis* (Pallen 2002) and is involved in producing a 6 kDa early secreted antigen target (ESAT-6) and a 10 kDa culture filtrate antigen (CFP-10), encoded by esxA and esxB (Stanley, Raghavan et al. 2003). This ESAT-like locus in MRSA252 is unique in that the *esx*B and *esa*C genes are absent in this strain but present in all the other sequenced *S. aureus* strains. Sequence comparison of genes from the two HA strains MRSA252 and Mu50 show similarity between many of its genes flanking the ESAT-6-like locus. The CA strains, MSSA476 and its close relative MW2, possess nine orthologs of SAV0294, a gene flanking the downstream terminal of the ESAT-6-like cluster in Mu50. SAV0294 protein also has significant homology to proteins encoded by 11 genes from another HA strain, COL.

Coagulase is an enzyme that is produced only by *S. aureus* and is used in differentiating it from other non-pathogenic strains. The gene encoding coagulase, coa, displays a wide variation among strains, and their allotypes share only about 65% to 88% amino acids. But presence of two distinct coa alleles only in closely related strains indicates a lateral gene transfer.

Another genomic islet gene is the collagen adhesion (*cna*) gene because it is devoid of any connection with mobile genetic elements (Gillaspy, Patti et al. 1997) and also is found in fewer strains.

Adhesion islands

Adhesion islands are genomic islands whose genes encode proteins that function in adhesion activities. The Cna protein belongs to the MSCRAMM protein family, whose main function is to adhere to the extracellular matrix of tissues. Because of its insertion between genes of the core genome the *cna* gene is part of a genomic island. The *cna* gene is believed to enhance virulence because it is associated with strains that cause severe necrotizing pneumonia and cartilage infections (Bentzmann, Tristan et al. 2004). Other important genomic islet genes are *fnb*A and *fnb*B that encode the fibronectin-binding proteins FnBPA and FnBPB. These belong to the MSCRAMM family as well and their genes found in most sequenced *S. aureus* genomes except the HA strain MRSA252 which has only *fnb*A (SAR2580). In MSSA476, *fnb*A (SAS2388) and *fnb*B (SAS2387) are flanked by a nucleotidyl transferase gene, SAS2386 and a gluconate permease gene, SAS2390. The same corresponding genes, SAR2579 and SAR2582, flank *fnb*A in MRSA252 (Chan, Sherman et al. 2006). Two gene products, *sar*T (SAS2385) and *sar*U (SAS2384), located in the same islet in MSSA476, belong to the Sar protein family involved in virulence (Cheung and Zhang 2002). The accessory gene regulator (*agr*) is an important complex in the expression of virulence proteins during *S. aureus* infection and is regulated by the combined activity of *sar*T and *sar*U along with *sar*A (Peng, Novick et al. 1988). The lack of this islet in MRSA252 suggests a different mode of virulence protein expression.

In MSSA476, a cell wall bound surface protein SasG (SAS2383) shows significant homology to a protein from *S. epidermidis* involved in biofilm formation (Hussain, Herrmann et

al. 1997) and also to a MRSA-specific plasmin-sensitive (PI) protein. All three of these are associated with adhesion to the nasal epithelial cells, an important stage prior to the colonization and infection of *S. aureus*.

Pseudogenes

Another core genome segment, the pseudogenes, consists of altered alleles that result from nonsense or frameshift mutations instead of genetic recombination. Among the HA-MRSA the evolution of vancomycin resistant strains has been observed and is associated with the pseudogenes. The HA strain Mu50 is a vancomycin-intermediate *S. aureus* (VISA) and is often compared to another closely related vancomycin-sensitive HA strain N315 to determine the significance of pseudogenes. Comparative studies involving these two strains have shown disruption of four genes through point mutations in Mu50, but not in N315. The four disrupted genes were unique to the VISA strain Mu50 (Wootton, Avison et al. 2004). Pseudogenes are thus considered an important area for study to better understand the origin of novel phenotypic traits through genotypic point mutations that can eventually alter an organism's overall pathogenicity.

Elements of the accessory genome

Bacteriophage and the Panton-Valentin Leukocidin (PVL) toxin

Three types of *S. aureus* prophages are encountered in the sequenced genomes and are designated ΦSa 1, ΦSa 2, and ΦSa 3 families. ΦSa 1 encodes the exfoliative toxin A (ETA) involved in scalded skin syndrome of *S. aureus* (Yamaguchi, Hayashi et al. 2000). None of the sequenced MRSAs carry this prophage in its active ETA producing form. The ΦSa 3 family of prophage carries a *sak* gene that encodes a plasminogen activator called staphylokinase and is found in strains MW2, MSSA476, MRSA252, N315, and Mu5O. The presence of this prophage in the genomes disrupts a β-toxin (*hlb*) encoding gene which is intact in most animal isolates. ΦSa 3 also carries several enterotoxin genes, the products of which cause food poisoning such as enterotoxin A, and also a few others like the *sep, seg2* and *sek2* (Holden, Feil et al. 2004; Lindsay and Holden 2004). However, one of the most potent toxins to have emerged in CA-MRSA strains is the PVL toxin. PVL has been implicated in a wide range of cases from life-threatening necrotizing pneumonia in children to skin and tissue infections among individuals from varying sectors of the community. MW2 and Newman are the two most invasive CA-MRSA strains that carry PVL. They are widespread in multiple sectors like gymnasiums, schools, and daycares and have led to increased mortality rates. While HA-MRSA strain, MRSA252, possesses a variant form of the ΦSa 2 prophage and does not make PVL toxin, the CA strain MSSA476 completely lacks ΦSa 2 and hence lacks PVL toxin. However, CA-MRSA MW2, which is suggested to have evolved from MSSA476, has the ΦSa 2 in a region that is occupied by SAS1429 in MSSA476. The attachment sites *att*L and *att*R required for prophage integration are found at the C-terminal end of SAS1429, a 750 amino acid hypothetical protein. The ortholog of SAS1429 in MW2 strain is split by the integration of ΦSa 2 in the middle of the

gene thus dividing the SAS1429 ortholog in MW2 into two separate coding sequences, MW1377 and MW1433. SAS1429 is flanked by SAS1426-28 on one side and SAS1430 on the other, and is also preceded by *srr*AB operon (SAS1432 and SAS1431) involved in oxygen dependent gene expression of a variety of virulence factors like toxic shock syndrome toxin (Yarwood, McCormick et al. 2001; Pragman, Yarwood et al. 2004). It is interesting to note that the integration of ΦSa 2 prophage, which harbors the PVL toxin gene, should take place right next the *srr*AB operon that modulates and controls the toxin expression. Though SAS1429 lacks a signal peptide its flanking genes SAS1426-28 and SAS1430 possess signal peptides in their protein sequences.

SCCelements

SCC*mec* is a genetic cassette that is categorized into six types designated SCC*mec* type I through VI. Within each type there are genetic variations that have been observed (Katayama, Ito et al. 2000; Ito, Katayama et al. 2001; Ma, Ito et al. 2002; Okuma, Iwakawa et al. 2002; Ito, Ma et al. 2004; Oliveira, Milheirico et al. 2006). SCC*mec* carries the methicillin resistance gene *mecA* encoding a penicillin binding protein PBP2a with reduced binding affinity for penicillin and other β-lactam antibiotics (Lim and Strynadka 2002; Chambers 2003). All types of *SCC* elements are found in the same location adjacent to the origin of replication *oriC* on the chromosome of *S. aureus* (Hiramatsu, Watanabe et al. 2004). Type I SCC element is the suggested to be the first to have arisen after introduction of methicillin in1959, followed by other types with additional genes conferring resistance to multiple antibiotics. Type I SCC element has only the *mec*A gene and is found in the hospital strain *S. aureus* COL. Types II and III SCC elements possess, in addition to *mec*A, other non-β-lactam resistance genes encoding resistance to macrolides, aminoglycosides, tetracycline, and heavy metals. While Types I, II and III are

found only in HA strains; types IV and V are usually encountered in CA strains, and easily undergo horizontal transfer by phage due to their relatively small size. The closely related CA strains, MW2 and MSSA476, both possess a type IV SCC element. However, the difference is that while MW2 has a *mec*A gene in its type IV SCC, MSSA476 has a fusidic acid resistance gene (SAS0043) (Holden, Feil et al. 2004; Lindsay and Holden 2004) in its Type IV SCC *mec*-like element, designated as SCC_{476}. Fusidic acid resistance is suggested to have developed as a result of frequent use of fusidic acid in the topical treatment of skin infections caused by *S. aureus* (Osterlund, Eden et al. 2002; Tveten, Jenkins et al. 2002; O'Neill, Larsen et al. 2004). Presence of protein domains within this SCC element have homology to domains found in another fibronectin-binding protein from *Listeria* (Gilot, Jossin et al. 2000; Gilot and Content 2002) . This evidence suggests a possibility that SCC_{476} may play a dual role of conferring fusidic acid resistance and also promoting bacterial adherence to the host fibronectin during infection. In addition, it also harbors the recombinase gene *ccrAB* or *ccrC*, the presence of which facilitates the excision and integration of SCC*mec* from one region to another on the chromosome (Katayama, Ito et al. 2000; Ito, Katayama et al. 2001; Ito, Ma et al. 2004). However, all types of SCC*mec* elements have been found to be integrated at a specific site of attachment*, attB*, present at the end of *orfX*, a hypothetical reading frame of unknown origin and function. The 15 bp nucleotide sequences that encode the five amino acids at the C-terminal end of *orfX* are the core of the *attB* site of the SCC*mecA* element. The presence of both the *orfX* and one of the recombinase genes, *ccrAB* or *ccrC,* is essential for a strain to become transformed into MRSA. When a SCC*mec* element is inserted, the 15 bp sequence comprising the core attB sequence repeats itself after the 15 bp sequence of the *orfX* and the new SCC*mec* insert is flanked by it on either side (Luong, Ouyang et al. 2002; Katayama, Takeuchi et al. 2003; Holden,

Feil et al. 2004; Mongkolrattanothai, Boyle et al. 2004). There are about 162 genetic backgrounds that comprise all *S. aureus* but MRSA is found in only 38 of these (Enright, Robinson et al. 2002).

The presence of a non *mecA SCC* element has been observed in *Staphylococcus aureus* MSSA476 which also harbors the recombinase gene *ccrAB* required for integration/excision activity. MSSA476 is unique and a rare exception because it is the only methicillin sensitive *Staphylococcus aureus* (MSSA) tested so far that has *ccrAB*, a feature characteristic of MRSA. Though some MSSA strains carry *orfX* gene with the 15 bp core *attB* sequence required for SCC*mec* insertion, the lack of a recombinase gene weakens their potential to acquire SCC*mec* element. Therefore MSSA476 marks a critical point in the evolution of novel strains possessing a recombinase and an *orfX* gene and serving as a source for other more invasive CA-MRSA strains such as MW2 and Newman. This idea is further strengthened by the fact that the two genes SAS0051-SAS0052 from MSSA476 and their orthologs MW0051-MW0052 from strain MW2 (Baba, Takeuchi et al. 2002; Holden, Feil et al. 2004), resemble by their homology enterotoxins and exotoxins from *Staphylococcus* and *Streptococcus* and are found flanking the right boundary of the SCC_{476} and the SCC*mec* elements of the two CA strains. The absence of these genes in HA strains, N315 and Mu50, indicate these are unique to the CA strains and show that MSSA476 could be the potential precursor CA strain that led to the emergence of CA strains like MW2, with additional characteristics acquired through shuffling of genes in the SCC element (Mongkolrattanothai, Boyle et al. 2004).

S. aureus pathogenicity islands (SaPI)

There are four types of SaPIs designated as SaPI(1-4) based on their insertion sites and the integrase homologies. The well-characterized SaPI2 is found in the two HA strains, Mu50

and N315, close to a ΦSa3 prophage. The phage related activities can cause excision and phage-packaging of SaPi2 (Ruzin, Lindsay et al. 2001). This promotes the dissemination of the SaPI2 family that carries genes encoding toxic shock syndrome toxin (*tst*) and enterotoxins. Another HA strain, *S. aureus* COL, carries SaPI1 which is found in the vicinity of *suf*B gene (SACOL0918) in close association with the ΦSa1 attachment sites. SaPI carries SACOL0908 encoding a putative β-lactamase (ear) protein. The COL strain carries only the ΦSa1 attachment sites but is not lysogenised. However, Mu50 carries the ΦSa1 prophage, as well as, a SaPI3 family protein but not SaPI1. The presence of a phage and SaPI3 and the potential to acquire the SaPI1 family near a *coa* allele makes this particular region a vulnerable zone for horizontal gene transfer in Mu50. This also explains why closely related strains possess distinct *coa* alleles while the distant strains share the same alleles (Hiramatsu, Watanabe et al. 2004).

In the CA strain MW2, a SaPI3 family is present in the same location of the genome as that of another SaPI3 family member in the HA strain Mu50. However, in MW2 the SaPI3 family has genes that encode a putative β-lactamase and two enterotoxins (Baba, Takeuchi et al. 2002) similar to that of the SaPI1 of the COL strain. The HA strain MRSA252 carries only a single SAPI4 family of pathogenicity islands, while MSSA476 completely lacks these pathogenicity islands, proving the high variability in these regions. The toxic shock syndrome (tst) toxin is the only clinically significant toxin associated with specific disease characteristics during infections. But due to its low incidence rate, *tst* alone cannot be used to evaluate the virulence enhancing role of pathogenicity islands. Hence in the virulence mechanisms of *S. aureus* the significance of pathogenicity islands is unclear and an area that deserves more study.

Genetic elements and islands (GEIs)

GEIs are of two types, vSaα and vSaβ positioned at 2 and 8 o'clock, respectively, on the chromosome map. They differ from the pathogenicity island SaPI2 by carrying a truncated transposase and unlike SAPI2 they lack any prophage association. Both vSaα and vSaβ harbor the DNA methylase gene, *hsd*M, and another gene *hsd*S that flanks *hsd*M and functions in the specificity of the methylase activity. Because of the presence of these methylation associated genes in the GEIs, vSaα and vSaβ are constantly transferred to a new genome without being excised. If excluded from the genome, the lack of methylation can allow restriction endonucleases to digest the genomic DNA (Kuroda, Ohta et al. 2001). These two elements also harbor a sequence of duplicated virulence genes that are putative in nature.

In vSaα, on the 5' end of the centrally located *hsd*M/*hsd*S, *set* genes are found in 9-11 tandem repeats and bear homology to exotoxin superantigens from other *S. aureus*. The 3' end is flanked by 3-9 tandem repeats of putative lipoprotein encoding genes, *lpl*. These exotoxins are suggested to play essential roles in destroying the host leukocytes (Kuroda, Ohta et al. 2001) contained within an abscess formed due to the regional inflammatory response induced by *S. aureus* infection.

In vSaβ, the *hsd*M/*hsd*S is located on the 3'-side next to the hypothetical transposase, and is flanked by the *spl* genes that are found in about five tandem repeats. The putative serine proteases (SplA-SplF) encoded by these genes carry a histidine active site signature sequence that is characteristic of the V8 family of serine proteases. The SplA-SplF proteins are significantly homologous to exfoliative toxins A and B (EtA and EtB), that are also glutamyl endopeptidases, and also to SspA, another glutamyl endopeptidase V8 serine protease protein encoded by the core genome. However, despite the absence of the *spl* genes by deletion in *S.*

aureus strain RN6390, experiments have shown that there has not been any reduction in the virulence of *S. aureus* (Reed, Wesson et al. 2001).

The vSaβ is further divided into types I and II (Baba, Takeuchi et al. 2002). The HA strains MRSA252, N315 and Mu50 carry type I with the exception of the COL strain which carries type II. The CA strains that have been sequenced carry type I. In type I vSaβ, the *spl* genes are in close proximity to the leukotoxin encoding genes *luk*D and *luk*E, and also to some putative enterotoxin genes. Interestingly, in the CA strains MSSA476 and MW2, the *spl* genes are separated from the *luk*D and *luk*E by an intervening sequence that harbors genes homologous to the lantibiotic synthesis genes involved in the synthesis of a peptide lantibiotic, epidermin, in *S. epidermidis*. This suggests that the CA strains MSSA476 and MW2 may have acquired this cluster of genes to fight off the competition posed by other normal flora of the skin by making the bacteriocin (Baba, Takeuchi et al. 2002). Also the presence of type I vSAβ in the first HA strains that evolved further strengthens this hypothesis because as more multiple drug resistant HA strains emerged, the need for this bacteriocin gene cluster became obsolete and the newer strains replaced it with leukotoxin and exotoxin encoding genes.

Bacteriocins and lantibiotics

Bacteriocins are peptides synthesized by some bacteria that have antibacterial or inhibitory effect against a narrow range of other bacteria. They are highly diverse in size, target, mechanism of action, and mode of immunity. Both gram positive and the gram negative bacteria produce these molecules. One widely studied bacteriocin is the colicin of *E. coli*. Colicin gene clusters are usually found on plasmids and they harbor separate genes encoding the colicin toxin, an immunity protein and a lysis protein that facilitates the extrusion of the synthesized colicin from the cell. Colicin synthesis and secretion are under the control of a SOS regulon which is

induced under stress (Braun, Pilsl et al. 1994; Cramer, Heymann et al. 1995; James, Kleanthous et al. 1996).

Gram positive bacteriocins are more diverse than those produced by the gram negative bacteria (Tagg, Dajani et al. 1976; Jack, Tagg et al. 1995). Their synthesis is not necessarily an activity that results due to stress. A specific protein transport mechanism, sometimes involving a *sec*-dependent pathway, exports the bacteriocins from the cell resulting in a less lethal event than in the gram negative bacteria. Their production is under the regulation of a bacteriocin-associated operon.

Classes of bacteriocins

Three classes based on the diverse production of bacteriocins by the lactic acid bacteria have been identified (Klaenhammer 1988). Class I are the lantibiotics named for their post-translational modification that that results in the addition of amino acids like lanthionine and β-methyllanthinone . There are two types of lantibiotics based on their mode of killing the target cell and unique structural characteristics (Jung 1991). Type A lantibiotics such as nisin are in the size range of 21-38 amino acids and kill the target cell by depolarization of the cell membrane causing pores and eventual lysis of the cell (Schuller, Benz et al. 1989; van Belkum, Hayema et al. 1989). Type B globular lantibiotics are in the size range of about 19 amino acids and kill the target by interfering with enzymatic activity involved in cell wall synthesis (Brotz, Bierbaum et al. 1995). An example of a type B lantibiotic is mersacidin.

Class II bacteriocins of the well studied lactic acid bacteria are heat-stable and comprise 30-60 amino acids (Jung 1991). They are further classified into class IIa and class IIb. Class IIa bacteriocins are similar in their mode of action to type A lantibiotics and kill the target by pore formation. All the class IIa bacteriocins identified thus far, pediocin AcH (Bhunia, Johnson et al.

1987), sakacin A (Schillinger and Lucke 1989), and leucocin A (Hastings, Sailer et al. 1991), harbor a conserved sequence of amino acids (YGNGVXaaC) and show inhibitory effect towards *Listeria*. Class IIb bacteriocins are a smaller group as compared to class IIa and include lacticin F (Muriana and Klaenhammer 1991) and lactococcin G (Nissen-Meyer, Holo et al. 1992) . Their mode of action is to form pores made of two different proteins in the target cell membrane.

Class III bacteriocins such as helveticins J and V (Joerger and Klaenhammer 1986; Vaughan, Daly et al. 1992) and lactacin B (Barefoot and Klaenhammer 1984) are generally larger than the other classes and heat-sensitive.

The Gram positive bacteriocins are more complex in their synthesis and involve a myriad of genes found in a cluster. A well characterized example is the nisin-producing genetic machinery comprised of *nis*A, encoding a prepeptide; *nis*B and *nis*C, encoding amino acid modifying proteins; *nis*P, encoding a leader-peptide cleaving enzyme; *nis*T, encoding a secretion-associated product; *nis*I and *nis*FEG, encoding immunity proteins; and *nis*R and *nis*K, encoding regulatory proteins (Buchman, Banerjee et al. 1988; Kaletta and Entian 1989; Engelke, Gutowski-Eckel et al. 1992; Kuipers, Beerthuyzen et al. 1993; Engelke, Gutowski-Eckel et al. 1994; Ra, Beerthuyzen et al. 1999). Most of these genes are clustered on transposons (Dodd, Horn et al. 1990) in plasmids but sometimes they are also found on the chromosome.

It is a general opinion that bacteriocins kill other closely related bacteria. However, type A bacterocins, nisin A and mutacin B-Ny266, kill a broad range of bacteria that includes *Streptococcus, Staphylococcus, Clostridium, Micrococcus, Listeria, Mycobacterium, Propionibaacterium, Corynebacterium*, and *Bacillus* (Mota-Meira, LaPointe et al. 2000). Clinically significant gram negative bacteria like *Neisseria, Campylobacter, Haemophilus*, and *Helicobacter* have also been shown to be killed by bacteriocins (Mota-Meira, LaPointe et al.

2000). Bacteriocin production starts during mid-log phase and peaks in the stationary phase (Buchman, Banerjee et al. 1988). The culture concentration and not the cell cycle is said to influence the expression of bacteriocins. As seen in the quorum sensing machinery, the bacteriocin production is dependent upon a two component regulatory system involving a response regulator and a sensor kinase (De Ruyter, Kuipers et al. 1996) . The *nis*R gene encodes the response regulator protein and *nis*K gene encodes the sensor kinase during the production of nisin.

Due to the antibacterial properties they possess, ribosomally synthesized bacteriocins are said to be secreted in order to antagonize the other bacterial flora that may occupy the same niche of the host. This potential to kill other bacteria has been tapped and some bacteriocins such as nisin and pediocin PA1/AcH are used in food preservation.

Staphylococcal bacteriocins

Bacteriocins produced by the *Staphylococcus* group of bacteria are called staphylococcins the best characterized being epidermin synthesized by *S. epidermidis*. Other staphylococcins include Pep 5, epilanciin K7, and epicidin 280.

The genes involved in the synthesis and production of lantibiotics in *Staphylococcus aureus* in general, and CA-MRSA in particular, is collectively called bacteriocins of *Staphylococcus aureus* (Bsa). Staphylococcin C55 is the only compound that has so far been proven to be a lantibiotic. But staphylococcin Au-26 produced by a vaginal isolate of *S. aureus* kills a broad range of targets like *Streptococcus pyogenes, Streptococcus salivarius, Streptocccus mutans, Staphylococcus hominis, Staphylococcus warneri, Lactococcus,* and *Neisseria* sp. Staphylococcin Au-26 and the Bsa genetic loci have been shown to be identical using mass spectrometry and mutagenesis (Daly, Upton et al. 2010).

Among other bacteriocins of *S. aureus*, aureocins A70 and A53, designated as class II bacteriocins, are the best characterized. While aureocin A70 is a bacteriocin, encoded on the plasmid pRJ6 and comprised of four related peptides, aureocin A53 is composed of 51 amino acids and is encoded on plasmid pRJ9. Since isoleucine is the first amino acid of both staphylococcin AU-26 and epidermin, made by *S. epidermidis*, it has been suggested that the two may be identical in their antibacterial activity. Sequencing results of the genes encoding these two lantibiotics indicated that they are highly homologous to corresponding regions of the type II vSaβ islands of many other sequenced strains of *S. aureus* linked to putative Bsa lantibiotics suggesting that the Bsa genes in the corresponding loci may be closely related to the well characterized epidermin and gallidermin produced by *S. gallinarum* . However, lack of homologues to BsaH or BsaT involved in the export of epidermin and galidermin suggests a different mode of export for lantibiotics encoded by *bsa* genes. The CA strains MW2, FPR3757, USA300, Newman, and MSSA476 harbor the *bsa* genes on their type II vSaβ genomic islands. The bovine mastitis associated strain, RF122, carries corresponding *bsa* genes that are related but not identical to those found in CA strains. But the HA-MRSA strains, MRSA252, N315, Mu50, and JH1, lack these genes in their types I or III islands.

Bacteriocins in general can be considered cytolytic virulence proteins because they provide the producer an ecological advantage in colonizing the host. Mutacins of *Streptococcus mutans*, streptococcins of *Streptococcus pyogenes* and staphylococcin C55 produced by *S. aureus* possess a cytolytic function that is no different from the actual cytolysin produced by the enterococci. The immunity proteins that each of these strains produce are specific and do not confer immunity to strains that make similar bacteriocins, hence production of these antibacterials constitutes a major factor in the pathogenesis of a single strain.

This study focuses on a unique gene that encodes a hypothetical exported protein SAS1738 found on the chromosome of a methicillin sensitive CA strain MSSA476. The protein SAS1738 was chosen because it is part of the accessory genome of MSSA476 and has relevant homologies to proteins from two other CA strains, MW2 and Newman, which makes it unique to the CA-MRSAs. MSSA476 is one of the first *Staphylococcus aureus* strains to be characterized a CA strain and is believed to be the first of its kind. Other CA strains like MW2 and Newman evolved later, acquiring additional genes encoding Panton Valentine Leukocidin (PVL) factors, and proteins that confer methicillin resistance. However, the continuous presence of the SAS1738 coding gene in all the recently emerged CAs seem to suggest that this protein plays an essential, significant role in the invasiveness of the CA strains.

We cloned and expressed the gene encoding SAS1738 in *E. coli* BL21 A1shot cells (Invitrogen). The protein was purified and tested by microbiological assay. Strong zones of lysis were observed against *Micrococcus luteus, Proteus vulgaris, and Pseudomonas aeruginosa.* No lysis was observed with the control samples. Based on the antibacterial properties displayed by SAS1738, we suggest that SAS1738 has a greater role in the infection pathway of the CA strains to aid in competition and antagonism of commensal microflora to occupy a certain niche and colonize the human skin and mucosa. While much work is yet to be done to understand the role of SAS1738 in the invasiveness of CA strains, it is clear that the effect of SAS1738 on other pathogens, especially *Pseudomonas aeruginosa* and *Proteus vulgaris,* well known for natural resistance to antibiotics commonly used in the clinical setting, make it a potential candidate for development as a treatment of certain bacterial infections in topical applications and possibly as a food preservative.

Part II

Caenorhabditis elegans

Caenorhabditis elegans is a nematode which serves as an efficient eukaryotic model for a wide range of studies. *C. elegans* metabolic pathways are conserved among the vertebrate eukaryotes making it a powerful system to determine the effects of toxins and other chemicals with respect to human biology. The presence of immune and nervous system and many common features that represent the cellular signaling pathways in humans and their sensitivities to heavy metals and human neuroactive drugs further justifies their widespread use in research (Nguyen, Alfonso et al. 1995). *C. elegans* is easy to grow; each adult worm is able to lay 300-1000 eggs every 3 to 5 days. Most worms are hermaphrodites, and thus do not require separate mating sessions in the laboratory, allowing for rapid experimental observations. The small size, approximately 1 mm in length, and transparent nature of the nematode are added advantages. Fusion proteins can be easily traced and protein expression can easily be visualized microscopically inside the worms. Another important area of research currently being explored with *C. elegans* is RNA interference studies.

Unusual movements of worms seen under the dissection microscope have been correlated with toxicity and this is one of the most important uses of these worms in research. Death due to toxic effects of the compound being studied can be determined by gently touching the worm with an eyebrow hair or a thin metal wire; lack of movement indicates death. Fluorescent dyes that can detect dead worms are sometime used to perform the above assay under a fluorescence microscope or using a fluorescent plate reader. *C. elegans* feeds on *E. coli* and the strain of *E. coli* that is commonly used in the laboratories for cultivation is the OP50 strain which is a uracil auxotroph. Both liquid and solid media can be used for growth.

Anatomy of *C. elegans*

Numerous studies have helped establish and characterize the anatomy of *C. elegans* (Brenner 1974; Byerly, Cassada et al. 1976; Sulston, Schierenberg et al. 1983). *C. elegans* goes through various larval stages before becoming an adult. Its long unsegmented body is cylindrical and divided into an outer and an inner layer separated by a pseudocoelomic cavity. The nervous system, muscular system, excretory system, cuticles and hypodermis are found in the outer layer; while the inner layer consists of the pharynx, intestine and gonads. The whole body is osmotically regulated by cells of the excretory system located in the posterior end.

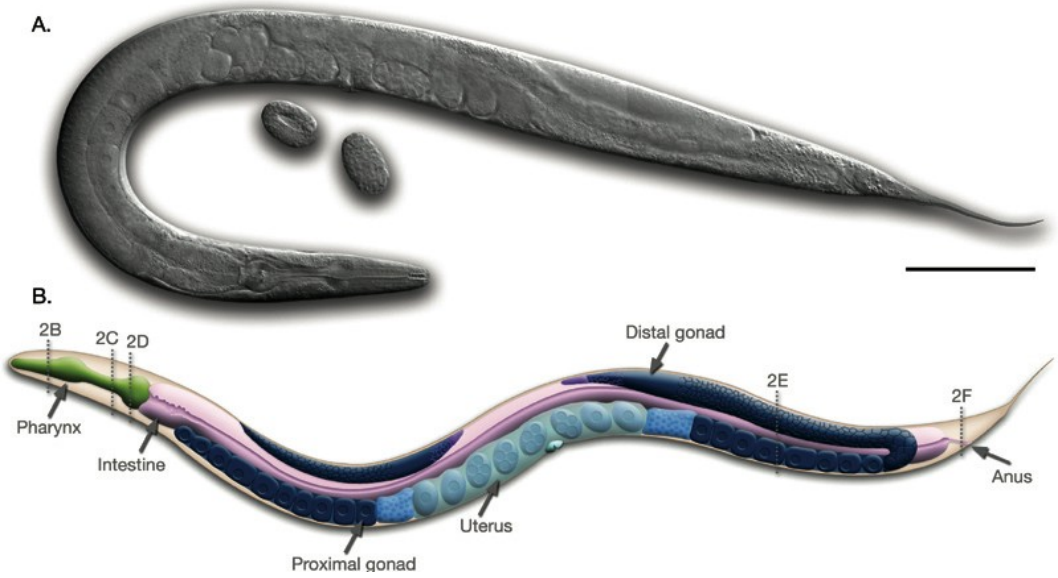

Figure 1 Anatomy of *C. elegans*.

A) An adult worm and two eggs. B) An adult worm showing various anatomical regions.

(www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro)

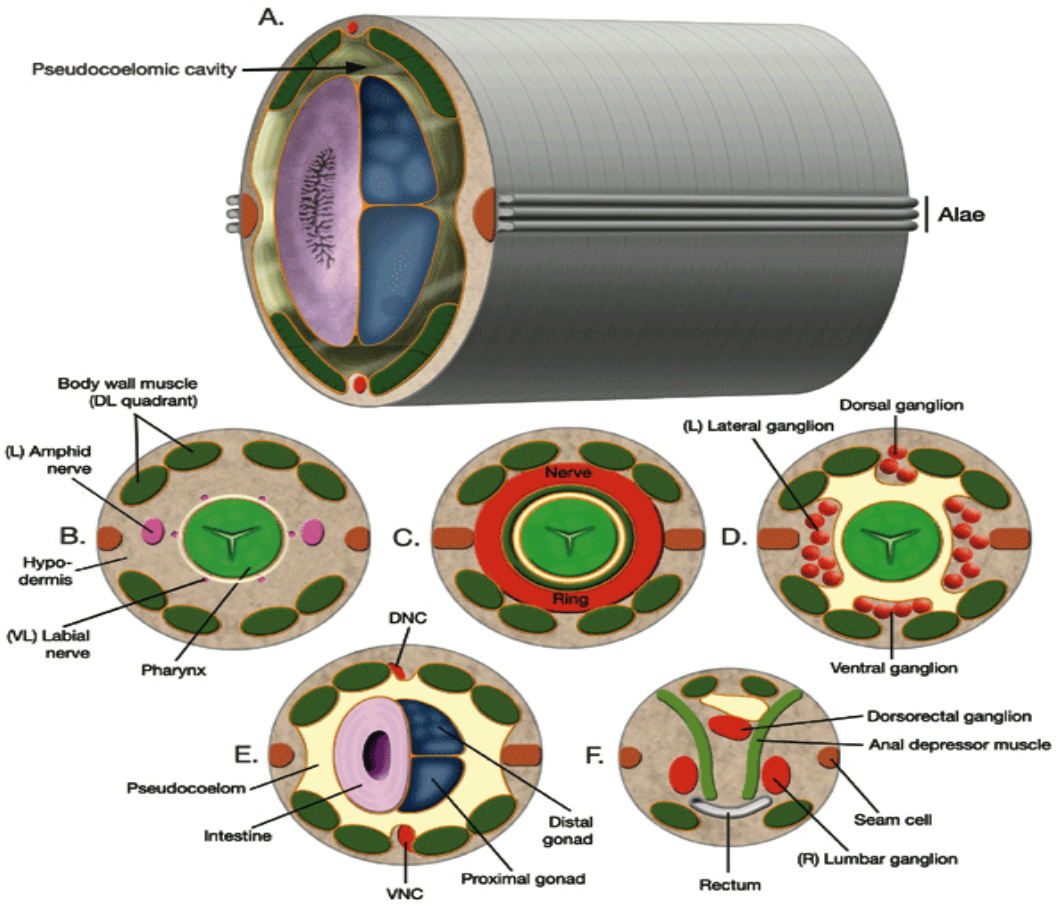

Figure 2. Cross sections of the *C. elegans*.

Figures A, B, C, D & E show cross sections of the worm at different locations. Pseudocoelomic cavity is seen in Fig A, separating the outer and the inner layer. Fig B, C and D show cross sections in the anterior, middle and the posterior ends of the head region. Fig E shows cross section of the body displaying dorsal and ventral nerve cords (DNC and VNC). Fig F shows section of rectum.

(www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro)

The hypodermis produces cuticle which comprises the outer layer. The nervous system consists of ganglia that are mostly concentrated around the pharynx in the head. They are also located in the mid-region of the body. The epithelial and the nervous system are separated by a basal lamina. Motor neuron signals influencing muscular movements are received by the muscular arms that come in contact with the nerve cords present in the nerve ring. The excretory system is involved in the expulsion of the waste from the body of the worm.

The pharynx of *C. elegans* functions independently and has its own muscular and nervous system. It is an organ that in involved in ingestion of food, and in passing the ground food to the intestine through an intestinal valve which connects to the intestinal lumen. Once inside the intestine the nutrients are absorbed by the microvilli that line the apical surface of the tubular intestinal lumen. The lumen is made up of 20 cells that have originated from a single somatic E blast cell lineage of cells. The cells that make the intestine are the only somatic tissue derived cells in *C. elegans*. The waste is then excreted out through the rectum, which is connected to the gut through a rectal valve.

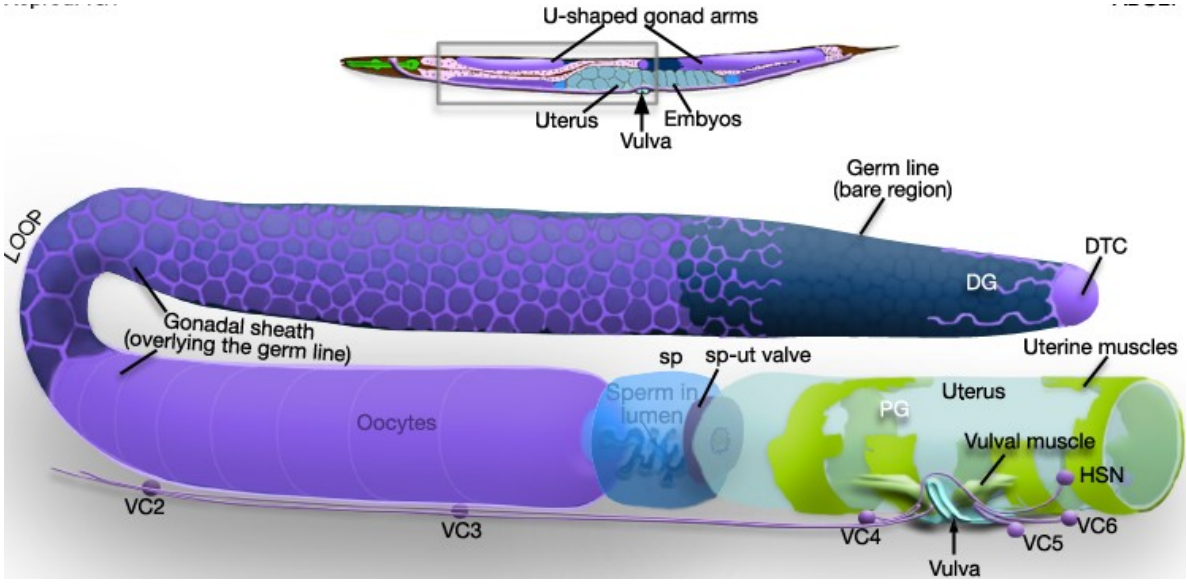

Figure 3. Reproductive system of *C. elegans*

(www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro)

Reproductive system of *C. elegans*

Somatic gonad, germ line, and the egg-laying apparatus comprise the three key parts of the reproductive system. The somatic gonad and the germ line form two U-shaped gonad arms that are connected by a uterus. Distal tip cell (DTC), gonadal sheath, spermatheca (sp), and a spermatheca-uterine (sp) valve together make up the somatic gonad which is distal to proximal in its organization (Fig 3). As the germ cells in the distal end, that are only mitotic in nature, move towards the proximal end, they enter the prophase stage of meiosis and reaches pachytene stage thus becoming differentiated in the proximal gonad. The hermaphroditic worms first produce sperm in the spermatheca before switching to a female somatic oocyte production. Self-fertilization of the female gametes by the sperm in the spermatheca roughly produces 300 embryos which move into the uterus, before the eggs are laid through an opening called the vulva.

Life cycle of *C. elegans*

There are two sexes in *C. elegans*, the predominant of which are the hermaphrodites (XX). Males (XO) comprise only 0.1% of the population and are generated as a result of an occasional non-disjunction in the germline of the hermaphrodites. However, hermaphrodites to high temperatures can increase the rate of male population generation to about 50%. Also mating between hermaphrodites and males can produce mutant offspring that can be stored at very low freezing temperatures. Under normal conditions, the worms possess a life span of approximately 3 weeks, but they can survive adverse environmental conditions by switching to a different lifestyle called the dauer stage. The dauer stage is facultative diapause stage which helps the worms survive longer than normal, increasing their life span 3 to 8 times that of their normal 3-week period.

The life cycle of *C. elegans* starts with the embryonic stage and proceeds to four larval stages (L1-L4) before attaining adulthood. The end of each larval stage is marked by a molt during which the cuticle of the previous larva is shed and a new one is synthesized. The first step of molting is the separation of the cuticle from the hypodermis (apolysis), the next step is the synthesis of a new cuticle, and the last step is the shedding of the old cuticle (ecdysis). The proteins synthesized during these 'molt' stages are stage specific and their production is higher in comparison to the non-molting stages. The microscopic structure of the cuticle varies during different stages. Prior to apolysis, pharyngeal pumping stops and the worm enters a stage of dormancy or immobility. This immobile or lethargus stage is divided into two phases. During the first phase, the cuticle begins to loosen around the rear end, the lips and the buccal cavity. The second phase is marked by further loosening of the cuticle from the body; the worm turns and flips longitudinally around on its axis. As the cuticle breaks down on both the posterior end on the intestinal side and at the dorsal end of the mouth, the larva forces itself out of a hole made near the head region.

The first cleavage of the embryo happens 40 min after fertilization and the eggs are laid 150 min after fertilization while still in gastrula stage. The larva that emerges goes through many different stages becoming a full grown adult as can be seen in Fig 4.

Larva 1

Five classes of motor neurons (VAn, VBn, VCn, ASn, VDn) are generated by the end of the L1 larval stage development. These 5 classes are produced from 13 precursors, W and P1-P13 (Sulston and Brenner 1974; Sulston and Horvitz 1977; Chalfie and White 1988). Also a ventral cord neuron (DDn) proceeds through the presynaptic phase which is inhibitory to the ventral wall muscles but is postsynaptic in its effect on the dorsal wall-activating neurons. Another class of motor neurons called VD motor neurons is generated during late L1 phase. At this point the DD motor neurons reverse in their function and become inhibitory to the dorsal muscle walls while being postsynaptic on the active neurons of the ventral wall muscles (White, Albertson et al. 1978; Walthall, Li et al. 1993).

In hermaphrodite worms, the two somatic gonad precursors Z1 and Z4 produce 12 cells, while the germline precursors Z2 and Z4 begin their long-lasting division that will continue to their adulthood (Kimble and Hirsh 1979).

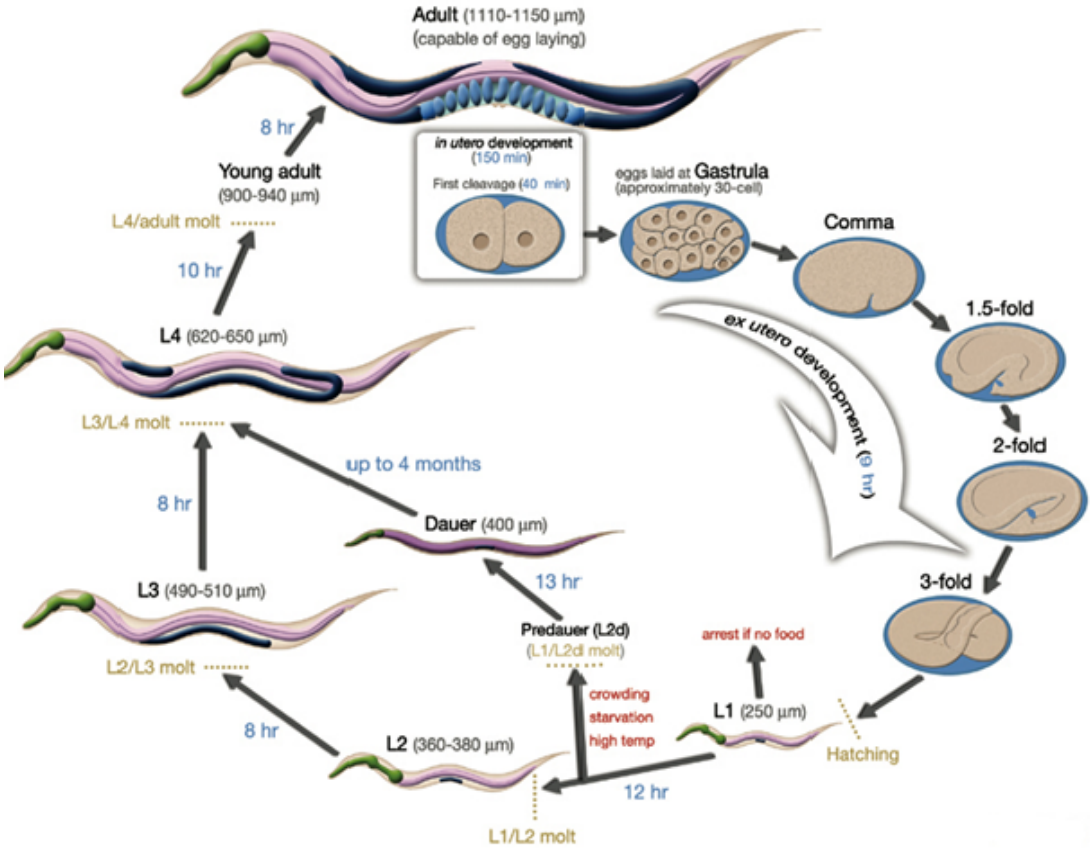

Figure 4. Life cycle of *C. elegans*. The different molting stages that a worm undergoes before attaining adulthood are shown in the figure.

(www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro)

L2 larva and dauer (L2d) larva

There is limited division during the L2 stage. Germline cells types Z2 and Z3 continue division resulting in quadruples. There is very little change in the Z1 and Z4 types. The somatic and germline lineages are basically interconnected until the stage of the L2/L3 molt is reached when there is a separation that leads to the formation of distal tip cell (DTC) and other gonadal structures.

The initial signal for the emergence of a dauer larva occurs in the middle of the L1 stage. Dauer stage larva will develop at the end of L2 stage if conditions are unfavorable. High temperatures, scarcity of food, and pheromones secreted during over-crowding of animals are factors known to induce the dauer stage. Under these conditions the worm does not molt into L3 but remains dormant, thickens it cuticle, shortens in size, stops feeding, ceases to move and does not age. Also there is shrinkage of the intestinal and pharyngeal lumen, a blockade of the buccal cavity by the cuticle and the complete lack of secretion by the granular glands of the excretory system. Gut cells turn dark and some microvilli appear in the intestine. As a result, entrance into a L3 stage under favorable conditions the normal life span is unaffected. When the dauer state is exited, feeding begins in about 3 hours and in about 10 hours, the worm molts into the L4 stage.

L3, L4 and the adult

The spermatheca and uterus develop from the somatic gonad precursors (total of 143 cells) in the L3 and the first part of the L4 stage (Kimble and Hirsh 1979). During L3, the growth of the distal tip cell (DTC) realigns and diverts in the dorsal direction. The vulva begins to develop from its precursors and vulval terminal cells develop by the early L4 stage.

The L3/L4 molt is characterized by germline meiosis producing sperm. Sperm development stops at the L4/adult molt during which the remaining germline cells differentiate

into oocytes. Egg-laying apparatus takes shape by a coordinated association of the vulval and uterus terminal cell generation, egg-laying neurons, and sex muscles.

An adult develops about 2 days after hatching, completing a reproductive cycle that lasts a total of 3 days (Byerly, Cassada et al. 1976). Oocytes are produced for about 4 days by the adult worm which can then continue to live for another 15 days. Each adult can lay up to 300 eggs.

Cholesterol and *C .elegans*

C. elegans does not possess cholesterol synthesizing enzymes and hence are auxotrophic for cholesterol (Hieb and Rothstein 1968; Chitwood and Lusby 1991). In the wild, they acquire cholesterol from animal feces and plant remnants. In the laboratory, they are provided with cholesterol in the nematode growth medium (NGM). As in other animal cells, cholesterol plays an essential role in the organization of the plasma membrane of nematodes. Inefficient molting and other defects in the reproductive cycle have been observed among worms grown in cholesterol-free medium (Yochem, Tuck et al. 1999). Cholesterol deprived worms have also been shown to display acceleration in their meiotic cell division by exiting the pachytene arrest, which is under the regulation of the Ras/MAP-kinase pathway (Church, Guan et al. 1995).

The mode of cholesterol uptake by the worms is not well understood. Whether its uptake is by the cuticle or the gut is still under investigation. The molecules involved in transporting cholesterol to various organs inside the worm have not been characterized, though gp330/megalin, belonging to the low-density lipoprotein (LDL) receptor superfamily is associated with this function (Yochem, Tuck et al. 1999). But its relatively high concentration in the hypodermis questions its role in the cholesterol absorption by the gut. A naturally occurring analog of cholesterol is a compound called dehydroergosterol (DHE) which possesses almost

identical activities as cholesterol (Schroeder, Jefferson et al. 1991; Schroeder, Woodford et al. 1995) and has been used to study the transport of cholesterol in these transparent worms using fluorescent microscopy. However, because of the inflexibility in its UV absorption and emission, it turned out to be a less successful candidate for the study than predicted. Cholesterol binding proteins have recently been studied with ease using a biologically active radiolabeled [^{3}H] photocholesterol. The use of DHE and [^{3}H] photocholesterol accumulation in *C. elegans* has primarily been found in the oocytes and the developing sperm (Matyash, Geier et al. 2001) . This stresses the importance of high level cholesterol storage in the oocytes for the growth and development of the embryo. However, the cholesterol level of sperm wanes in comparison with that of oocytes suggesting that the level of cholesterol in the sperm is insignificant for the development of the embryo.

Pathogen susceptibility in *C. elegans*

C. elegans displays diverse behaviors in the presence of bacteria. While some bacteria serve as its food, others can be pathogenic to these worms and induce activation of the innate immune system pathways to fight the pathogens. Studies using two different strains of worms to compare their behavior in response to pathogen exposure using *Pseudomonas aeruginosa* have shown that the N2 strain (isolated in Bristol, England) of worms took longer (90±13 hours) than another strain CB4856 (50±7.8 hours) in its susceptibility towards *Pseudomonas aeruginosa* for 50% lethality (Reddy, Andersen et al. 2009). A G-protein coupled receptor encoding gene *npr-I* is associated in the susceptibilities of these two strains. This receptor is closely related to the neuropeptide Y receptor of mammals. The N2 strain showed increased *npr-I* activity as compared to strain CB4856. This increased *npr-I* activity, caused by a polymorphism in its *npr*-I gene, is suggested to be responsible for the better pathogen fighting ability of the N2 strains (Reddy, Andersen et al. 2009).

CHAPTER 2

MATERIALS AND METHODS

Bacterial strains

S. aureus strains including MSSA476, MW2 and Newman were procured from the network on antimicrobial resistance in *Staphylococcus aureus* (NARSA), Eurofins Medinet, Virginia, USA. *Micrococcus luteus, Pseudomonas aeruginosa,* and *Proteus vulgaris* were purchased from American Type Culture Collection (ATCC). Glycerol stock cultures were made by adding 200µl of 80% glycerol to 800µl of overnight cultures of bacteria grown in tryptic soy broth (Difco). Stocks were stored at -70ºC. Before performing a bioassay, overnight cultures were prepared by inoculating a loopful of the glycerol stock into 3 ml Tryptic soy broth (Difco) and incubated at 37ºC.

Genomic DNA extraction and purification from *S. aureus* MSSA476

Overnight cultures of MSSA476 were used for DNA extraction using Invitrogen Pure Link Genomic DNA kit. All buffers and reagents were from the kit. Overnight cells were harvested by centrifugation at 10,000 x g for 2 min, and the pellet obtained was resuspended in 180µl of the lysozyme digestion buffer, which was incubated at 37ºC for 30 min after vortexing for 2 sec. To this, 20 µl proteinase K and 200 µl genomic lysis/binding buffers were added, mixed and incubated at 55ºC for 30 min. This was followed by addition of 96-100% ethanol to

the lysate and the mixture was vortexed to obtain a homogeneous solution. Purification of the extracted DNA was carried out by adding the lysate to the spin column and centrifuged at 10,000 x g for 1 min. After discarding the filtrate from the collection tube, the spin column was replaced and centrifuged for 1 min at 10,000 x g after adding 500 µl of wash buffer. The filtrate was discarded, 500 µl wash buffer 2 was added and the centrifugation was repeated at 16,000 x g for 3 min. The spin column was removed and placed over a sterile microcentrifuge vial. Pure Link Genomic elution buffer (25-200 µl) was added to the column and incubated for 1 min at room temperature. The purified DNA was collected in the microcentrifuge vial.

PCR

Primers were designed using the Invitrogen primer design tool for Gateway cloning which simulates the recombination that occurs in nature between the lambda bacteriophage and the *E. coli* genome (Zinn, Mellon et al. 1982; Bushman, Thompson et al. 1985; Landy 1989). Both the forward and reverse primers were designed to carry, at their 5' ends, a 25 base pair sequence known as attachment sites *att*B1 and *att*B2 (Weisberg and Landy 1983) . These *att* sites were stabilized by further adding four guanine residues on their 5' ends. The *att* sites are sites of attachment for various recombination proteins during recombination. The *att*B1 and *att*B2 sites recombine with *att*P1 and *att*P2 sites on the donor vector before inserting the gene of interest (Hartley, Temple et al. 2000). In order to fuse SAS1738 to a 6x His tag sequence, the stop codon was excluded in the reverse primer. However, there is a stop codon on the expression vector immediately after the 6x His tag sequence.

PCR was performed using a Promega kit, and as per Promega's protocol. The 50 µl reaction volume consisting of 8 µl $MgCl_2$, 10 µl 5X GoTaq buffer, 2 µl PCR nucleotide mixture, 0.25 µl each of the forward and reverse primer to a concentration of 0.5 µM , 0.25 µl of GoTaq

DNA polymerase, 1 µl purified DNA template (approximately 0.5 µg per 50 µl) and 28.25 µl distilled water. Primer sequences and PCR program parameters are given in Table 1.

PCR amplification was confirmed by performing a 1% agarose gel electrophoresis (Laemmli 1970). Agarose gel (1%) was prepared by adding 0.3 g agarose (Fisher Scientific) to 30 ml 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3 at 25ºC). The mixture was heated in a microwave for about 50 sec until the agarose melted and the solution was homogeneous. The hot solution was poured into a gel block with both of its open ends sealed with tape. A comb of appropriate length was inserted in the gel 1 mm from the base to make wells. After solidification of the gel, the comb was gently removed and the tape was removed. The gel was placed along with the block inside a chamber and was filled with 1X TAE buffer sufficient to cover the top of the gel. 5 µl of 1 Kb DNA marker (Invitrogen) was loaded into a well for size comparison. PCR reaction mixture (3 µl), previously mixed with GoTaq green gel loading buffer was loaded into a well. Electrophoresis was carried out at 100 volts (constant voltage) for about 45 min until the dye was approximately 0.5 cm from the bottom of the gel. The gel was transferred to a UV transilluminator and the amplified bands were observed. The amplified band corresponded to about 0.6 Kb lengths because of the addition of *att* B sites.

The amplified PCR product carrying the *att* B sequences were purified from the PCR reaction mixture by adding 150 µl of TE buffer (10 mM Tris-HCl/1 mM EDTA buffer, pH 8.0) and 100 µl of 30% PEG 8000/30 mM $MgCl_2$ to the 50 µl PCR reaction mixture. The vial was mixed well using a vortex and centrifuged for 15 min at 10,000 x g. The supernatant was removed and the pellet was dissolved in 50 µl of TE, pH 8.0. The amount of the PCR amplified DNA to be used in the first step of cloning (the BP reaction) was determined by the formula (50 fmol) x (N) x (660 fg/fmol) x (1 ng/10^6 fg) where N is the size of the amplified fragment; the size of the

amplified product was 639 bp (564 bp was the gene of interest, 50 bp was the *att* B sites, 8 bp was the guanine residues and 17 bp was from the sequence upstream of the gene of interest that includes the ribosome binding site). From the formula 21.087 ng/µl of the amplified DNA was needed for BP reaction. The concentration obtained from the fluorometer reading was 11 ng/µl. Therefore, 2 µl of the PCR amplified DNA was used for BP reaction.

Table 1.

Oligonucleotides and program settings used in PCR

Primer sequences	**PCR program parameters**
The attachment sites are shown in bold. Forward 5'**GGGGACAAGTTTGTACAAAAAAG** **CAGG**CTCCAAAGGAGCAATAAAAAT GAATAC 3' Reverse 5'**GGGGACCACTTTGTACAAGAAAG** **CTGGGT**CATCAATTGTGATTTTGTTG AT 3'	1) 95ºC – 2 min 2) 94ºC – 50 sec 3) 55ºC – 50 sec 4) 68ºC – 90 sec 5) Cycle 5x to 2 6) 95ºC – 30 sec 7) 55ºC – 30 sec 8) 72ºC – 90 sec 9) Cycle 25x to 6 10) 72ºC – 5 min 11) 4ºC – 0 min (END)

Cloning and Expression of SAS1738

Cloning was performed using Invitrogen Gateway Cloning Technology, which uses a site-specific recombination feature found in bacteriophage lambda (Zinn, Mellon et al. 1982; Bushman, Thompson et al. 1985; Landy 1989). This technique involves cloning of the gene in two separate steps called the BP and LR recombination reactions.

The BP reaction involves recombination between the *att*B PCR product and the *att*P sequence bearing donor vector, pDONR^{TM} 221, from Invitrogen

The reaction was carried out in a microcentrifuge vial containing 2 µl PCR product, 1 µl donor vector, 5 µl TE buffer at pH 8.0. To this mixture, 2 µl BP clonase II was added and the vial was incubated at 25ºC overnight. Following incubation, 1 µl of proteinase K was added to the vial and was incubated for another 10 min at 37ºC, to remove the enzymes from the mixture. The recombination products are the entry clones (Fig1) that have the gene of interest flanked by *att*L1 and *att*L2 sites on either end inserted in place of chloramphenicol and *ccd*B resistance genes thus knocking out both (Bushman, Thompson et al. 1985; Landy 1989).

The entry clones generated after the BP recombination reaction were transformed into One Shot OmniMAX 2-T1 chemically competent *E. coli* (Invitrogen) cells that were CcdB protein resistant (Bernard and Couturier 1992; Miki, Park et al. 1992; Bernard, Kezdy et al. 1993) by first thawing the vial on ice, adding 2 µl of the BP reaction mixture and incubating on ice for 30 min. The cells were subjected to heat shock by placing the vial at 42ºC for 30 sec and immediately replacing it in ice. SOC medium (250 µl), kept at room temperature, was added and the vial incubated at 37ºC for 1 hour in a shaker incubator set to 225 rpm. After a 1:10 dilution of the transformation mixture in Luria Bertoni (LB) broth, 20 µl and 100 µl were spread on LB agar plates containing 50 µg/ml kanamycin and incubated overnight at 37ºC. Five colonies were

individually picked for the successful insertion of the gene of interest. Screening was done by inoculating the five colonies into separate vials containing 50 µl PCR mixtures, except this time it contained an additional 1 µl of distilled water to substitute for 1 µl DNA template not used in this reaction. After PCR, a 1% agarose gel showed the bands corresponding to the fragment of interest (639 bp). Also the five colonies were streaked on chloramphenicol containing LB agar plates and none of the colonies grew, indicating that they were true transformants. The five colonies were grown in LB broth containing 50 µg/ml kanamycin and the entry clone plasmids were extracted using Zymoclean plasmid miniprep kit. The purified plasmid was used for the next cloning stage to obtain expression clones (the LR reaction).

The LR reaction involves recombination between the entry clones with *att*L sites and the destination vector pET-DEST42 (Invitrogen) carrying *att*R sites

The entry clones (50-150 ng/µl) were used in the second recombination reaction with the destination vector pET-DEST42 containing a C-terminal 6x His tag. The reaction was carried out in a vial containing 5 µl of the entry clone from the BP reaction, 1 µl of the pET-DEST42 (150ng/µl), and 2 µl TE buffer, pH 8.0. To this mixture LR clonase II enzyme (Invitrogen) was added and the vial was incubated overnight at 25ºC. This was followed by addition of 1 µl Proteinase K and incubation for an additional 10 min at 37ºC. This reaction was catalyzed by the LR Clonase enzyme II (Invitrogen) which catalyzes recombination between the *att* L and att R sites of the entry clone and pET-DEST42 vector. The expression clone obtained after the reaction bears *att*B sites on either end of the gene of interest. The expression of SAS1738 will be mediated by the Lac operon and the T7 promoter present upstream of the gene of interest. SAS1738 will be expressed as a fusion protein bound to a C-terminal V5 epitope and 6x His

sequence, so that the expressed protein can be either purified using Histidine binding Nickel or Cobalt columns or can be detected using anti-V5 antibodies.

The expression clones generated after the LR recombination reaction were transformed into One Shot OmniMAX 2-T1 chemically competent *E. coli* (Invitrogen) cells as described above, but using the LR reaction mixture. The clones were screened using LB agar plates containing 100 µg/ml ampicillin. Five colonies were individually picked for the successful insertion of the gene of interest. Screening was done by inoculating the five colonies into separate vials containing 50 µl PCR mixtures, except this time it contained an additional 1 µl of distilled water to substitute for 1 µl DNA template not used in this reaction. The resulting colonies were subjected to PCR as described above and agarose gel electrophoresis was used to visualize the resultant PCR product. Electrophoresis showed bands corresponding to the fragment of interest (639 bp). Also the five colonies were grown in LB broth containing 100 µg/ml ampicillin and the expression clone plasmids were extracted using Zymoclean plasmid miniprep kit. The purified plasmid was used for the next transformation step with *E. coli* BL21-AI One Shot® competent cells.

Transformation and protein expression in *E. coli* BL21-AI One Shot® competent cells

The expression clones were transformed into *E. coli* BL21-AI One Shot® competent cells that were derived from the BL21 group of expression cells (Studier and Moffatt 1986; Grodberg and Dunn 1988). Transformation was carried out by the same procedure as described above. *E. coli* BL21-AI One Shot® competent cells have a T7 RNA polymerase gene in the *ara*B locus of the *ara*BAD operon. The *ara*B is replaced by the T7 RNA polymerase gene which in turn is regulated by the *ara*BAD promoter. Hence, to induce expression L-arabinose was added (Lee, Butler et al. 1987).

Protein expression

Five colonies were individually picked for the successful insertion of the gene of interest. Screening was done by inoculating the five colonies into separate vials containing 50 µl PCR mixtures, except this time it contained an additional 1 µl of distilled water to substitute for 1 µl DNA template not used in this reaction. After screening of colonies from ampicillin plates and confirming the successful insertion of the gene of interest, a single transformed colony forming unit was inoculated into 5 ml LB broth containing 100 µg/ml ampicillin and incubated at 37ºC overnight or until the OD_{600} was between 0.6-1.0. A 1:20 dilution (OD_{600}=0.05-0.1) of this culture was made using LB broth with 100 µg/ml ampicillin and was incubated in a shaker incubator at 37ºC for about 2 hours until the mid-log phase OD_{600} reached ~ 0.4. The mid-log phase culture was divided into two equal parts containing 10 ml each, one to be induced, and the other an un-induced control. To one tube L-arabinose and IPTG were added to give final concentrations of 0.2% arabinose and 1 mM IPTG, respectively. To the other tube glucose was added to a final concentration of 0.1% in order to repress basal level expression of SAS1738 (Miyada, Stoltzfus et al. 1984; Lee, Francklyn et al. 1987). A 1 ml sample from each tube was aliquoted into fresh sterile microcentrifuge vials every hour from the start of induction and placed in the -20^{o}C freezer to stop induction. Samples were collected for four hours (Sambrook and Gething 1989).

15% Sodium dodecyl sulfate-Polyacrylamide gel (SDS-PAGE) electrophoresis and Western Blot

Two gels, each comprised of 10 ml resolving gel and 5 ml stacking gel, were prepared. One was for staining with Coomassie brilliant blue and the other for Western Blot.

SDS-PAGE gels were prepared as per the procedure described by Laemmli (Laemmli 1970). The resolving gel (10 ml) consisted on the following: 2.5 ml 1.5 M Tris-HCl (pH 8.8) buffer, 4.33 ml acrylamide/bisacrylamide (30%/0.8% w/v), 100 µl 10% SDS, 3 ml distilled water, 50 µl 10% ammonium persulfate and 10 µl TEMED. Stacking gel (4%) consisted of the following components: 670 µl acrylamide/bisacrylamide (30%/0.8% w/v), 1.25 ml 0.5 M Tris-HCl (pH 6.8) buffer, 50 µl 10% SDS, 3 ml distilled water, 25 µl 10% ammonium persulfate, and 10 µl TEMED.

Tank buffer was prepared using the following method: to prepare a total volume of 2000 ml, 28.8 g glycine, 6 g Tris base, and 20 ml 10% SDS were added to 1000 ml distilled water. After adjusting the pH to 8.3 with HCl, the volume was increased to 2000 ml with distilled water.

The 10 ml of resolving gel were carefully added into the space created by two glass plates separated by spacers. The resolving gel was allowed to polymerize after which the stacking gel was prepared and poured onto the solidified resolving gel. An appropriate comb was inserted into the still liquid stacking gel and allowed to polymerize. The 1ml fractions collected from the un-induced and induced samples were centrifuged at 12,000 rpm (11,750 x g) for 1 min. After discarding the supernatant, the pellet was resuspended in 100 µl of 1X SDS treatment buffer and the mixtures were heated to 90ºC for 10 min to denature the proteins. The mixture was cooled to room temperature and centrifuged at 9,000 rpm (6,610 x g) for 5 min. Samples (15 µl) from induced and un-induced reactions were loaded onto the SDS-PAGE gel. A 5 µl aliquot of protein standard marker sample (14,000 - 66,000) was loaded for comparison. The gel was placed in the tank buffer and electrophoresis was carried out at 150 V (constant voltage) for ~ 1 hour. One gel

was stained with Coomassie brilliant blue overnight followed by destaining with methanol/acetic acid while the other was used for Western blot.

Novagen His bind® AP Western reagents kit was used to perform Western blot

Blocking solution (3% bovine serum albumin (BSA) was prepared fresh in 1X Tris buffered saline (TBST) (150 mM NaCl, 10mM Tris-HCl (pH 7.5), 0.1% Tween-20) by adding 3 g of BSA to 100 ml of 1X TBST. Two liters of transfer buffer were prepared by dissolving 7.25 g Tris-Base and 33.5 g glycine in 1000 ml distilled water. Methanol (600 ml) was added to the mixture before the volume was brought to 3 L with distilled water after adjusting the pH to 8.0.

A Bio-Rad transblot system was used to perform Western blot as per the nitrocellulose gel transfer procedure of Towbin (Towbin, Staehelin et al. 1992). The SDS gel that was not subjected to Coomassie blue staining was placed on a nitrocellulose membrane and sandwiched in Whatman filter paper. This sandwich was again sandwiched between two pieces of sponge, clamped and placed in the Western Blot apparatus. The entire setup was then immersed in SDS tank buffer and protein transfer was carried out electrophoretically at 100 milliamps (constant current) overnight. The current was increased to 200 milliamps for another hour the following day. The nitrocellulose membrane was carefully removed and subjected to a series of washing and blocking steps as follows: two 10 min washes with 15 ml 1X TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.5), 1 hr incubation in 15 ml blocking solution at room temperature, two 10 min washes with 20 ml TBSTT (1X TBS, 0.05% Tween-20, 0.2% Triton X-100), a 10 min wash with 1X TBS, 1 hr incubation in 10 ml of 1:1000 dilution of anti-His monoclonal antibody prepared by adding 10 µl of antibody (Invitrogen) to 10 ml of blocking solution, two 10 min washes with 20 ml 1X TBSTT, a 10 min wash with 15 ml TBS, 1 hr incubation with 8 ml 1:5000 dilution of goat anti-mouse IgG antibody conjugate prepared in blocking solution, and five 10 min washes

with 20 ml 1X TBSTT. All washing and binding steps were carried out with gentle rocking on a shaker. The membrane was transferred to a clean container and the developing solutions were added. Glycerol stocks of colonies that were deemed positive by the Western Blot were prepared and stored at -70ºC for further use.

Protein purification was carried out as per a Novagen protocol for His-bind purification kit

Overnight cultures were prepared by inoculating a loopful of glycerol stock from -70ºC into LB broth containing 100 µg/ml ampicillin. A 1:20 dilution (OD_{600}=0.05-0.1) of this culture was made using LB broth with 100 µg/ml ampicillin (total volume 400 ml) and was incubated in a shaker incubator at 37ºC for about 2 hours until the mid-log phase (OD_{600} ~ 0.4). This mid-log phase culture was divided into two equal parts containing 200 ml each, one to be induced and the other an un-induced control. To one part, L-arabinose and IPTG were added to give final concentrations of 0.2% arabinose and 1 mM IPTG, respectively, for induction. To the other un-induced control, glucose was added to a final concentration of 0.1% to repress basal level expression of SAS1738. Both samples were incubated for 4 hours at 37ºC with constant shaking at 200 rpm (Miyada, Stoltzfus et al. 1984; Lee, Francklyn et al. 1987). The cells were harvested by centrifuging at 10,000 x g for 10 min. The supernatant was discarded and the pellet was drained completely by tapping the container on a tissue. To the pellet, 80 ml 1X binding buffer (40 ml per 100 ml culture volume) was added. The samples were briefly sonicated for resuspension and centrifuged for 15 min at 5,000 x g. The supernatant was discarded and the pellet was resuspended in 40 ml 1X binding buffer (20 ml per 100 ml culture volume). The samples were sonicated for three 6 min cycles at a constant duty cycle of 30% to release trapped proteins. After centrifugation at 5000 x g for 15 min, the pellet was resuspended in 10 ml (5 ml

per 100 ml culture volume) 1X binding buffer containing 6 M guanidine-HCl. This was followed by incubation on ice for 1 hr and centrifugation at 16,000 x g for 30 min. The supernatant from both samples was used for the purification process by column chromatography.

Column preparation

Two Novagen chromatography columns were prepared for the induced and un-induced samples. Sterile distilled water was added to the dry column and by pushing the column top with the gloved finger proper flowing through the column was ensured. His-Bind resin present in the form of slurry was mixed by inverting the container and 1 ml was pipetted into the column, resulting in 0.5 ml of a settled bed volume of resin. The resin was allowed to pack under gravity flow of storage buffer. When the storage buffer was almost gone, the following washes were carried out to charge the resin and maintain equilibration: 3 volumes (1.5 ml) of sterile distilled water, 5 volumes 1X charge buffer ((50mM $NiSO_4$) (2.5 ml)) and 3 volumes 1X binding buffer ((0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9)(1.5 ml).

Column chromatography

After 1X binding buffer was drained to the top of column bed, the 10-ml samples were loaded into the column, followed by a series of washes carried out with 10 volumes (5 ml) 1X binding buffer and 6 volumes (3 ml) 1X wash buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris-HCl (pH 7.9)). The bound protein was eluted by adding 6 volumes (3 ml) 1X elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9)), and the eluate was collected as 1 ml fractions.

Dialysis

Eluate fractions from both the induced and un-induced samples were subjected to dialysis to separate the recombinant protein from salts and imidazole. Dialysis was carried out using

porous membrane tubing (Spectra/Por) with the molecular weight cutoff (MWCO) of 12,000-14,000 daltons. Two 1.5 hour buffer changes were carried out before the setup was left for overnight dialysis with constant stirring at 4ºC. After dialysis, the samples were carefully removed by unclamping one end of the tubing and letting the sample flow into a sterile micro-centrifuge tube. The collected samples were concentrated using Centriplus YM 3 concentrators (Millipore) by centrifuging at 3000 x g for 1 hour. The concentrated sample from the retention vial was collected and filtered through a sterile 0.22 µm membrane filter to remove any bacterial contaminants.

Bioassays for antibacterial activity

Overnight cultures of bacteria were produced by inoculating 5 ml tryptic soy broth (Difco) with aliquots from 80% glycerol freezer stocks and incubating at 37ºC. The cultures were diluted with sterile tryptic soy broth (Difco) to match a 0.5 MacFarland turbidity standard. Bacterial lawns were prepared using the diluted cultures on Mueller-Hinton agar (Difco) plates. The plates were divided so that one half was tested with un-induced sample and the other with induced sample. 10 µl purified protein samples (~ 50 µg/ml) from induced and un-induced cultures were dispensed onto the lawns, respectively. The plates were incubated at 37ºC overnight.

Preparation of nematode growth media (NGM) and seeding of NGM with *E. coli* OP50

The N2 strain of *C. elegans* was purchased from University of Minnesota *C. elegans* Genomic Center (CGC). The worms were maintained on *E. coli* OP50, a pure culture of which was obtained from CGC.

NGM plates were prepared by adding 3 g NaCl, 2.5 g peptone and 17 g agar to 975 ml distilled water. This mixture was autoclaved at 121^0C for 50 min and cooled to about 55^0C. To

this cooled solution, the following sterile solutions were added and shaken to mix well: 1 ml (1 M) $CaCl_2$, 1 ml (5 mg/ml) cholesterol in ethanol, 1 ml (1 M) $MgSO_4$, and 25 ml (1 M) KPO_4 buffer (pH 6) (108.3 g KH_2PO_4, 35.6 g K_2HPO_4, H_2O to 1 liter) . This NGM agar medium in its molten state was poured into sterile Petri plates and allowed to solidify at room temperature (Brenner 1974). The plates were held at room temperature for 2 days to ensure no contamination. A fresh overnight culture of *E. coli* OP50 was used to seed the NGM plates (Hirsh, Oppenheim et al. 1976). The overnight *E .coli* OP50 culture (50 µl) was spread on the center of the NGM plate with a sterile loop to obtain a circular lawn of about 60 mm diameter. The seeded plates were allowed to incubate at room temperature overnight.

Platinum wire bent in the shape of a hook and attached to the holder of an inoculating needle was used for the transfer of worms. The surface and tip of the wire were flattened with a heavy rod and the edges were rubbed with sand paper to remove sharpness to avoid inflicting injury to the worms. The individual worms were gently transferred to the *E. coli* OP50 lawn using a dissection microscope. Plates stored at 16^0C can be stored longer than those kept at 20^0C. However, the growth rate is 1.3 times faster at 20^0C than at 16^0C; plates were routinely incubated at 20^oC (Maniatis 1982).

Freezing of *C. elegans* for long term use

The method used for storage of *C. elegans* was provided by CGC and uses a soft agar freezing solution to store stocks at -80^0C. This method allows for easy recovery of worms by inoculating a loopful of the frozen stocks onto a fresh plate. Freshly starved L1 and L2 larvae are the stages of worms that survive the best at -80^0 C (Brenner 1974).

Soft agar freezing solution was made by adding 0.58 g NaCl, 0.68 g KH_2PO_4, 30 g glycerol, and 0.56 ml 1 M NaOH, and 0.4 g agar to distilled water sufficient to bring the volume

to 100 ml (Avery 1993). The solution was mixed and autoclaved at 121^0 C for 20 min and cooled to about 55^0 C.

NGM plates containing freshly starved L1 and L2 larvae were obtained by letting the NGM plate with worms sit at room temperature until the *E. coli* OP50 was exhausted. This provides a great number of the L1 and L2 larvae for long-term storage. Each plate was washed with 600 µl of S buffer (129 ml 0.05 M K_2HPO_4, 871 ml 0.05 M KH_2PO_4, 5.85 g NaCl), the washings were collected in a sterile test tube and kept on ice for 15 min. An equal volume (600 µl) of soft agar freezing solution that was held in a water bath maintained at 55^0C was added to the test tubes. The test tube contents were then mixed well and 1 ml from this homogeneous solution was dispensed under aseptic conditions into sterile cryovials of approximately 2 ml capacity. The cryovials were stored in a -80^0C freezer for future use.

Toxicity and behavior assay of *C. elegans* as a model host during treatment with SAS1738

Two sets of NGM plates were prepared. To one set of plates 0.2% arabinose and 1 mM IPTG were added for induction. To the other set of plates glucose was added to a final concentration of 0.1% in order to repress basal level expression of SAS1738.

Toxicity assays were performed by first growing overnight cultures of *E. coli* OP50 in LB broth and *E. coli* BL21-AI One Shot® carrying the plasmid containing SAS1738 encoding gene fused to 6 X His in LB broth containing 100 mg/ml ampicillin. After overnight incubation both the *E. coli* OP50 and the *E. coli* BL21-AI One Shot® were diluted 1:20 times with LB broth to an OD_{600} of 0.05-0.1. The diluted cultures were then incubated with constant shaking for about 2 hours until the OD_{600} reached about 0.4 (mid-log phase). At this stage 50 µl culture from the *E. coli* OP50 tube was spread on a NGM plate containing no IPTG and arabinose to obtain a circular lawn. Another 50 µl was spread on a NGM plate containing 0.2% arabinose and 1 mM

IPTG. Likewise, circular lawns using 50 µl of *E. coli* BL21-AI One Shot® grown to mid-log phase were obtained on NGM plates with and without arabinose and IPTG. The plates were incubated at room temperature overnight. The following day, 3 adult worms were transferred aseptically to each of the four plates under a dissection microscope. The plates were incubated at 20^0 C for 3-4 days and assessed by counting the number of worms in the treated, untreated, and control plates.

GFP fusion to track SAS1738 inside *C. elegans*

A pGFP plasmid vector purchased from Clonetech was used for GFP fusion studies. This vector was converted to a gateway cloning destination vector by using Invitrogen Gateway vector conversion kit system. Restriction digestion of about 5 µg of pGFP vector was carried out with SmaI enzyme which induced a single blunt ended cut in the 5' MCS of the vector thus linearizing it to possess two blunt ends. The 5' phosphates were removed by using calf intestinal alkaline phosphatase (CIAP) to prevent self ligation by adding 4 µl of 10 x CIAP buffer, 1 pmol of DNA, 39 µl of sterile water, and 1 µl of CIAP into a 1.5 ml microcentrifuge vial and incubating at 50^0 C for 1 hr. The mixture was then incubated at 65^0C for 15 min to heat-inactivate the CIAP.

Ligation reaction was carried out by adding 1-5 µl dephosphorylated DNA (20-50 ng), 2 µl (5X) T4 DNA ligase buffer, 2 µl gateway reading frame cassette (10 ng) carrying the *att*R sites, 1 µl T4 DNA ligase (1 unit), and sterile water to a final volume of 10 µl. The mixture was incubated at 16^0C overnight.

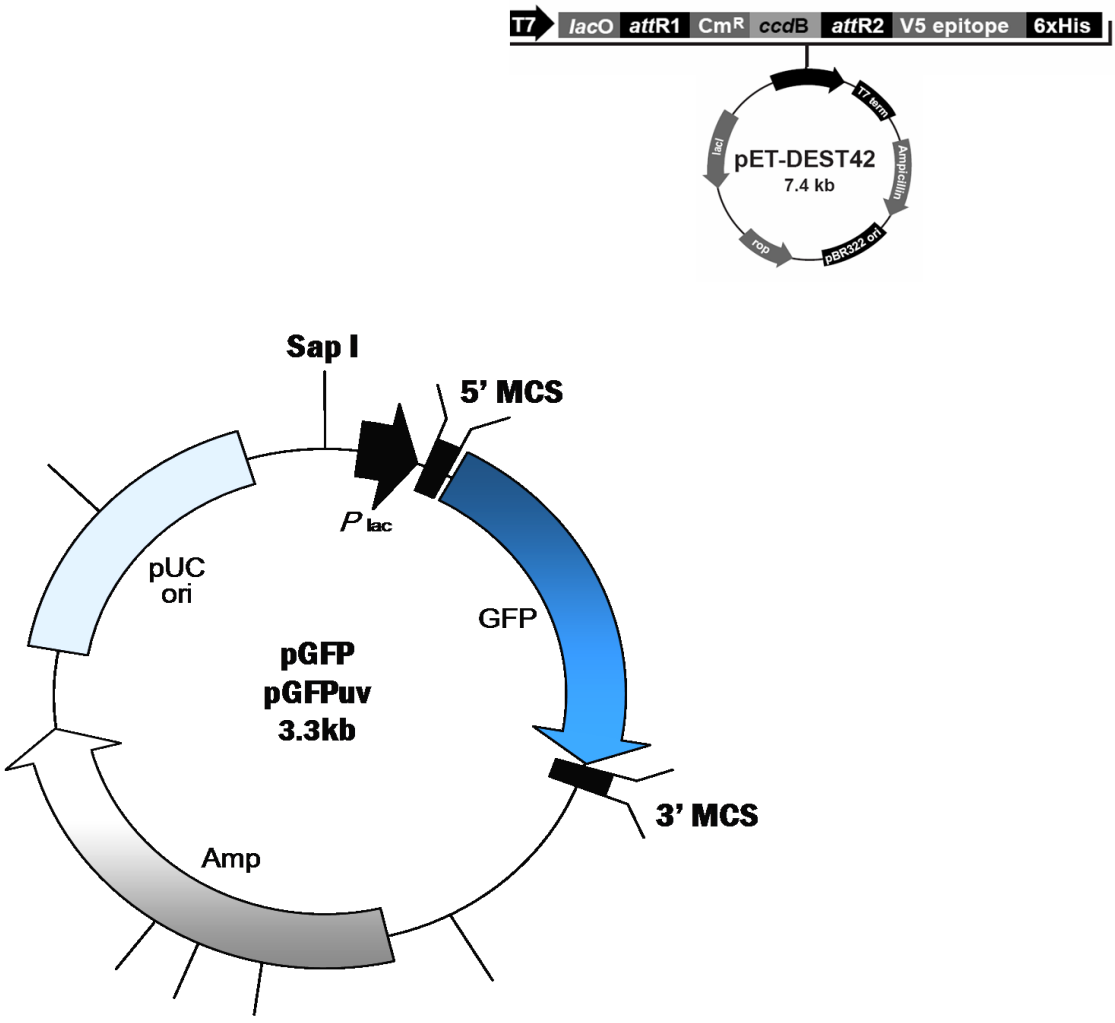

Figure 5. pGFP vector (Clontech) and pET-DEST42 vector (Invitrogen).

SmaI restriction site on the 5'MCS of pGFP vector was nicked to linearize the vector for conversion to a destination vector with attR sites as described above.

LR reaction between the entry clone carrying SAS1738 gene and the gateway destination vector carrying GFP gene (pDEST-GFP) and transformation of the same into *E. coli* **BL21 A1 shot competent cells.** The LR reaction and transformations were performed as described above. The transformants were isolated and grown in LB for GFP-fused protein expression.

Feeding *C. elegans* with *E. coli* BL21 A1 shot cells expressing GFP-fused SAS1738

GFP tracking of SAS1738 was carried out by first feeding to the worms bacteria expressing the protein.

This assay was performed by first growing overnight cultures of *E. coli* OP50 in LB broth and *E. coli* BL21-AI One Shot® carrying the plasmid containing SAS1738 fused to GFP in LB broth containing 100 mg/ml ampicillin. After overnight incubation, both the *E. coli* OP50 and the *E. coli* BL21-AI One Shot® were diluted 1:20 times with LB broth to an OD_{600} of 0.05-0.1. The diluted cultures were incubated with constant shaking for about 2 hrs until the OD_{600} reached about 0.4 (mid-log phase). At this stage 50 µl of *E. coli* OP50 culture was spread on a NGM plate containing no IPTG to obtain a circular lawn. Another 50 µl was spread on a NGM plate containing 1 mM IPTG. Likewise, circular lawns were produced using 50 µl of *E. coli* BL21-AI One Shot® grown to mid-log with and without IPTG. The plates were incubated at room temperature overnight. The following day, 3 adult worms were transferred aseptically to each of these four plates under a dissection microscope. The plates were incubated at 20^0 C for 3-4 days. Worms were picked individually from both the control and the test plates and observed under a fluorescence microscope after treating them with 3 % formaldehyde.

Toxicity assay of *C. elegans* using purified SAS1738

The purified, dialysed and filtered protein sample containing SAS1738 was used for this assay. On a clean and dry microscope glass slide 20 µl of the purified sample containing approximately 50 µg/ml of the protein SAS1728 was dispensed on one end. On the other of the slide, end an equal volume of the purified sample from the control uninduced culture was dispensed with a sterile micropipette. Adult *C. elegans* growing on *E. coli* OP50 were gently removed one at a time, with a bent metal wire under aseptic conditions. One worm each was

transferred into the 20 µl sample dispensed on both ends of the slide. The slide was constantly under observation with the naked eye. When the movement of the worm slowed down or stopped, the slide was placed under a compound microscope at 10 x magnification to ascertain the death of the worm by looking for lack of pharyngeal pumping action. A total of five trials were carried with each trial lasting for 60 min. The worms were transferred one at a time in order to make sure that there were no larval stages being transferred and also to reduce the chances of inflicting external physical harm during transfer, which would produce erratic experimental results.

CHAPTER 3

RESULTS AND DISCUSSION

The results obtained for individual experiments are reported in the order they were performed and the overall summary based on the antibacterial assay and *C. elegans* toxicity assay is discussed later.

Result

BP recombination

The entry clones generated after BP recombination reaction (Fig 6) had SAS1738 encoding gene inserted into the pDONR™ 221vector in place of the *ccd*B and Cm^R genes. The transformants failed to grow on LB agar plates containing 30 µg/ml chloramphenicol. This observation indicated a successful BP recombination reaction had occurred. Transformants were isolated from LB agar plates containing 50 µg/ml kanamycin and subjected to PCR for confirmation of the SAS1738 gene.

Electrophoresis of the PCR reaction mixtures revealed bands corresponding to the size of *SAS1738* gene, 639 Kb (Fig 7). Out of the ten transformants picked, nine showed bands corresponding to the size of the *SAS1738* gene (~ 639 kb) as determined using a standard 1 kb DNA marker.

LR recombination

The expression clones generated after the LR recombination reaction (Fig 8) had the SAS1738 encoding gene inserted into the pET-DEST42 vector in place of the *ccd*B and Cm^R genes. The transformants failed to grow on LB agar plates containing 30 µg/ml chloramphenicol. This observation indicated a successful LR recombination reaction had occurred. Transformants were isolated from LB agar plates containing 100 µg/ml ampicillin and subjected to PCR for confirmation of the SAS1738 gene.

Electrophoresis of the PCR reaction mixtures revealed bands corresponding to the size of *SAS1738* gene, 639 Kb (Fig 9). Out of the ten transformants picked, nine showed bands corresponding to the *SAS1738* gene (≈ 639 kb) as determined using a standard 1 kb DNA marker.

Western Blot

Following protein expression the induced and uninduced samples were subjected to Western blot technique as described earlier. Strong purple bands developed in less than a minute in lanes 2 and 3 (Fig 11). Protein bands were seen only in the induced sample but not in the uninduced.

Lane 1shows the separation of the standard protein marker displaying three bands corresponding to 14 kd, 20 kd and 24 kd respectively (Fig 11). The size corresponding to the SAS1738 was 24 kd (Fig 11) which can be seen in lanes 2 and 4. Lane 2 represents protein sample collected after four hours of induction while lane 3 shows sample collected after two hours of induction. Lane 4 was loaded with standard protein marker and lane 5 was used for protein collected from uninduced sample after 4 hours. No band was seen in lane 5.

However, two strong bands were seen, in lanes 2 and 3, one corresponding to 24 kd and the other to 20 kd, suggesting a possible posttranslational processing inside *E. coli*. Loss of the 42 amino acid long signal peptide from SAS1738 reduces its molecular size from 24 kd to approximately 20 kd, which was evident from the gel (Fig 11). Since western blot involved lysing the cells and extracting proteins from the inclusion bodies the samples are likely to contain both the processed and the unprocessed proteins within the *E. coli* and also from the medium in which it has been growing. The anti-his antibody binds to the 6xhis tag that is towards the c-terminal end of SAS1738 and hence bands representing both the processed and the unprocessed can be seen on the western blot.

Bioassay for antibacterial activity

Zones of lyses were observed on the *Micrococcus luteus* lawn (Fig. 12) when 10 µl of the filtered, purified and dialysed sample containing SAS1738 protein was spotted. No zones were observed in regions where filtered samples of the *E. coli* BL21 lysate and elution buffer were spotted.

A zone of lysis also was observed on a lawn of *Proteus vulgaris* (Fig. 13) when purified SAS1738 was spotted. However, the lysis observed with *Proteus vulgaris* was not as strong as with *M. luteus*. No zone was observed in the area where purified sample from the control uninduced culture was spotted.

Toxicity and behavior assay of *C. elegans* as a model host during treatment with SAS1738.

NGM Plates were observed after incubation at 20^0C for three days. In presence of 1 mM IPTG and 0.2% arabinose the first generation showed a substantial decrease in the number of worms on NGM plate with *E. coli* BL21 cells carrying the SAS1738 gene.

The number of worms on NGM with *E. coli* OP50 was higher and almost equal both in presence and absence of 0.2% IPTG and 0.2% arabinose, indicating that IPTG or arabinose had no effect on the growth and development of the worms. The total number of worms on *E. coli* OP50 was 5 times higher than that estimated on NGM with *E. coli* BL21 cells growing in presence of 1 mM IPTG and 0.2% arabinose, suggesting that SAS1738 inhibited the replicative life cycle of *C. elegans* causing a decline in their number after the first generation over a period of 4 days. The results obtained were constant with all the five trials performed.

On the NGM plate where *E. coli* BL21 was not induced by IPTG and arabinose, the number of *C. elegans* was almost identical to the number observed on *E. coli* OP50 plates (Fig 14). Five-fold fewer *C. elegans* were observed on plates containing *E. coli* BL21 induced by IPTG and arabinose suggesting expression of SAS1738 inhibited growth of the worms.

In addition to the reduced population size, *C. elegans* growing in presence of SAS1738 also displayed bordering (Fig 14 & 15), a phenomenon where the worms mostly concentrate on the edges of the bacterial lawn. No bordering phenomenon was observed in absence of SAS1738 (Fig 14 & 15). The number of *C. elegans* on *E. coli* BL21 growing in absence of IPTG and arabinose is higher and shows no bordering (Top left). Bordering is seen, and the numbers are reduced when SAS1738 expression in *E. coli* BL21 is induced by 1 mM IPTG and 0.2 % arabinose (Top right). The number of worms is high and no bordering appears on NMG with *E. coli* OP50.

This behavioral pattern may be a result of pathogen susceptibility which makes the worms prefer low oxygen concentrations found at the edges of the bacterial lawn (Reddy, Andersen et al. 2009). By moving to the border the worms avoid increased contact with the bacteria in the center of the lawn. The center of the lawn has a high concentration of bacteria presumably making SAS1738, which may be sensed as a potential toxin by the worms. Conversely SAS1738 might have induced a change in the behavior and driven the worms towards the edges as was evident from the results. This difference in aerotaxis behavior (movement in different directions that have varying oxygen concentrations) between *C. elegans* growing in presence and absence of SAS1738 combined with a 5-fold reduction in population size in presence of SAS1738 strongly suggests that SAS1738 might be a toxin that could induce behavioral changes in *C. elegans* and affects their replicative cycle, causing a decline in their numbers.

Inhibitory effect of SAS1738 on *C. elegans* growth.

Results from the five trials of experiments are shown to demonstrate the consistent 5-fold reduction in the number of worms when grown on *E. coli* BL21 expressing SAS1738 compared to growth on *E. coli* OP50 and the uninduced *E. coli* BL21(Fig 16). The number of worms on uninduced *E. coli* BL21 and *E. coli* OP50 were approximately 800 in number after 4 days while the number of worms on induced *E. coli* BL21 was about 200 in all the five trials.

Confirmation of successful ligation of the gateway reading frame cassette into the 5' MCS of pGFP vector by PCR and gel electrophoresis

The expected molecular size of the new insert was approximately 1.7 kb because PCR primers were designed such that the forward primer included bases specific to the reading frame and the reverse primer spanned bases upstream from the stop codon of GFP encoding gene. The five transformants that were picked from a LB agar plate containing 100 µg/ml ampicillin showed the appropriate 1.7 kb fragment after PCR. Lane 1 represents standard 1 kb DNA marker showing bands corresponding to different sizes as shown in figure 15. Lanes 2-6 represent samples after PCR from the five transformants and each of them displayed a band corresponding to approximately 1.7 kb size (Fig 17).

Toxicity and behavioral assay of *C. elegans* when grown on *E. coli* expressing the GFP-fused protein SAS1738.

The number of *C. elegans* was higher in the presence of *E. coli* OP50 compared to the numbers on NGM containing *E. coli* expressing SAS1738 fused to a GFP protein (Fig 18 and Fig 19). The number of *C. elegans* feeding on GFP-fused SAS1738 expressing *E. coli* BL21 was lower and their behavior was similar to those displayed when the worms were grown on *E. coli* BL21 expressing 6 x his-fused SAS1738. This study indicated that the GFP-fused SAS1738 retained its inhibitory effects on the growth and replicative cycle of *C. elegans*.

GFP fusion tracking of SAS1738 inside *C. elegans.*

GFP-fusion tracking of SAS1738 was done in order to see which anatomic centers inside the worms had the highest concentration of SAS1738. In addition, this was also a test to show that addition of a GFP tag did not reduce the inhibitory effect on the worms. Since there was a similar decline in the number of worms after feeding them with GFP-fused SAS1738, it indicated that SAS1738 was inhibitory to the growth of worms even when fused to GFP. On the contrary this test was also an indication that the 6Xhis tag in itself did not confer any inhibitory effect on the worms since.

The first generation of worms were observed under a fluorescence microscope after three days of incubation at 21^0 C. Fluorescence inside the worms were areas where GFP-fused SAS1738 were concentrated which was evident in most areas but the strongest was in the pharyngeal zone and in the reproductive system (Fig 20). Intestinal lumen showed a reasonable amount of fluorescence as well.

Toxicity assay of *C. elegans* using purified SAS1738

Five trials of the toxicity assay were performed and the results were plotted with time on the y-axis and the number of trials on the x-axis. Death of worms was based on complete lack of movement and cessation of pharyngeal pumping action. The death times recorded for each trial were 5 min, 10 min, 20 min, 7 min and 6 min when treated with purified SAS1738 (Fig. 21). The average death time was 8 min. No deaths occurred when worms were treated with a purified uninduced control sample. Each trial was performed for a total of 60 min. Each assay was carried out for a total of 60 min and hence the plot with respect to untreated worms flattens though the worms in the sample remained alive even at 60^{th} min (Fig 21).

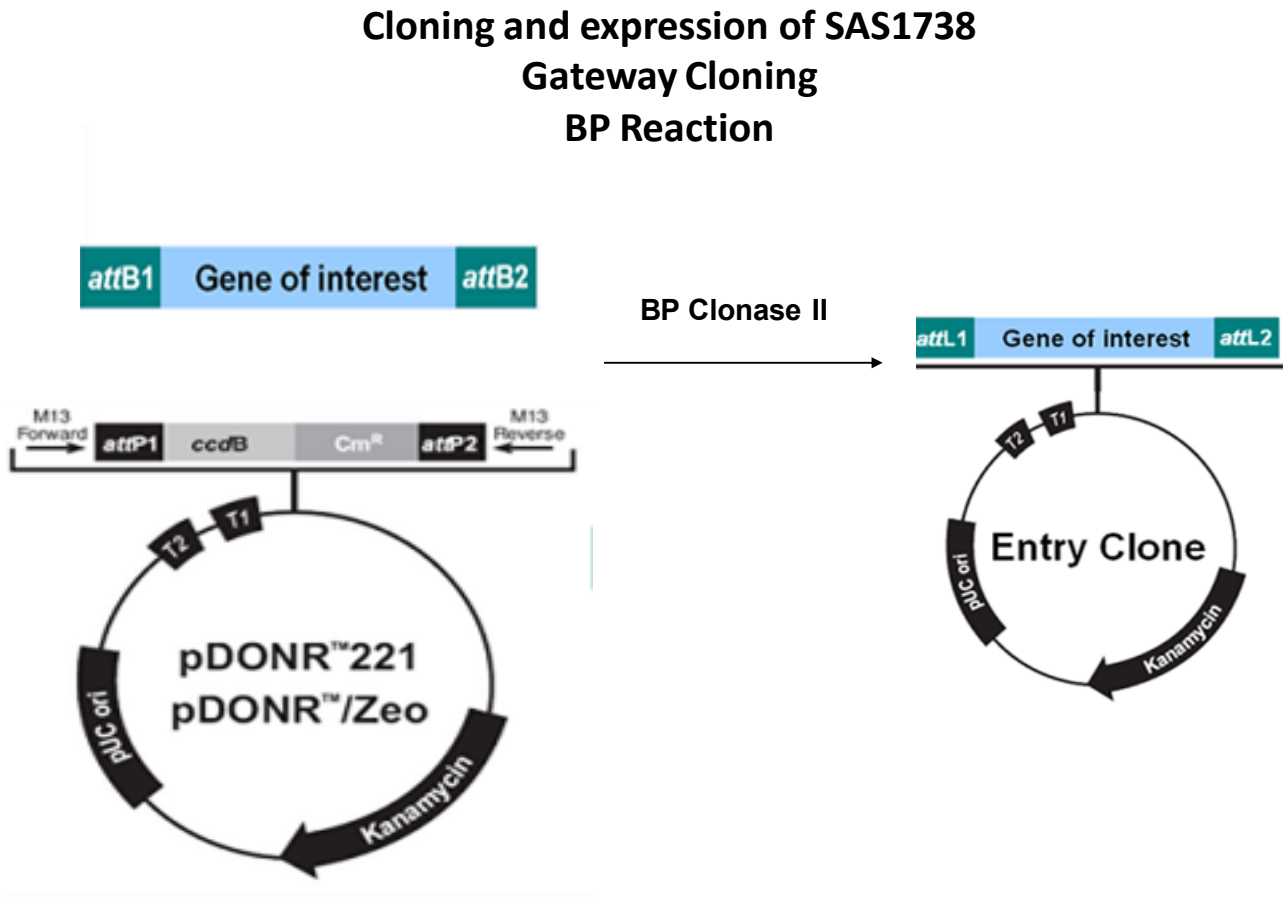

Figure 6. BP recombination reaction.

In BP recombination reaction, attB sites on the amplified sequence containing the gene of interest recombine with attP sites on the pDONR 221 vector producing a recombination product (entry clone) bearing att L sites.

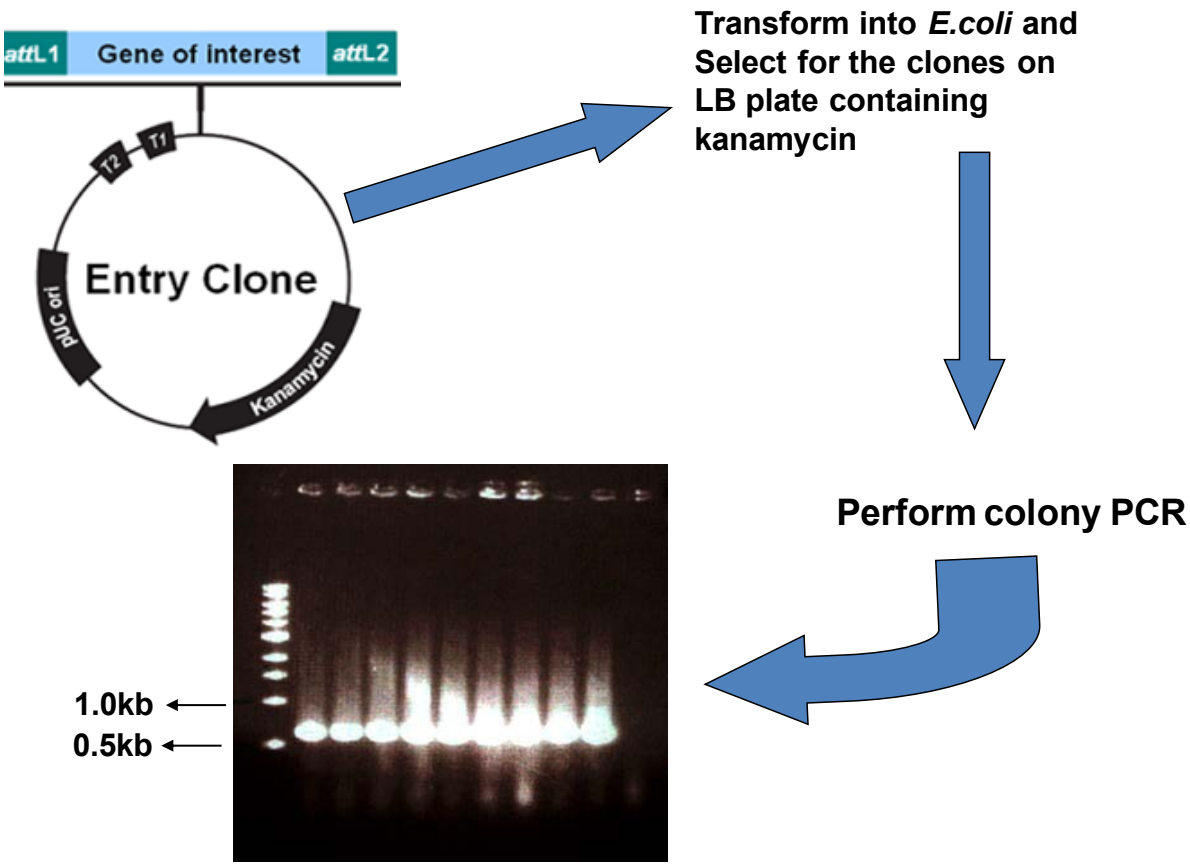

Figure 7. Confirmation of entry clones on 1% agarose gel .

The entry clone after being transformed into One Shot OmniMAX 2-T1 chemically competent *E. coli* (Invitrogen) cells were selected on kanamycin plates (40 µg/ml). PCR was performed and the corresponding band sizes were obtained representing the gene of interest.

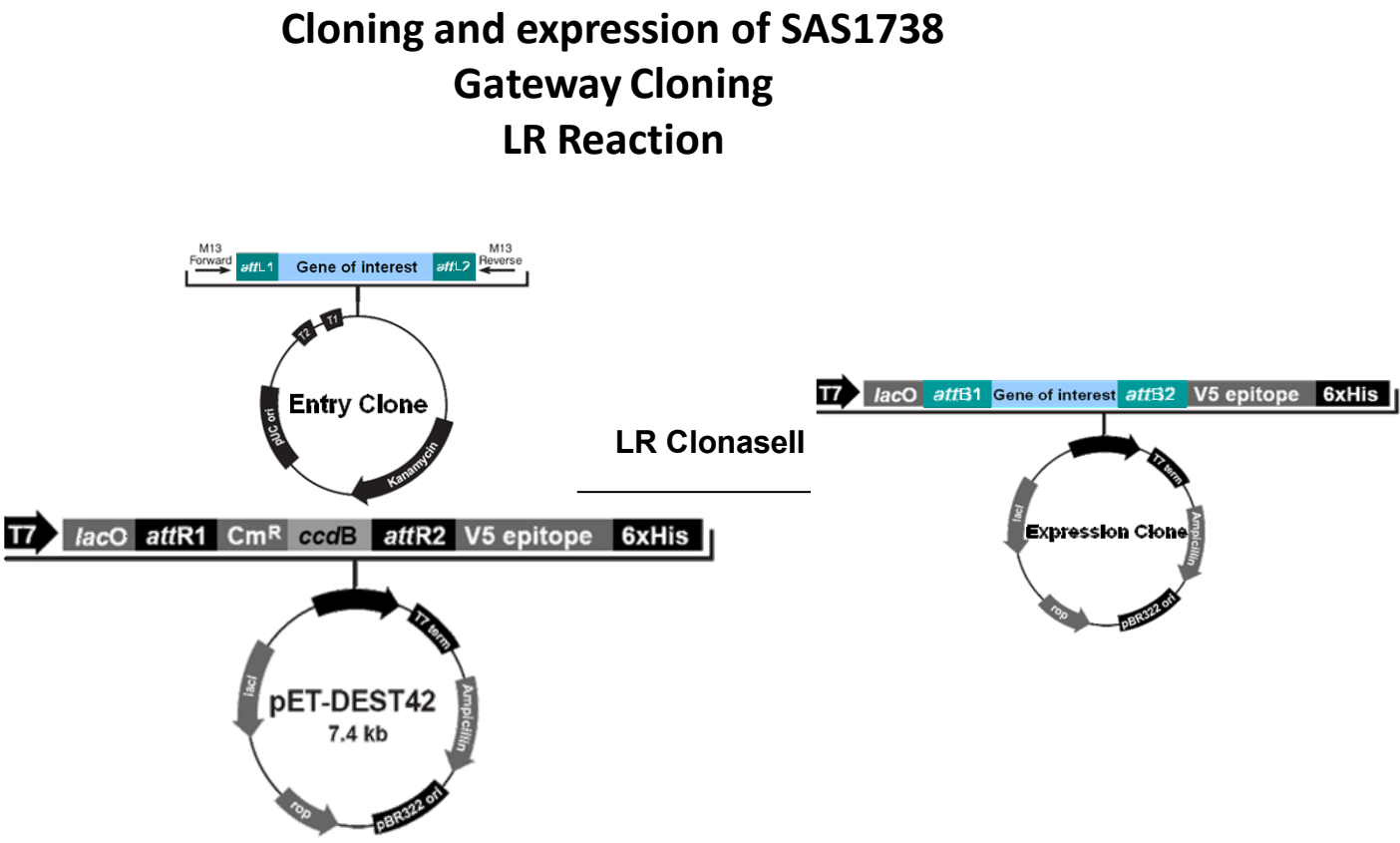

Figure 8. LR recombination reaction.

The entry clone with att L sites was recombined with pET-DEST 42 vector carrying att R sites for LR recombination. The LR clonase II enzyme catalyses the recombination, producing an expression clone with the gene of interest in place of cloramphenicol resistance gene, and fused to V5 epitope and a 6 His tag sequence. The LR recombination reaction produces expression clones that bear *att* B sites.

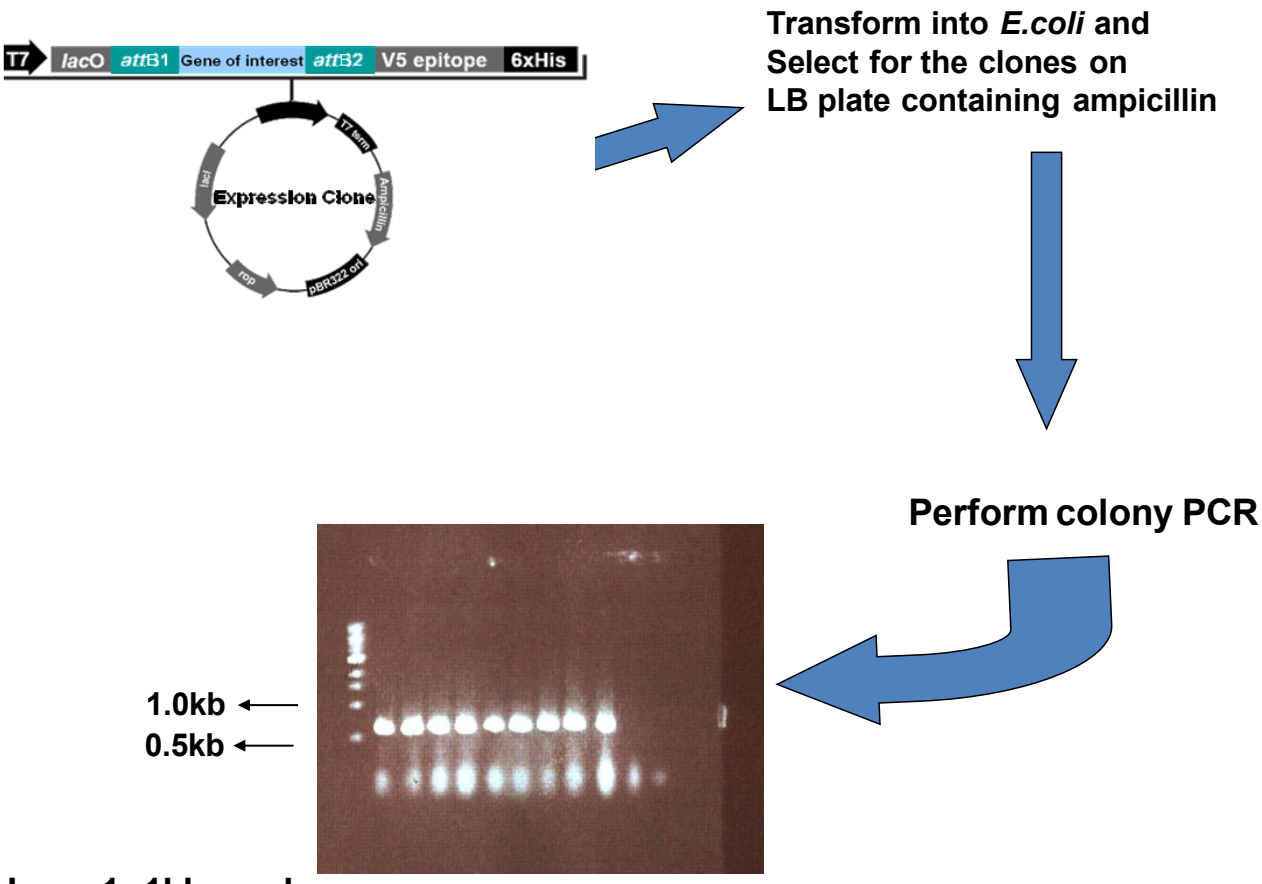

Figure 9. Confirmation of SAS1738 containing expression clones on 1% agarose gel.

The presence of the SAS1738 gene in expression clones transformed into *E. coli* BL21 A1 shot *E. coli* was confirmed by PCR and the expected 0.6 kb on gel electrophoresis is shown.

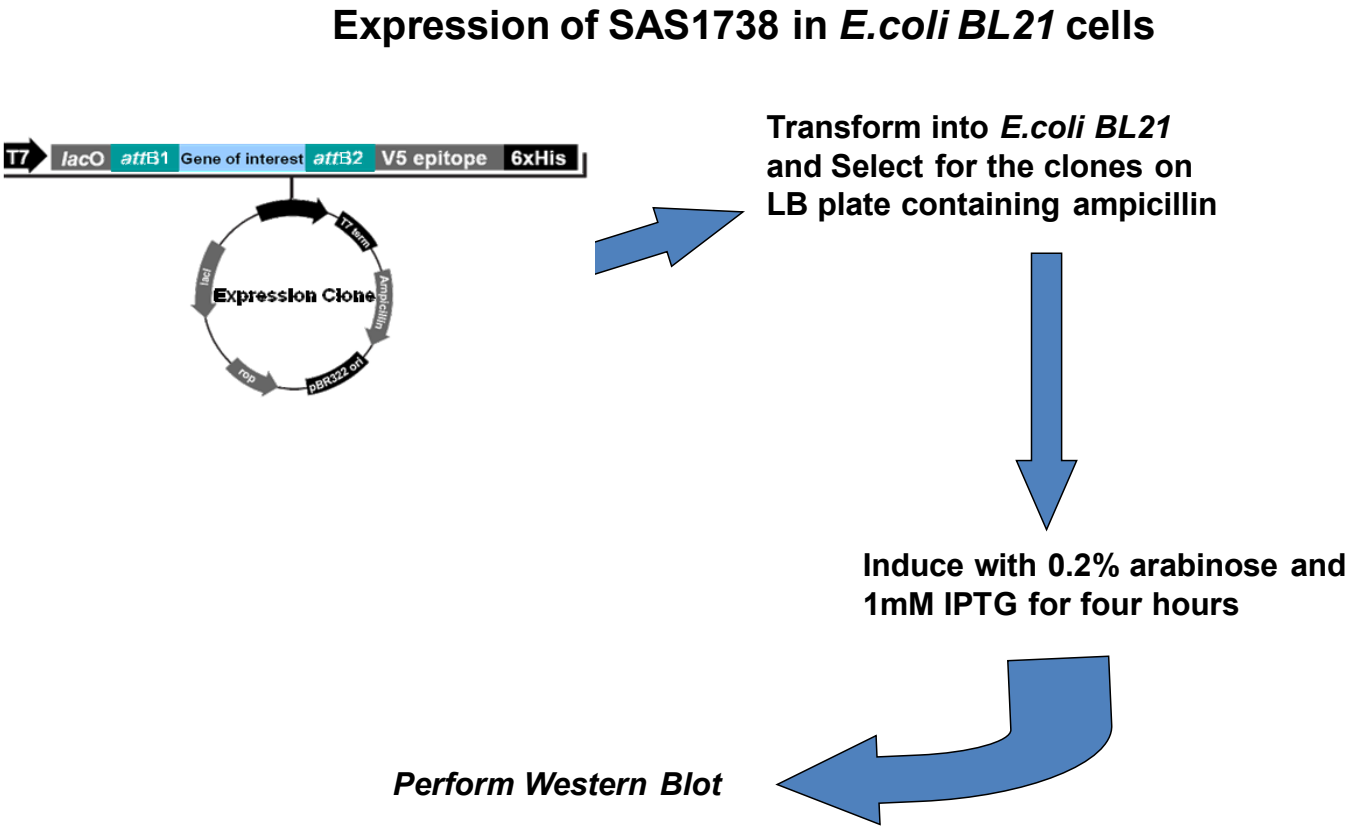

Figure 10. Transformation and expression of expression clones into *E. coli* BL21.

After confirmation the *E. coli* BL21 cells were grown in LB containing 0.2% arabinose and 1 mM IPTG and induced. Expression of SAS1738 was confirmed by Western Blot.

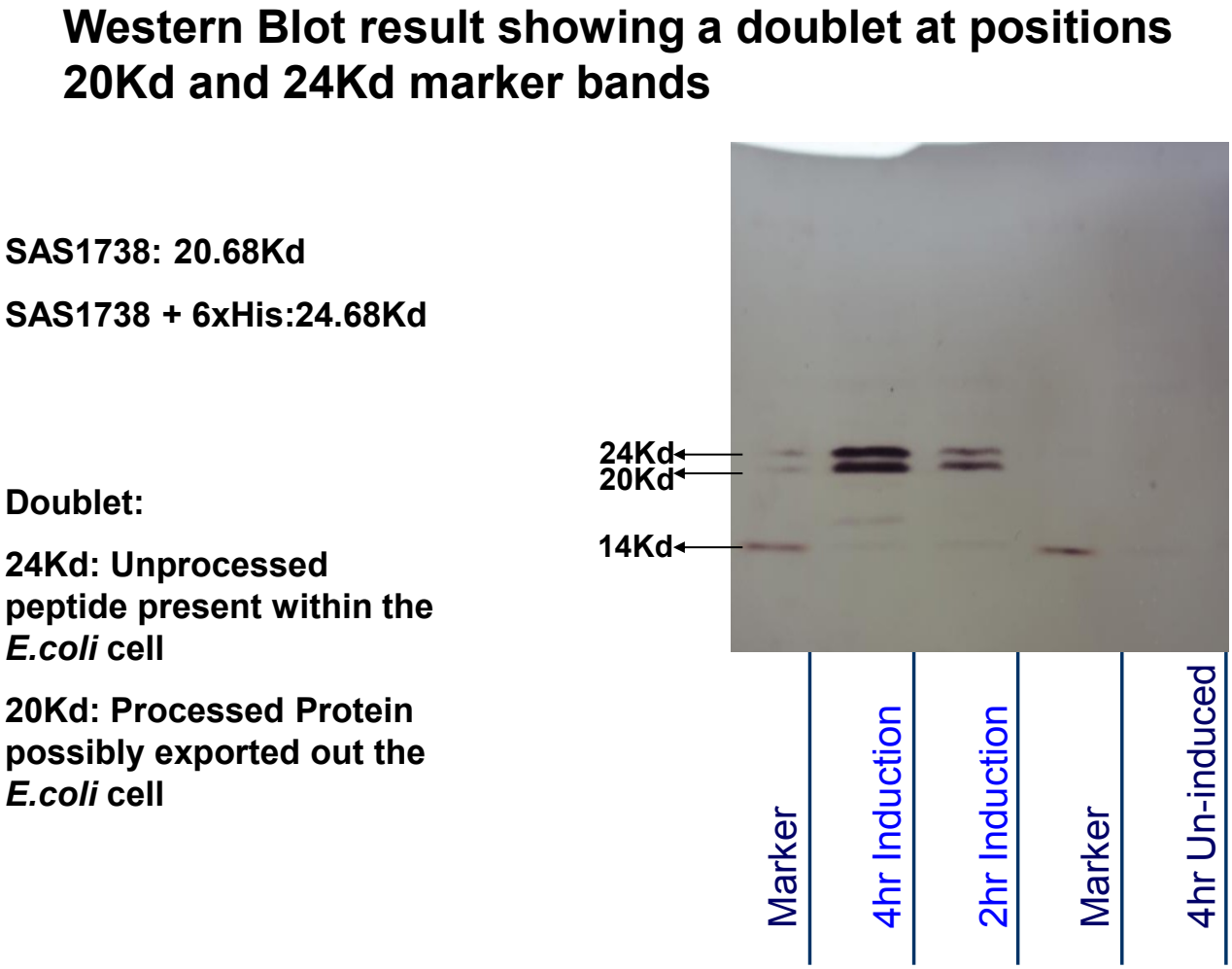

Figure 11. Western Blot.

Anti-his monoclonal antibody was used to determine the expression of SAS1738.Strong bands are seen with the sample collected at the 4th hour of induction and a slightly strong band is seen with the 1st hour induced sample. No band is observed in the uninduced 4th hour sample. Two bands are seen one corresponding to 25 kd and the other to 20 kd suggesting a possible post-translational processing of the protein in *E. coli*. Loss of the 42 amino acid signal peptide sequence after processing will reduce the protein size by 4.5 kd.

Zones of lyses were observed on plates with *Micrococcus luteus*.

Figure 12. Antibacterial assay results.

Zones were observed on Micrococcus luteus lawn withpurified, dialysed and filtered SAS1738. No zone is seen in the two controls, one with purified, dialysed, and filtered sample of uninduced solution, and the other using filtered elution buffer.

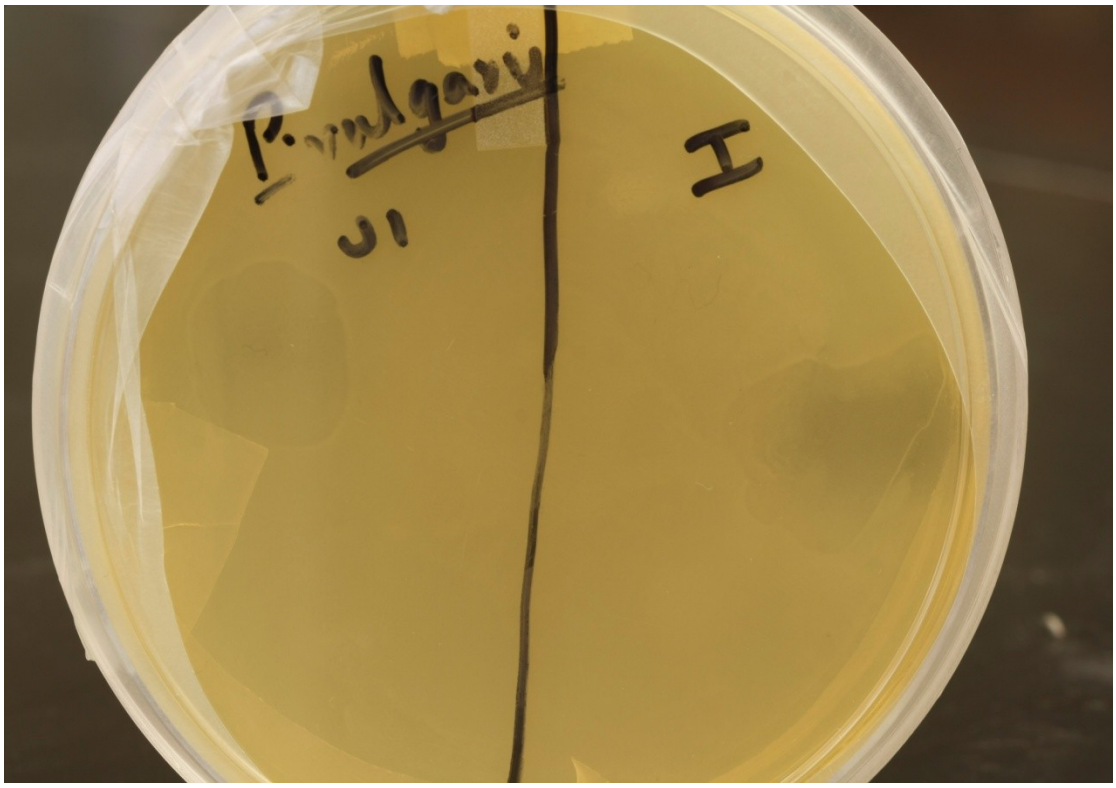

Figure 13. Antibacterial assay.

A Zone of lysis was observed on the lawn of *Proteus vulgaris* (right side of the petridish) when purified SAS1738 was spotted. No zone was lysis was observed on the area where purified sample from an uninduced culture was spotted.

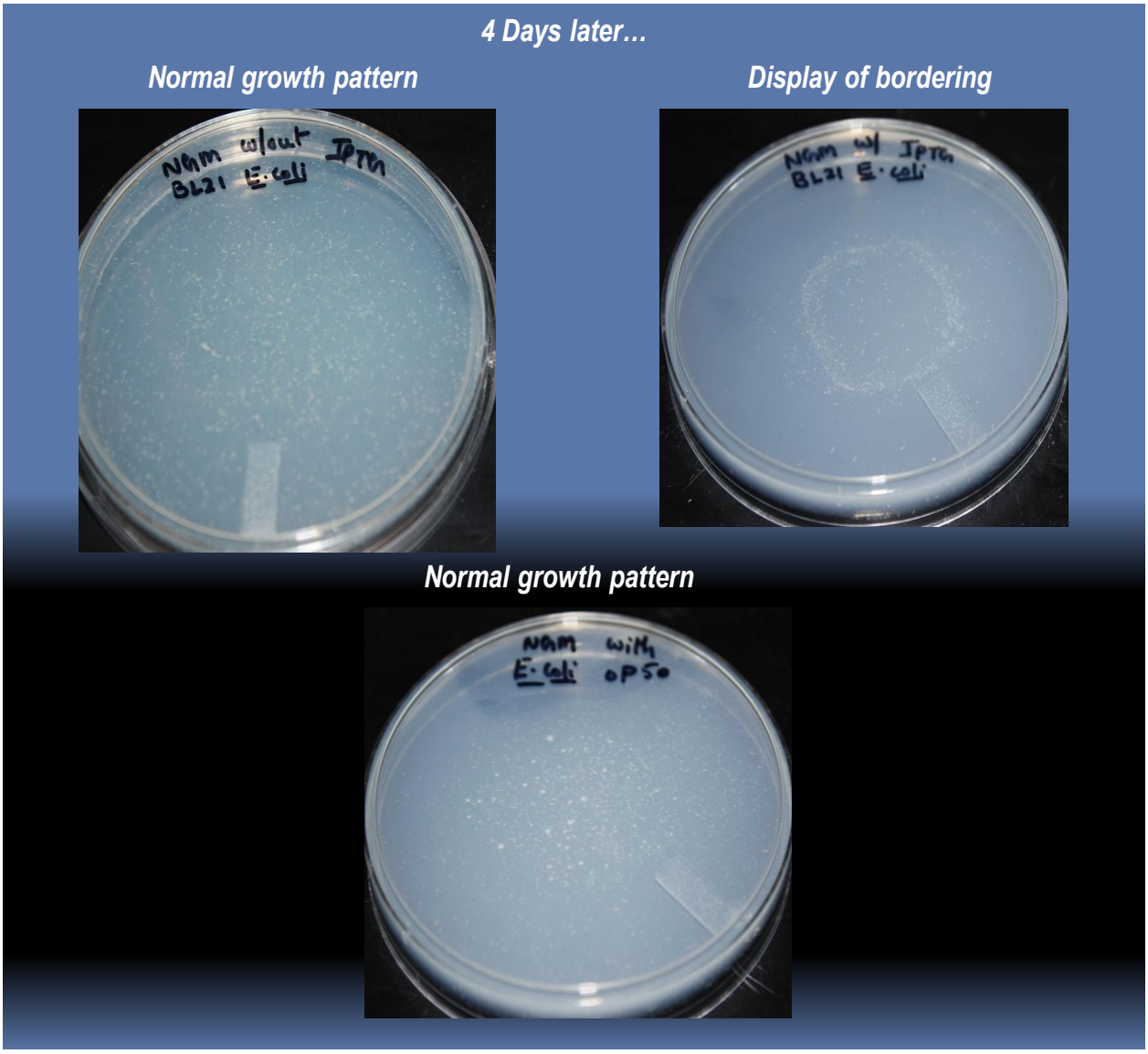

Figure 14. Toxicity assay using *C. elegans*.

The number of *C. elegans* on *E. coli* BL21 growing in absence of IPTG and arabinose is higher and shows no bordering (Top left). Bordering is seen, and the numbers are reduced when

SAS1738 expression in *E. coli* BL21 is induced by 1 mM IPTG and 0.2 % arabinose (Top right). The number of worms is high and no bordering appears on NMG with *E. coli* OP50.

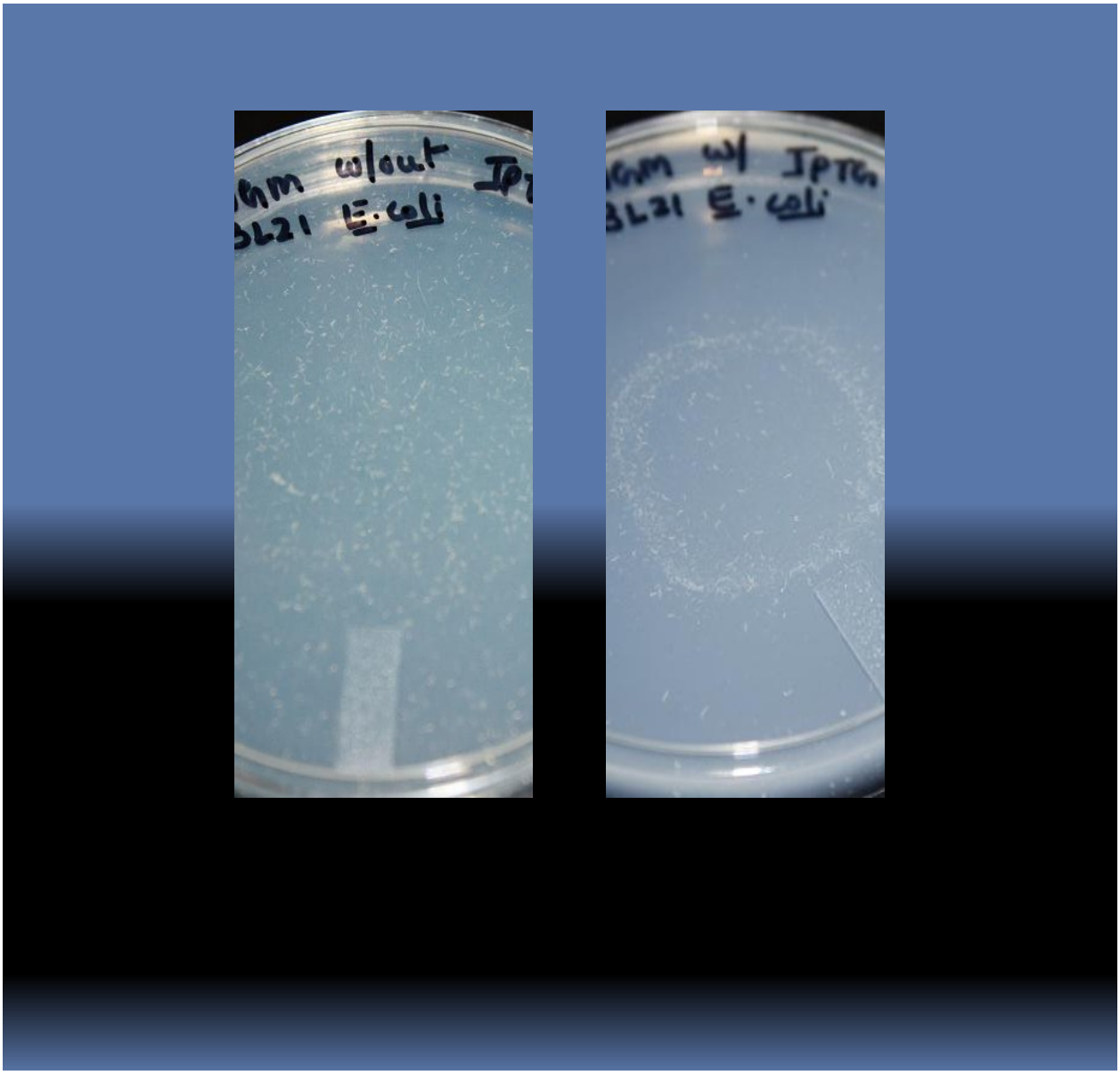

Figure 15. NGM plates displaying bordering phenomenon.

NGM plates showing the behavioral pattern of *C. elegans* in presence and absence of 1 mM IPTG and 0.2 % arabinose . Bordering is displayed on NGM in presence of SAS1738 (top right) and no bordering is seen in absence of SAS1738 (top left).

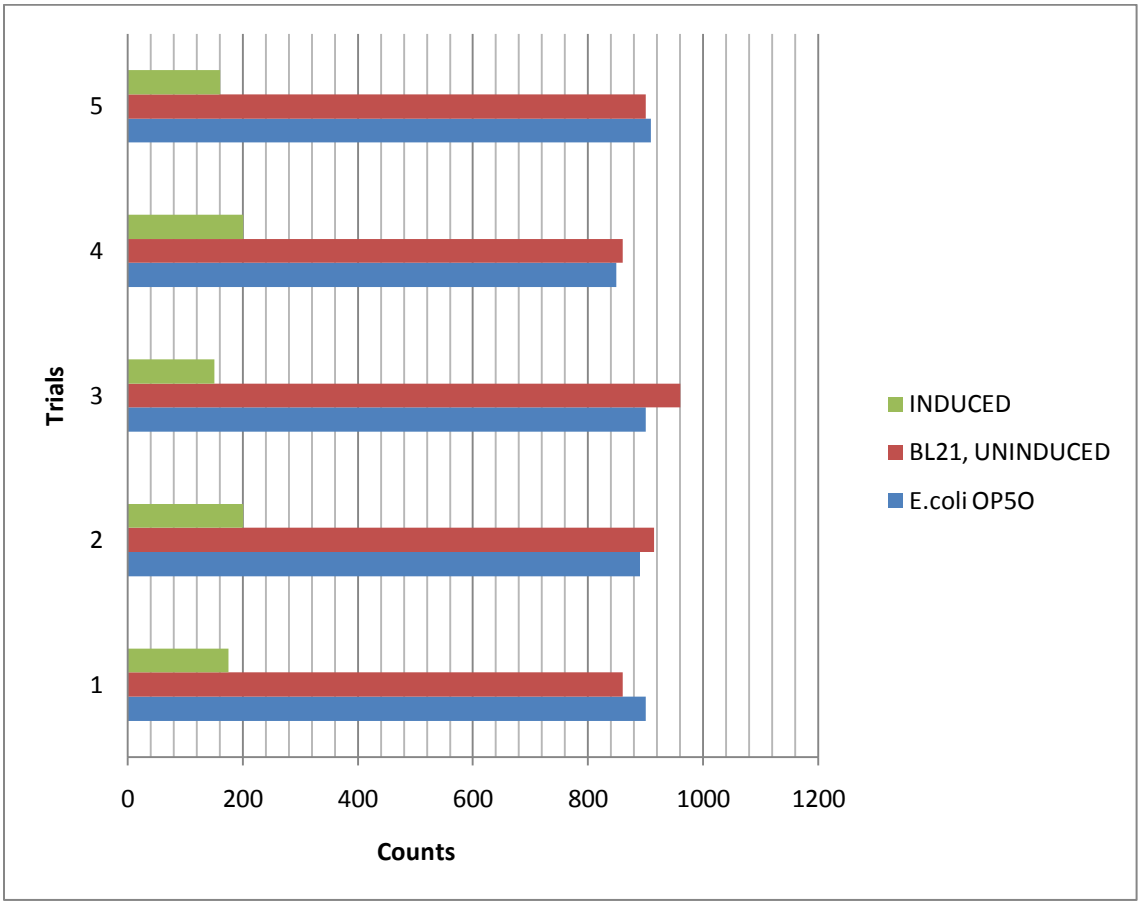

Figure 16. Inhibitory effect of SAS1738 on *C. elegans* growth.

The green bar represents the number of worms consuming *E. coli*BL21 expressing SAS1738. Red bar represents the number of worms when grown on *E. coli* BL21 not producing SAS1738. Blue bar represents the number of worms growing on *E. coli* OP50.

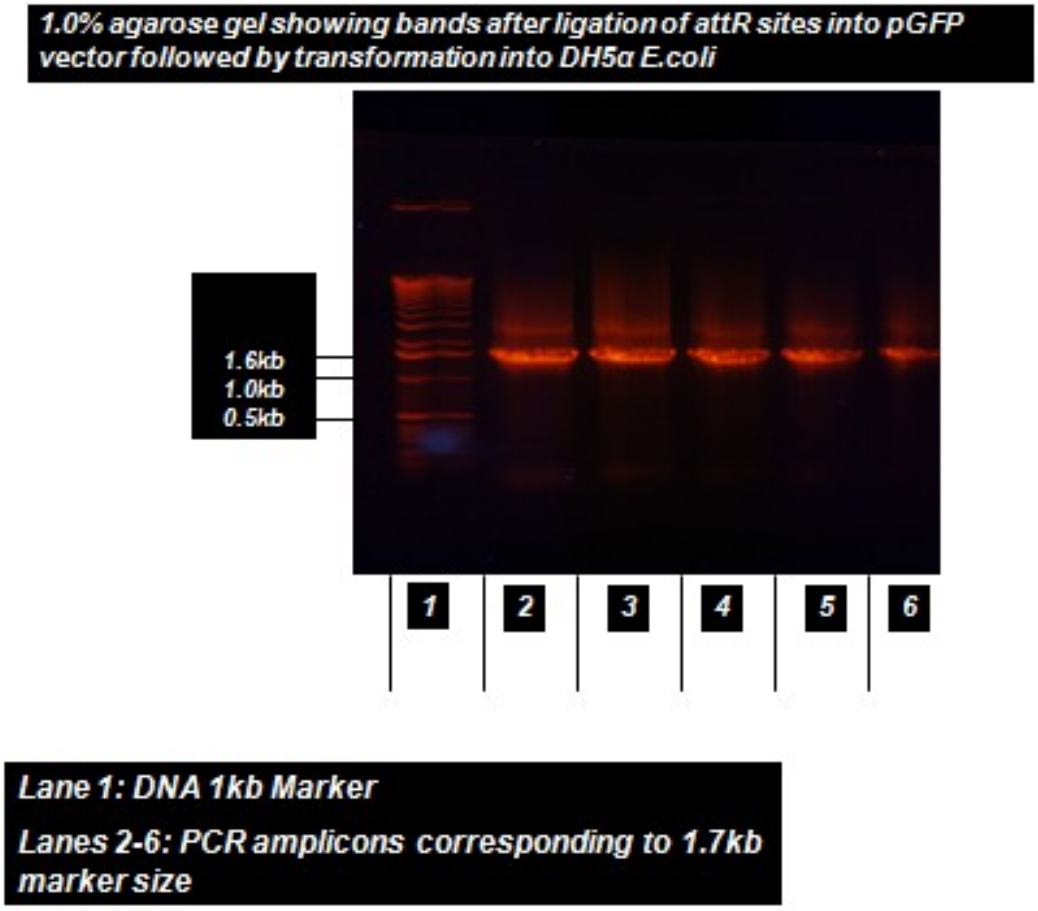

Figure 17. 1% agarose gel confirming the insertion of reading frame cassette into pGFP vector.

Confirmation of successful ligation of the gateway reading frame cassette into the 5' MCS of pGFP vector by PCR and gel electrophoresis. Bands corresponding to 1.7 kb are the expected size and confirm the insertion of the *att* R containing reading frame cassette in the 5' MCS of the pGFP vector.

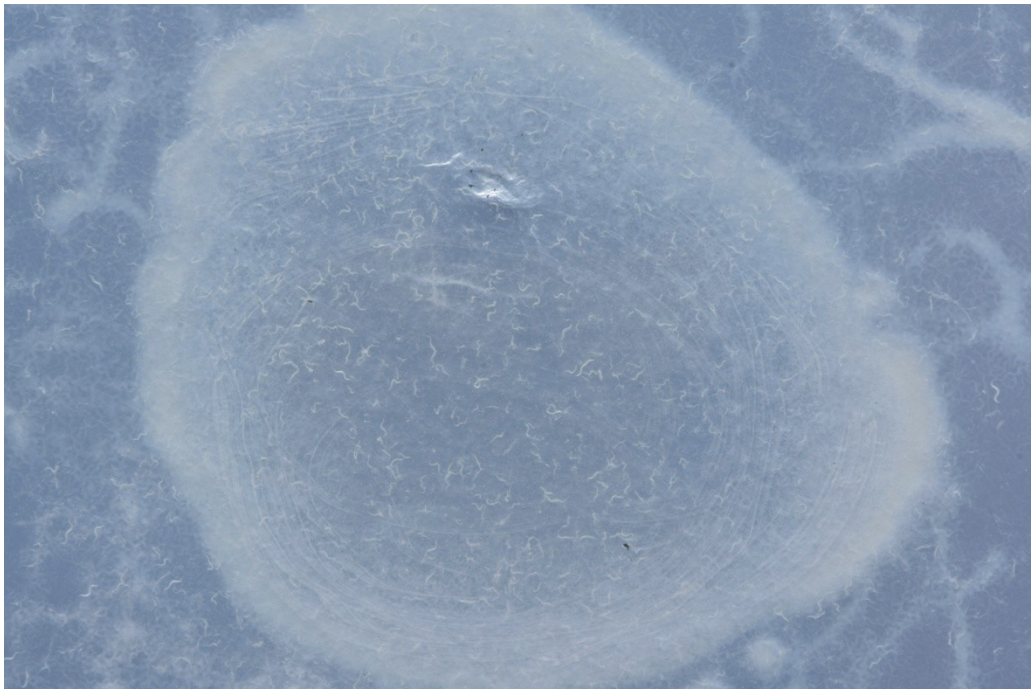

Figure 18. Growth of *C. elegans* on *E. coli* OP50.

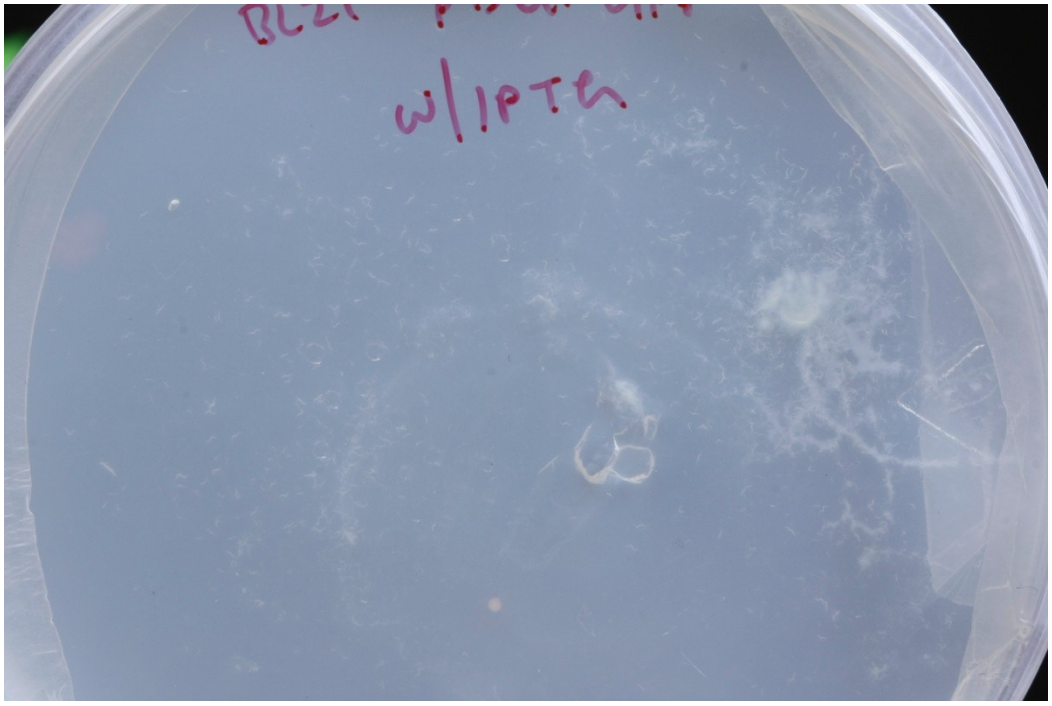

Figure 19. Growth of *C. elegans* on *E. coli* BL21 expressing the GFP-fused SAS1738.

Figure 20. Fluorescence microscopic results of worms fed with GFP-fused SAS1738.

Fluorescence is seen inside the worms after they were fed GFP-fused SAS1738 expressing *E. coli* BL21. Fluorescence is seen throughout the body, but is strong in pharyngeal and reproductive areas.

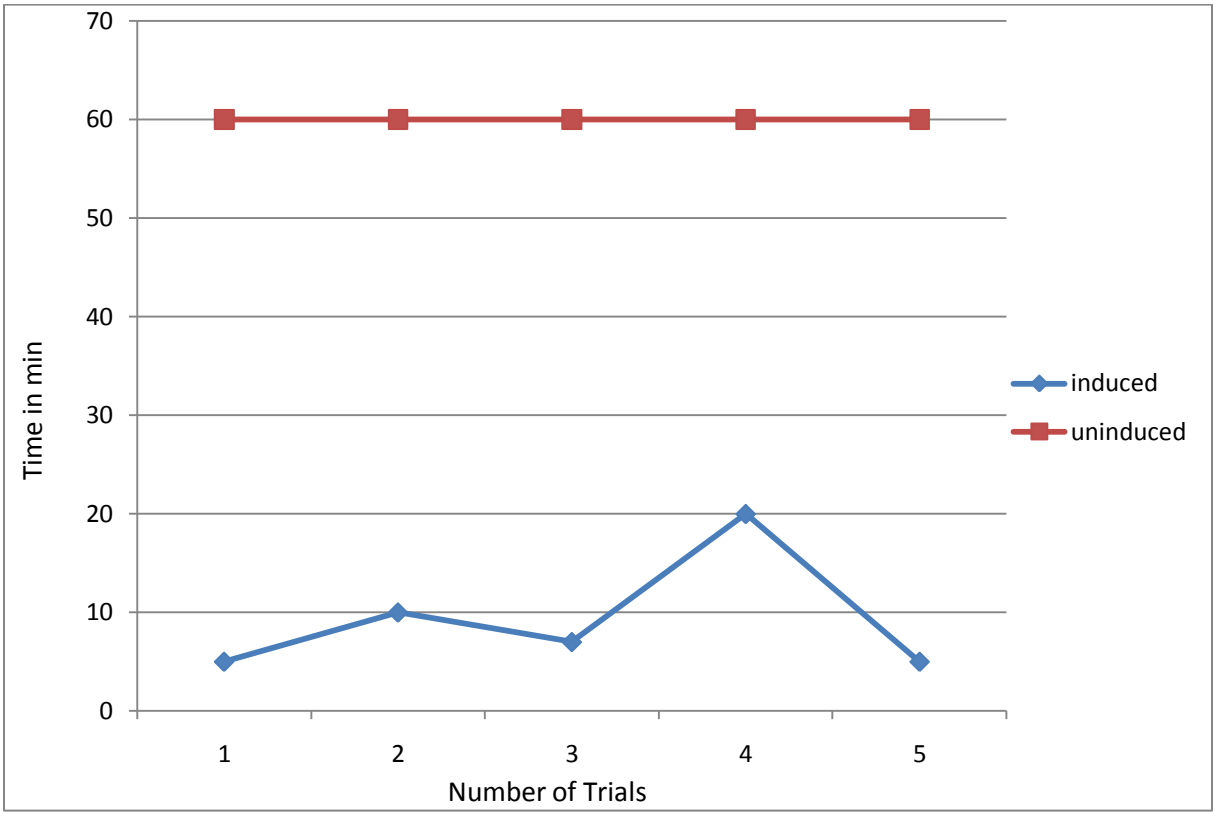

Figure 21. Toxicity assay of *C. elegans* using purified, dialysed and filtered sample containing SAS1738.

Toxicity assay results plotted with the number of trials of the assay on the x-axis against time in minutes on the y-axis. The blue diamond represents time points at which *C. elegans* died when treated with purified SAS1738 from an induced sample. The red squares represent time points at which *C. elegans* died when treated with purified uninduced control sample.

Discussion

The antibacterial activity of SAS1738 against *Micrococcus luteus* and *Proteus vulgaris* in addition to its growth inhibition of *C. elegans* stresses the importance of the roles that hypothetical proteins of CA strains may play during an infection process in humans. The death of adult *C. elegans* in response to SAS1738 further enhances the above hypothesis. The results obtained from the study clearly show that SAS1738 can function in multiple ways and can inhibit the growth of both prokaryotic and eukaryotic cells. There are about 73 uncharacterized hypothetical proteins in CA strains and their potential as virulence factors can be assessed by testing them against *C. elegans*. Use of *E. coli* BL21 cells to express a single gene encoding a hypothetical protein such as SAS1738 is a method that has not been employed before. This novel method of feeding a toxin resistant strain of *E. coli* BL21cells, expressing a particular gene in order to determine the effects of its protein product is feasible and easy to perform. Also, the purified protein can be used in a killing assay of *C. elegans* and its effects inside a eukaryotic system can be assessed.

Community associated MRSA infections have been a major public health concern since their emergence. The increased invasiveness of these strains has made them one of the fastest growing emerging infectious agents. Though they are not as resistant to a wide range of antibiotics as are the HA-strains, they are more invasive than the HA strains. Furthermore, CA-MRSA has overcome regional barriers and is present in many places, including gymnasiums, daycares, schools, athletic training centers. The acquisitions of novel genes, some of which encode major virulence factors like the PVL, have contributed to the high mortality rate from infections caused by the CA strains. But how they overcome the competing commensal flora of the human skin before they can become systemic is still not clear. However, from the results

obtained from our study it is evident that CA strains have an advantage over HA strains in that they inhibit the growth of normal flora by producing bacteriocins or antibacterial peptides. The high-specificity of these antibacterial peptides prevents any cross-immunity from occurring, thereby making the strains that are producing them dominate over others that occupy the same niche (Daly, Upton et al. 2010).

The presence of numerous as yet uncharacterized proteins encoded by hypothetical genes found in locations that lie in close proximity to known toxins or virulence genes suggest that the CA strains have invasive mechanisms that deserve further study. This may shed more light on their mode of pathogenesis and help in locating targets for drug designs. The killing of *Micrococcus luteus*, a normal gram positive bacteria found on the skin, nose, and in the nares, and a close relative of *Staphylococcus aureus*, strengthens the hypothesis that environmental competition with closely related species by producing secreted proteins is one of the features that makes the CA strains unique from their HA counterparts. However, mere competition with the normal commensal flora does not make a strain more invasive to its human host. What factors make some individuals more vulnerable to CA infections than others is constantly debated. The *C. elegans* toxicity assay results suggest that SAS1738 may play a dual role of inhibiting other commensal bacteria on the human skin as well as induce a change inside the eukaryotic system as evidenced by the 5-fold population reduction observed among worms treated with SAS1738. This dual function of SAS1738 of acting as an antibacterial peptide and as a virulence factor shows the complexity in the pathogenesis cycle of these bacteria.

The less complex type IV and V SCC elements confer more flexibility to the CA strains thus enhancing their ability to adjust to newer environments faster than HA strains. Presence of a myriad of secreted protein-encoding genes is an added advantage. However, how most of these

secreted proteins act on the host cell and what induces their expression are some of the questions that are currently addressed in most research studies.

The mechanism by which SAS1738 causes lysis and ultimately cell death of *Micrococcus luteus* is not known, but the reduction in *C. elegans* population size and presence of SAS1738 mostly in the pharyngeal and the reproductive system seems to indicate the involvement of lipids. Given the fact that *M. luteus* and *Staphylococcus aureus* are differentiated by an antibiotic susceptibility test using bacitracin which kills *M. luteus* but not *S. aureus*, it is possible that a similar lipid involved in the bacitracin susceptibility is involved here as well. Also most lipids involved in cholesterol biosynthesis are concentrated in the pharynx and reproductive regions of *C. elegans*, and fluorescence from GFP-fused SAS1738 mostly in these regions is another indication of the involvement of lipids. Cholesterol in oocytes is essential for the developing embryos, and the reduced worm population in response to SAS1738 further strengthens the possibility of the involvement of lipids in cholesterol synthesis in this decline in worm population.

The *npr-1* gene in nematodes is associated with the differences in the pathogen susceptibility observed among different strains of *C. elegans* (Reddy, Andersen et al. 2009). It has been shown in a previous study that the N2 strain of worms was more resistant than most other strains like CB4856 when exposed to a pathogen because they had an increased *npr-1* gene activity (Reddy, Andersen et al. 2009). However, in our study N2 strains that were shown to have a higher *npr-1* activity in fighting infections showed susceptibility to SAS1738 as evidenced by a significant reduction in their total number after the first generation. The other strains that were shown to be less effective than N2 in their pathogen fighting abilities were not

tested with SAS1738 in our study. It would be interesting to see how those strains might react to SAS1738.

Bordering, which was shown to occur only in the CB4856 strains but not in N2 strains in previous study that used *Pseudomonas aeruginosa* strain PA14 (Reddy, Andersen et al. 2009), was postulated to be a behavioral pattern induced by the pathogenic environment created by the bacterial lawn. In our experiments, the N2 strains showed bordering and a substantial reduction in number in response to SAS1738. What change SAS1738 induces inside the N2 strains and changes its oxygen requirements as seen by a change in its aerotaxis behavior, and pushes it to the border is not clear.

Alternatively, a change in the behavior as a result of stress induced by SAS1738 may lead to a reduction in the egg-laying and hence low population rate. Another possibility is that by spending more time on the edges, the worms are not acquiring enough nutrients for the proper development of embryos and results in a decrease in their hatchlings. Either way the effect of SAS1738 is detrimental to the worms.

GFP-fused SAS1738 fed worm plates exhibited similar reduction in the worm population and displayed similar bordering pattern as seen with the 6xhis-fused SAS1738 fed worms. This study was carried out to locate SAS1738 inside the worms and also to test if the GFP tag reduced the effect of SAS1738. However, similar results obtained with GFP-fusion study inadvertently showed that the 6xhis tag in itself did not confer any inhibitory effect when fused with SAS1738. Toxicity testing using purified SAS1738 exhibited earlier killing of the adult worms when the worms were introduced into a 20 µl filtered and exposed to the sample containing SAS1738 at a concentration of approximately 50 µg/ml. The assay lasted 60 min and the worms treated with an uninduced control sample remained alive. Though the time points at which death of the worms

occurred when treated with SAS1738 varied with each trial, with the average time of death being 8 min from the initial time of contact, treatment with SAS1738 resulted in death nonetheless. The rapid pharyngeal pumping action of the worms in liquid medium can possibly increase the uptake of SAS1738, which is being constantly ingested, unlike on a bacterial lawn where there are relatively non-toxic regions that the worms can occupy. In two of the trials, numerous eggs were seen in the sample after the worm was confirmed dead. In this study, the effect of SAS1738 was determined only on the adult worms, not the eggs or the larvae. But from the plate assay described earlier based on a drastic decline in the number of worms after the first generation it is clear that SAS1738 affects the normal development of the eggs.

Future study using a shuttle vector carrying the SAS1738 gene insert to transform a competent strain of *Staphylococcus aureus* for expression should be considered. This in combination with another experiment using site-directed mutagenesis for gene knock-out study would be interesting to further determine the effects of SAS1738 when expressed inside a *Staphylococcus aureus* system. Furthermore, this study would provide substantial data to delineate the activity of SAS1738. However, future study involving testing of SAS1738 on human cell lines will be another exciting area to explore to assess the role of SAS1738 during an actual MRSA infection in humans.

SAS1738 is one of the many hypothetical proteins that are produced by CA-MRSA, most of which are as yet uncharacterized. This study stresses the importance of studying hypothetical proteins in CA-MRSA in order to better understand the versatile nature of this fast growing pathogen that is constantly evolving.

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